Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
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Lipid and choline supply affect Atlantic salmon enterocyte steatosis. In vivo and in vitro studies

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2 Abbreviations and definitions

1. OA: Oleic acid
2. S: Stomach
3. PI: Pyloric intestine
4. PC: Pyloric caeca
5. MI: Mid intestine
6. DI: Distal intestine
7. GALT: Gut associated lymphoid tissue
8. PRR: Pattern recognition receptor
9. PAMP: Pathogen-associated molecular pattern
10. TLR: Toll-like receptor
11. NLR: NOD-like receptor
12. CLR: C-type lectin receptor
13. PGRP: Peptidoglycan recognition protein
14. FA: Fatty acid
15. FFA: Free fatty acid
16. TAG: Triacylglycerol
17. DAG: Diacylglycerol
18. PL: Phospholipid
19. PTL: Pancreatic triacylglycerol lipase

20. BAL: Bile-activated triacylglycerol lipase
21. PLA2: Phospholipase A2
22. SFA: Saturated fatty acid
23. MUFA: Monounsaturated fatty acid
24. LD: Lipid droplet
25. HDL: High density lipoprotein
26. LDL: Low density lipoprotein
27. VLDL: Very low-density lipoprotein
28. OSIPI: Organosomatic indices of the pyloric intestine
29. TGC: Thermal growth coefficient
30. DMC: Differentially methylated cytosines

3 List of papers

Paper I

Published in *Journal of Nutrition science 12, e61*.

Effects of dietary lipid level and environmental temperature on lipid metabolism in the intestine and liver, and choline requirement in Atlantic salmon (Salmo salar L) parr.

Daphne Siciliani, Trond M. Kortner, Gerd M. Berge, Anne Kristine Hansen, Åshild Krogdahl

Paper II

Submitted to *Scientific Reports*

Effects of dietary lipid source and fish size on induced steatosis in two Atlantic salmon (Salmo salar L) populations.

D. Siciliani, A, Hubin, B. Ruyter, E.M. Chikwati, V.G. Thunes, E.C. Valen, A.K.G. Hansen, H. Hanssen, T.M. Kortner, Å. Krogdahl

Paper III

Submitted to *Epigenetics*

Epigenetic changes in pyloric caeca of Atlantic salmon fed diets containing increasing levels of lipids and choline.

Anusha K. S. Dhanasiri, Daphne Siciliani, Trond M. Kortner, Åshild Krogdahl

Paper IV

Manuscript

A fish intestinal in vitro model for investigation of lipid metabolism and steatosis.

Daphne Siciliani, Bente Ruyter, Guro Løkka, Kirsti Elisabeth Præsteng, Matteo Minghetti, Trond M. Kortner

4 Abstract

The shift of farmed Atlantic salmon from a marine- to plant-based diet the last decades has revealed challenges concerning the dietary requirements necessary to ensure fish health. Of the nutrients lacking in plant feeds, choline has been identified as essential to guarantee efficient lipid transport and metabolism. The distinctive sign of choline deficiency is an excessive presence of unabsorbed fat accumulating within the intestinal enterocytes, a condition known as steatosis. The results of the first attempts to define choline requirement for post-smolt Atlantic salmon were published recently. However, it can be expected that choline requirements vary with life stages and environmental and dietary conditions, but information on such relationships is lacking. Besides being fundamental for lipid transport, choline has gained particular interest due to its important role in DNA methylation processes. So far, very little is known regarding the effects of variation in DNA methylation in fish, but it is likely that disturbances in methylation processes may lead to increased disease risk. In this thesis, two studies, resulting in three papers, were conducted to investigate how choline requirement in farmed Atlantic salmon may be influenced by different production conditions, and how dietary choline level may interfere with DNA methylation. However, our understanding of the cellular mechanisms responsible for nutrient interactions with fish digestive physiology is still fragmentary. To address this, the third study in this thesis focused on in vitro studies of lipid metabolism. This approach is an important tool to facilitate further research into the basic functions of the digestive tract.

In the first study, we investigated the effects of increasing dietary lipid levels on steatosis symptoms. This study involved Atlantic salmon parr that were fed choline-deficient diets. The fish were raised at two different environmental temperatures: 8 and 15 °C. The results showed that fish raised at 15 °C had higher feed intake and growth performances compared to those raised at 8 °C. Choline deficiency throughout all the diets induced steatosis in each experimental group. The increasing lipid level worsened the severity of the condition, as shown by the selected indicators i.e., the

organosomatic index of the pyloric intestine (OSIPI), the enterocyte lipid accumulation, and the expression of the biomarker genes (*plin2*, *apoAIV, apoAI and pcyt1a*). Simultaneously, the severity increased with increasing water temperature, presumably as a consequence of the higher feed intake and therefore lipid supply to the fish. The fatty acid (FA) composition of the pyloric caeca mirrored that of the diets, while no clear effect was observed in the FA composition of the liver. Taken together, these results confirmed the importance of choline in lipid transport across the intestine, while demonstrating the relevance of dietary lipid level and water temperature as important drivers for intestinal lipid accumulation.

In the second study, we assessed the effects of lipid source on steatosis symptoms. This study involved two Atlantic salmon populations differing in size. Small fish showed higher feed intake and therefore growth performances compared to large fish. Simultaneously, the increasing level of rapeseed oil strongly influenced lipid digestibility and therefore lipid supply to the fish. The higher lipid supply, given by the increased feed intake and by the higher lipid digestibility, worsened the severity of enterocytes vacuolation. Total digestible FA increased with increasing rapeseed oil level in the diet. This result was clearest for mesenteric fat, followed by the pyloric intestine tissue and then by the liver. Taken together, these results were in accordance with the results from the first study and suggest that the lipid uptake is the main factor influencing steatosis symptoms.

The epigenetic study, which was based on samples collected in the first study, in which Atlantic salmon parr were fed four different diets with lipid levels increasing from 16% to 28%, and with insufficient choline content. Pairwise analyses of genome-wide DNA methylation patterns in pyloric caeca samples were conducted to compare different dietary impacts on the fish. The first comparison involved fish fed diets with 25% vs 16%, with similar, insufficient choline content, 1.9 g/kg feed. The second comparison focused on fish fed diets with substantial difference in choline levels (1.9 g/kg vs 2.3 g/kg), i.e., 22%, but with quite similar lipid content, 28 vs 25%. The results revealed that increasing dietary lipid level induced methylation differences in genes involved in membrane transport, signalling pathways, and microRNA important for

regulation of lipid homeostasis. Increasing choline level affected genes related to FA biosynthesis and transport, lipolysis, lipogenesis as well as important immune genes. Our observations confirm the role of choline in epigenetic regulation in Atlantic salmon, as reported in other vertebrate groups.

The aim of the third study was to establish an in vitro steatosis model mimicking the lipid accumulation conditions observed in the Atlantic salmon intestine. A newly developed cell line derived from the distal intestine of the rainbow trout (RTdi-MI) was exposed to increasing concentrations of oleic acid (OA): 31.25 μM, 250 μM. Cells were sampled at 24 h, 72 h and 168 h (1 week) after exposure. The results showed that cell viability was not significantly influenced by the high concentration of OA or by exposure time. However, both factors affected the morphology of the cells. Cells became more rounded because of increasing intracellular lipid droplets (LD), which also compressed the cells' nuclei towards the periphery of the cells. Such morphology resembles that of preadipocytes observed in other studies. The lipid quantification analysis confirmed not only that the cells accumulated OA in parallel to the OA exposure, but also that such accumulation increased with increasing exposure time. In addition, all cells, including the control cells, showed signs of fatty acid metabolic activity. The molecular results showed that, similar to what happens in vivo, all the cells expressed a set of genes involved in lipid metabolism and synthesis. The assessed genes were upregulated with increasing exposure time, and *plin2* especially, which correlates with severity of steatosis in in vivo situations, was upregulated with increasing OA concentration in the medium. Taken together, these results confirm that RTdi-MI cells are able to accumulate OA and that their molecular mechanisms resemble those of the in vivo Atlantic salmon intestine.

In consideration of the role of choline in lipid transport and methylation processes, the present thesis generates new knowledge for the urgently needed studies defining choline requirement in Atlantic salmon for optimal growth and health. In addition, the conducted work has paved the way to study fish lipid metabolism on in vitro platforms.

5 Norsk sammendrag

Endringene i sammensetningen av fôr til laks i oppdrett de siste tiår, fra marine til vegetabilske ingredienser, har avdekket kunnskapsmangler når det gjelder tilførsel av næringsstoffer som sikrer god fiskehelse. Kolin er et av næringsstoffene som finnes i begrensende mengder i plantebaserte fôr, og som er essensielt for transport og metabolisme av fett. Det karakteristiske symptomet på kolinmangel er en unormal akkumulering av fett i tarmens enterocytter, en tilstand kjent som steatose. De første forsøkene som er gjennomført for å få kunnskaper om kolinbehovet hos postsmolt oppdrettslaks, ble nylig publisert. Det er sannsynlig at behovet varierer gjennom livsstadier hos oppdrettslaksen og påvirkes av miljømessige og fôringsmessige variasjoner, men kunnskap om slike forhold mangler. I tillegg til å være grunnleggende for fettransport, har kolin fått økt oppmerksomhet på grunn av sin viktige rolle i DNA-metyleringsprosesser, dvs epigenetiske prosesser. Hittil er lite kjent om effektene av variasjon i DNA-metylering hos fisk, men det er sannsynlig at forstyrrelser i metyleringsprosesser kan føre til økt sykdomsrisiko.

Dette doktorgradsarbeidet omfatter tre studier. To av disse, som er presentert i tre av artiklene i denne avhandlingen, hadde som mål å undersøke hvordan kolinbehovet hos oppdrettslaks kan påvirkes av ulike produksjonsforhold, og hvordan fôrenes kolininnhold kan påvirke DNA-metylering. Det tredje studiet hadde som mål å øke kunnskapene om cellulære mekanismer og næringsstoffers interaksjon med fiskens fordøyelsesfysiologi. I dette ble en in vitro-cellemodell for studier av lipidmetabolisme i fisketarm etablert. Denne modellen kan bli et viktig verktøy for videre forskning rundt de grunnleggende funksjonene i fiskens fordøyelsessystem.

I det første studiet ble effektene av økende fettinnhold, fra 16 til 28 %, i et fôr med mangelfullt innhold av kolin, på symptomer på steatose undersøkt hos parr av atlantisk laks som ble holdt ved to temperaturer: 8 og 15 °C. Resultatene viste, som forventet, at fisken ved 15 °C hadde høyere fôropptak og raskere vekst enn de ved 8 °C. Kolinmangelen i fôrene forårsaket steatose i alle forsøksgrupper. Økende

fettinnhold i fôret forverret alvorlighetsgraden av tilstanden, som vist, for eksempel for organosomatisk indeks av pylorus-tarmen (OSIPI), lipidakkumulering i enterocyttene og uttrykket av biomarkørgener (*plin2*, *apoAIV*, *apoAI* og *pcyt1α*). Alvorlighetsgraden økte også med økende vanntemperatur, antagelig som en konsekvens av økt fôropptak og dermed økt lipidtilførsel til fisken. Fettsyresammensetningen i pylorus-blindsekker reflekterte fôret, mens det ikke ble observert noen tydelig effekt på fettsyresammensetningen i lever. Samlet sett bekreftet studien viktigheten av kolin for effektiv fettransport i tarm, samtidig som den viste at fettinnholdetet i fôr og vanntemperatur er viktige faktorer for alvorlighetsgraden av steatose i tarmen, og derved for kolinbehovet.

I det andre studiet ble effektene av andelen av fiskeolje og rapsolje i fôret, på symptomer på steatose indusert av kolinmangel studert. I denne studien inngikk to forskjellige populasjoner av atlantisk laks med forskjellig startvekt, smoltifiseringsforløp (S0 og S1), og genetisk bakgrunn. Disse populasjonene ble henholdsvis kalt liten og stor fisk. Liten fisk hadde høyere fôrinntak per kg kroppsvekt, og dermed høyere relativ tilvekst sammenlignet med stor fisk. Andelen rapsolje i fôret økte fettfordøyeligheten, og dermed fett-tilførselen, kraftig. Den høyere fett-tilførselen, som var resultat både av økt fôropptak og av høyere fettfordøyelighet, forverret alvorlighetsgraden av fettakkumuleringen i enterocyttene. Samlet sett var disse resultatene i samsvar med resultatene fra det første eksperimentet og antyder at fett-tilførsel er den viktigste faktoren for alvorlighetsgraden av steatosesymptomene.

Effekter av grad av kolinmangel på epigenetiske prosesser ble studert basert på prøver fra det første studiet, dvs fra parr av atlantisk laks fôret med fire forskjellige fôr med økende lipidnivåer fra 16 % til 28 %, og med utilstrekkelig tilskudd av kolin. To parvise analyser av genomvid DNA-metyleringsmønstre i prøver fra pylorus-blindsekker ble gjennomført. En for fisk som ble gitt fôr med 25 % og 16 % fett og likt, men utilstrekkelig kolininnhold, 1.9 g/kg, den andre for fisk som ble gitt fôr med forskjellig kolinnivåer (1,9 g/kg vs 2,3 g/kg), og relativt likt fettinnhold (28 vs 25%).

Resultatene viste at økende innhold av fett i fôret resulterte i metyleringsendringer i noen gener involvert i membrantransport, signalveier, og mikroRNAer som er viktige for regulering av lipidhomeostase. Økende kolinivå påvirket gener for fettsyresyntese og transport, lipolyse og lipogenese samt viktige immungener. Disse observasjonene bekrefter rollen til kolin i epigenetisk regulering hos atlantisk laks, slik det er rapportert også for høyere virveldyr.

Målet med den tredje studien var å etablere en *in vitro* steatosemodell som kunne etterligne forholdene under lipidakkumuleringen i tarmsystemet hos atlantisk laks. En nyutviklet cellelinje fra baktarmen hos regnbueørret (RTdi-MI) ble eksponert for økende konsentrasjoner av oljesyre (OA): 31.25 μM og 250 μM. Celleprøve ble tatt 24, 72 og 168 timer (1 uke) etter eksponering. Resultatene viste at celleviabiliteten ikke ble betydelig påvirket, verken av den høye konsentrasjonen av OA, eller av eksponeringstiden. Imidlertid påvirket begge faktorene morfologien til cellene. Cellene ble mer avrundet på grunn av økende intracellulære lipiddråper (LD), og cellekjernene ble forskjøvet mot periferien av cellene. En slik morfologi ligner den som ses hos preadipocytter observert i andre studier. Kvantitative fettanalyser bekreftet at cellene akkumulerte OA som følge av OA-eksponeringen, og også at en slik akkumulering økte med økende eksponeringstid. I tillegg viste alle cellene, inkludert kontrollcellene, tegn til fettsyremetabolisme. De molekylære resultatene viste at cellene uttrykte et sett med gener involvert i lipidmetabolisme og -syntese på samme måte som det som skjer in vivo. De analyserte genene ble oppregulert med eksponeringstiden, og spesielt var *plin2*, som korrelerer med grad av steatose in vivo, oppregulert av økende OA-konsentrasjonen i cellemediet. Samlet sett bekrefter disse resultatene at RTdi-MI-celler er i stand til å akkumulere OA, og at deres molekylære mekanismer ligner de i tarmen til atlantisk laks.

Denne avhandlingen har generert ny og viktig kunnskap om kolins rolle i lipidtransport og metyleringsprosesser som er nødvendig for design av forsøk som må gjennomføres for å definere kolinbehov hos laks for optimal vekst og helse. I tillegg har arbeidet etablert en *in vitro*-plattform for videre studier av lipidmetabolisme i tarm hos fisk.

6 Sinossi

In natura, il salmone è un predatore che si ciba principalmente di altri pesci piu' piccoli. Negli allevamenti tuttavia, oltre al pesce, il salmone atlantico viene nutrito con mangimi a base di soia, grano ed oli vegetali. Tuttavia questi alimenti sostitutivi risultano spesso carenti in alcuni nutrienti che hanno assicurato, finora, il benessere animale. La colina, essenziale per il trasporto e metabolismo lipidico, e' tra quei nutrienti non sufficientemente presenti nei mangimi a base vegetale. L'inadeguato apporto di colina puo' causare eccessivo accumulo di grasso all'interno degli enterociti, una condizione nota col nome di steatosi e comune in diverse specie.
Uno studio condotto recentemente ha indicato il fabbisogno di colina necessario ad eliminare i sintomi di steatosi in salmoni in fase post-smolt.
E' tuttavia possibile che tale fabbisogno vari durante la vita dei salmoni, e che sia influenzato da fattori ambientali e nutrizionali.
Oltre ad essere fondamentale per il trasporto lipidico, la colina ha guadagnato particolare interesse negli ultimi anni grazie al suo ruolo di donotare di gruppi metili durante i processi di metilazione del DNA. Le informazioni disponibili riguardo gli effetti della metilazione del DNA nei pesci sono molto limitate ad ora. E' tuttavia possibile che un aumento di tali processi possa accrescere il rischio di malattie.
Per la stesura di questa tesi sono stati condotti due esperimenti fini allo studio dei fattori che potrebbero influenzare il fabbisogno di colina, e alla ricerca dei possibili effetti della colina sulla metilazione del DNA.
In aggiunta, un terzo esperimento in vitro e' stato condotto col fine di studiare i meccanismi cellulari e molecolari responsabili delle interazioni tra metabolismo lipidico e fisiologia intestinale.

Nel primo esperimento, sono stati testati gli effetti del livello di lipidi nel mangime, in combinazione con la temperatura dell'acqua. I salmoni, in stadio evolutivo parr. sono stati cresciuti a due diverse temperature ambientali, 8 e 15 C°, e alimentati con tre diete contenenti un crescente livello lipidico, 16%, 20%, 25%. Tutte le diete contenevano un basso livello di colina. Il basso livello di colina ha indotto un'eccessivo

accumulo lipidico nei cieci pilorici di tutti gli animali. Tale condizione e' stata peggiorata dall'aumento del contenuto di grasso nel mangime, come testimoniato dai principali biomarkers di steatosi: l'indice organosomatico dei ciechi pilorici (OSIPI), il quantitativo di gocce lipidiche accumulate negli eneterociti e l'espressione di un gruppo di geni marker (*plin2, apoAIV, apoAI e pcyt1α).* Per quanto riguarda gli effetti della temperatura dell'acqua, i sintomi di steatosi sono stati aggravati a 15 C°, consequentemente ad un aumento di appetito e quindi di apporto lipidico causato dall'alta temperatura. Il profilo degli acidi grassi nei ciechi pilorici ha rispecchiato quello della dieta, mentre il fegato non ha riportato particolari effetti o differenze. Nel complesso questi risultati confermano il ruolo della colina nel favorire efficiente trasporto lipidico, e mettono in luce l'importanza del livello di grasso nel mangime e della temperatura ambientale come fattori influenzanti l'accumulo lipidico.

Nel secondo esperimento, salmoni atlantici provenienti da due allevamenti differenti, e aventi quindi diverso background, razza e taglia, sono stati alimentati con sei diete contenenti crescente livello di olio di colza e decrescente livello di olio di pesce. I pesci piu' piccoli, avendo un maggiore appettito, hanno mostrato maggiori perfomance di crescita e un alto apporto lipidico rispetto ai pesci piu' grandi. Allo stesso tempo, il crescente livello di olio di colza ha incrementato la digeribilita' dei grassi, incrementando l'apporto lipidico. Il crescente apporto lipidico, dato sia da una maggiore assunzione di cibo che dall'alta digeribilita' dei grassi ha contribuito ad aggravare i sintomi di steatosi. Nel complesso questi risultati confermano, ancora una volta, che l'apporto lipidico e' imprincipale fattore influenzante l'accumulo di grasso nei ciechi pilorici.

Nel terzo esperimento, salmoni atlantici in stadio parr sono stati alimentati con quattro diete contenenti un crescente quantitativo di grasso, dal 16% al 28%, ed insufficiente apporto di colina, compreso tra 1820 g/Kg e 2310 g/Kg. Le due diete conteneti il piu' alto e il piu' basso livello di grassi e simile livello di colina (16% vs 25%) sono state analizzate ai fini di mettere in luce gli effetti dei grassi sul metiloma intestinale degli animali. Allo stesso modo, le due diete contenenti simile livello lipidico ma differente livello di colina (1940 g/Kg vs 2310 g/kg) sono state analizzate

ai fini di verificare se la colina avesse indotto metilazione del DNA nei ciechi pilorici. Il pi' alto apporto lipidico ha indotto la metilazione di alcuni geni responsabili del transporto di membrana, pathways di signalling e microRNA. Il diverso livello di coline nella dieta, invece, ha indotto l'ipometilazione di geni coinvolti nella sintesi e transporto degli acidi grassi e nella lipolisi e lipogenesi. In aggiunta, anche alcuni geni regolatori della risposta immunitaria sono stati influenzati dal livello di colina. Nel complesso, questo studio conferma il ruolo della colina nella regolazione dei processi di metilazione del DNA nel salmone atlantico, come dimostrato per altre specie di vertebrati.

Nel quarto esperimento descritto in questa tesi, una nuova linea cellulare ottenuta dall'intestino distale della trota iridea e' stata esposta ad un crescente livello di acido oleico (31.25 e 250μM) per un periodi di 24, 72 e 168h (1 settimana) col fine di produrre un modello in vitro di steatosi e studiare l'accumulo e il metabolismo lipidico. Prima di tutto, i risultati hanno mostrato che l'acido oleico non influenza in modo significativo la vitalita' cellulare. Tuttavia, in parallelo all'aumento dell'acido oleico nel medium di coltura, le cellule hanno accumulato un crescente quantitativo di gocce lipidiche e granuli di stress nel citoplasma. Tale accumulo lipidico ha influenzato la forma e dimensione delle cellule, che dopo 168h alla piu' alta esposizione di acido oleico erano diventate rotonde e con un'area molto elevata. L'accumulo lipidico e' confermato dai risultati della quantificazione degli acidi grassi. I risultati mostrano infatti che la concentrazione di acido oleico al quale le cellule sono state esposte rispecchia il quantitativo di acido oleico ritenuto dalle stesse. In aggiunta, la totalita' degli acidi grassi all'interno delle cellule era maggiore nei fosfolipidi e trigliceridi rispetto agli acidi grassi liberi, dimostrando un tentativo, da parte delle cellule, di produrre lipoproteine. Dal punto di vista molecolare, i nostri risultati hanno dimostrato che le RTdi-Mi possiedono gli stessi geni coinvolti nel metabolismo lipidico delle trote iridee in vivo. In secondo luogo, l'espressione genica conferma non solo una relazione dose-risposta tra *plin2*, importante gene coinvolto nella formazione delle gocce lipidiche, e il livello di acido oleico nel medium; ma anche un aumento dell'espressione di tutti i geni in risposta al crescente tempo in coltura.

Questi risultati dimostrano che linee cellulari di intestino possono essere usate per riprodurre le stesse dinamiche di accumulo lipidico che avvengono in vivo.

Considerando l'importante ruolo della colina nel trasporto lipidico e nei processi di metilazione, la presente tesi offre nuove informazioni da applicare a lavori futuri. Inoltre, l'esperimento condotto in vitro rappresenta il primo passo verso lo studio del metabolismo lipidico dei pesci su piattaforme in vitro.

7 Introduction

In recent decades, the aquaculture industry has attained global relevance. When it comes to farming carnivorous fish species, the attempts to satisfy the market demand have led to high exploitation of marine resources used in feed formulations. Concurrently, the competition for fishmeal and fish oil from other sectors (human consumption, pet food etc.) has drastically increased (Naylor et al., 2000; Naylor et al., 2009), forcing the aquaculture industry to pursue alternative feed ingredients that are both economically viable and environmentally sustainable.

In the specific case of Atlantic salmon, in parallel to a shift in dietary materials, the diets have gradually become more energy dense. In the 1970s the percentage of lipids in salmon diet was ~10% and nowadays the level has increased to over 30% (Bjørgen et al., 2020; Clarke and Bostock, 2017; Aas et al., 2019; Ytrestøyl et al., 2020). The lipid fraction of today's salmon diets is mainly derived from plant materials, such as rapeseed oil (Aas et al., 2019). These simultaneous alterations in the diet have created concerns over the health of farmed fish, since the incidence of several production-related intestinal disorders has increased with the decreasing use of fishmeal in the feed (Bjørgen et al., 2020; Krogdahl et al., 2010). For instance, many plant ingredients contain antinutrients. One such antinutrient is saponin, an anti-nutritional factor found in soybeans and other legumes. Saponin has been shown to induce inflammatory reactions in the distal intestine of several fish species, including salmonids (Gu et al., 2016, 2018; Urán et al., 2008; van den Ingh et al., 1991). Additionally, plant ingredients often contain insufficient or imbalanced nutrient supply according to fish nutrient requirements. Therefore, although plant raw materials represent a feasible alternative to marine ingredients, certain nutrients must be supplemented to the feed in order to develop complete and balanced diets which satisfy fish requirements (Collins et al., 2012; Hartviksen et al., 2014).

One challenge is that Atlantic salmon nutrient requirements are not well defined yet, and the development of a complete diet is far from being achieved (Jobling, 2011). One production-related disorder associated with the lack of knowledge over nutritional requirements in Atlantic salmon is excessive accumulation of dietary lipid in the intestinal tissue, referred to as intestinal steatosis, which results from fish being fed a plant-based diet containing high lipid levels. In severe cases, intestinal steatosis may rather be called lipid malabsorption syndrome (LMS).

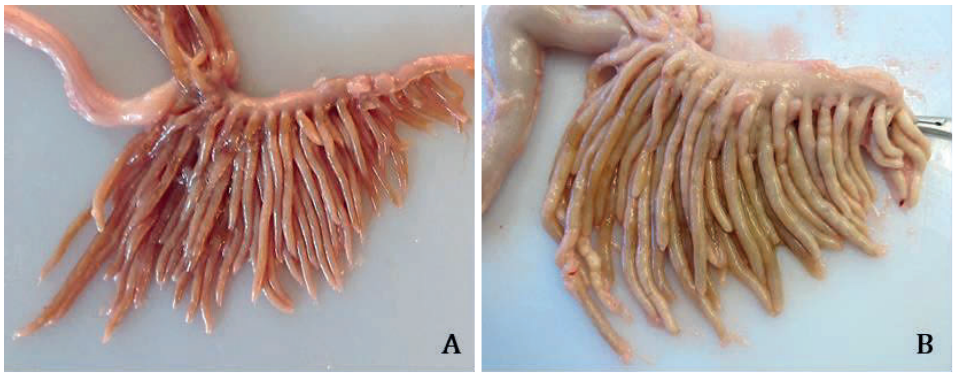

Figure 1. *Macroscopical appearance of Atlantic salmon's pyloric intestine with annexed pyloric caeca. Figure A represents a healthy condition, whereas Figure B shows clear steatosis symptoms.*

The first signs of this condition were reported about a decade ago, when Atlantic salmon farmers observed whitish fish feces floating (i.e. steatorrhea) on the water surface around the cages. Although severe LMS symptoms are no longer observed, the characteristic signs of steatosis can still be detected in commercial salmon farms (Krogdahl et al., 2022).

The pyloric caeca of affected fish exhibit a distinctive whitish and swollen appearance (Figure 1), while histologically, an excessive amount of lipid droplets can be detected in the enterocytes (Figure 2).

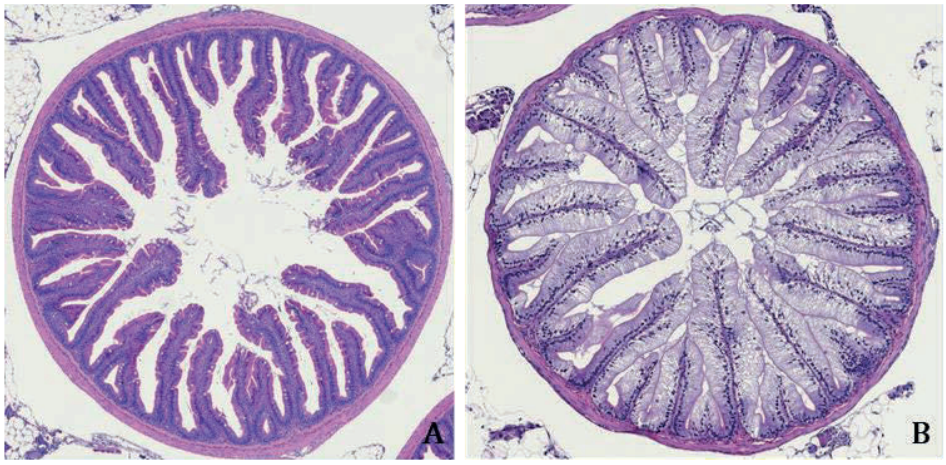

Figure 2. *Histological preparation of two transversal cuts of a healthy (A) and affected (B) pyloric caecum.* Photo: Elvis M. Chikwati

Previous studies have suggested that intestinal steatosis may be the result of deficiency in one or more essential nutrients (Krogdahl et al., 2020). As a confirmation to this hypothesis, Hansen et al. (2020a) demonstrated an inverse relationship between steatosis symptoms and dietary choline level and estimated choline requirement in post-smolt Atlantic salmon.

Choline is an organic water-soluble compound, and, among its many roles, it is part of the hydrophilic head of the phosphatidylcholine molecule. Phosphatidylcholine participates in the formation of lipoproteins, which transport lipids from the enterocytes to the bloodstream.

At the beginning of this thesis, however, questions about whether choline requirement may be influenced by different production-related conditions remained unanswered. Additionally, choline has been demonstrated to play an important role as methyl group donor in DNA methylation processes, which raises even more questions concerning the importance of this compound in nutri-epigenomic studies. The present work addresses these questions, with the aim to investigate which factors may influence choline requirement in Atlantic salmon, but also to dig deeper into the role of choline in the nutri-epigenomic framework. Another important issue is related to limited available information concerning the molecular and cellular mechanisms underlying the interaction between nutrients and intestinal physiology.

Therefore, this thesis also describes a first approach to the use of newly developed intestinal fish cell lines to investigate lipid metabolism.

The following chapters provide background information necessary to understand the subsequent discussion of this study. Firstly, the gross anatomy and histomorphology of the intestinal tract of the Atlantic salmon is described, together with the main functions of the intestine. Respectively, digestive and absorption function, barrier function, immune function and microbiota are discussed. The lipid classes are then explained, and a detailed description of lipid digestion and absorption mechanisms in fish is given. In light of the importance of choline in lipid transport, the introductory section of this thesis offers an overview of choline's molecular and metabolic functions, including its importance in DNA methylation processes and estimation of requirement. Finally, a brief introduction to in vitro approaches to fish intestinal cell cultures and lipid metabolism studies is given.

7.1 The alimentary tract of Atlantic salmon- structure and function

The alimentary tract of animals is a complex system, not only a site of nutrient digestion and absorption, but also the most extensive layer of defense against pathogens and other alien components. Furthermore, the intestine acts as a selective filter between the intestinal lumen and the circulatory system.

7.1.1 Anatomy and histomorphology

In general terms, the alimentary tract of Atlantic salmon can be divided into three sections: pre-gastric, gastric and post-gastric section. The pre-gastric section includes the mouth (where the feed ingestion occurs), the pharynx, and the esophagus (a thick-walled tube which increases in diameter when large amounts of feed are ingested). The gastric portion, also called stomach (S), initiates solubilization, mechanical and enzymatic breakdown of the feed particles producing the so-called chyme. After the first digestion has taken place in the stomach, the obtained chyme enters the post gastric section, or intestine via the pyloric sphincter. The intestine is

divided into three segments: the proximal or pyloric intestine (PI), the mid intestine (MI) and the distal intestine (DI) (Buddington et al., 1997).

The PI is the section where most of the nutrient digestion and absorption occur (Bakke-McKellep et al., 2000; Denstadli et al., 2004). Annexed to the PI there are the pyloric caeca (PC), which are finger-like protrusions surrounded by adipose tissue.

The morphology of the MI is very similar to the PI, except for the absence of the PC, and the DI is characterized by a wider diameter and a darker pigmentation due to high vascularization (Figure 3).

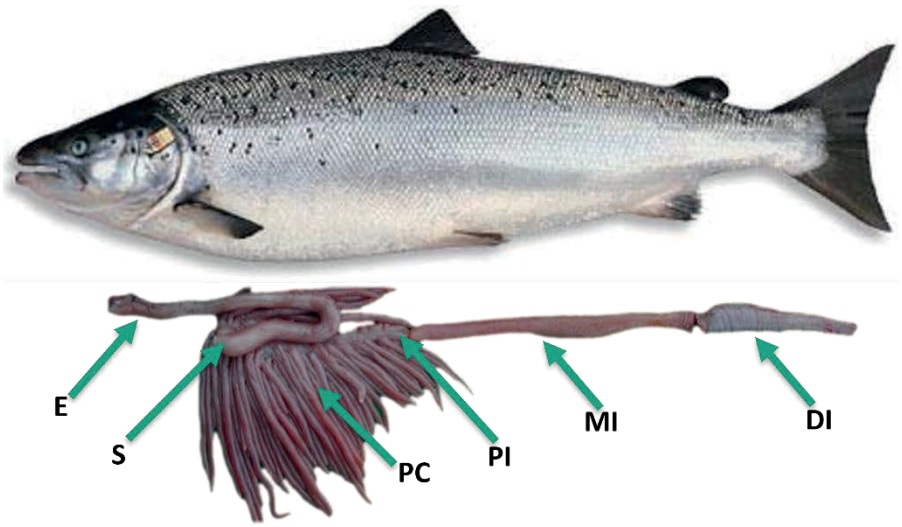

Figure 3. *The alimentary tract of Atlantic salmon. Abbreviations: E, esophagus; S, stomach; PC, pyloric caeca; PI, pyloric intestine; MI, mid intestine; DI, distal intestine. Photo: Åshild Krogdahl modified by Daphne Siciliani*

The PI and the annexed PC are characterized by the presence of irregular mucosal folds arranged longitudinally with respect to the length of the intestine. These folds are wider in the PI and smaller in the pyloric caeca. The folds of the MI, on the other hand, do not show clear orientation. Concerning the DI, it presents complex circular folds transversally oriented with respect to the intestinal tract, and simple folds in between the complex ones. In addition, the complex folds exhibit secondary longitudinal folds (Løkka et al., 2013). Part of the nutrients and other molecules not fully digested and absorbed in the PI and MI may be further metabolized and absorbed in the DI. The DI also seems to have important immunological functions

(Bjørgen et al., 2020; Løkka et al., 2013). The anus, located at the most distal part of the digestive tract, regulates the excretion of undigested food, endogenous digestive components, and metabolic waste products, including those released from the liver into the intestine.

From a histomorphological perspective, the intestinal tract is composed of four main layers, mentioned from the outermost layer, respectively: tunica serosa, tunica muscularis, tunica submucosa and tunica mucosa (Figure 4). The tunica serosa consists of mesothelial cells and connective tissue. The tunica muscularis underneath comprises a circular and a longitudinal layer of muscular tissue, which ensures the peristaltic movements and allows the passage of the chyme all along the intestinal tube. The tunica submucosa has an extra layer of connective tissue known as stratum compactum which supports the further internal layers. Finally, the innermost layer, the tunica mucosa, is composed of a single layer of epithelial cells and the lamina propria.

Along the whole intestine, the epithelium presents several types of cells including enterocytes, goblet cells, rodlet cells, intraepithelial lymphocytes and enteroendocrine cells. The enterocytes, columnar cells with elongated nuclei, are the most abundant, characterized by finger-like extensions on the apical side called microvilli, which constitute the brush border membrane. Depending on the area of the intestine in which they are located, enterocytes may differ slightly: for instance, those in DI present large supranuclear vacuoles, while those from PI and MI do not.

All along the intestinal tract, the mucosa is covered by a mucus layer produced by the goblet cells located in the epithelium. The mucus may present acidic, basic or mixed pH depending on the area of the intestine where the goblet cells are located. The function of the mucus is twofold: providing a first physical and chemical barrier against external threats, and supporting nutrient digestion, absorption and intestinal homeostasis by containing antimicrobial substances, such as lysozymes, lectins, antimicrobial peptides and immunoglobulins.

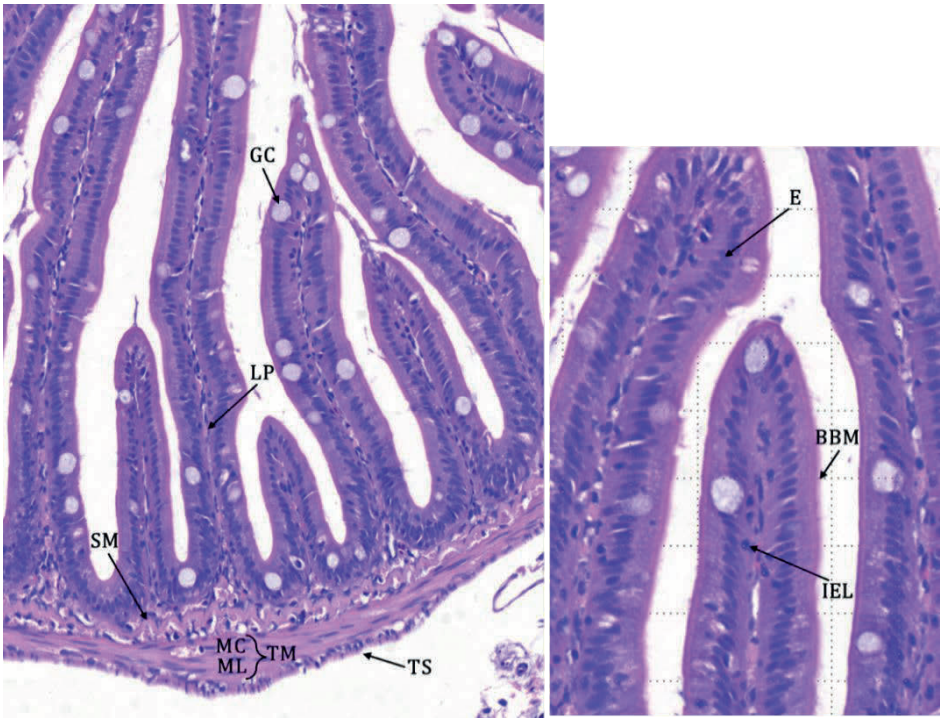

Figure 4. *Normal histomorphological representation of the pyloric intestine of Atlantic salmon stained with haematoxylin and eosin. The images are representative of a longitudinal cut. Abbreviations: LP, lamina propria; SM, tunica submucosa; TM, tunica muscularis; MC, muscularis circularis; ML, muscularis longitudinalis; TS, tunica serosa; E, enterocyte; BBM, brush border membrane; GC, goblet cell; IEL, intraepithelial lymphocyte. Photo: Elvis Chikwati*

Rodlet cells are a group of cells widely distributed throughout the fish body, including in the intestinal epithelium, which participate in osmoregulation and ion transportation. Additionally, according to several studies, rodlet cells may also play a

key role in the fish immune apparatus (Reite, 2005; Silphaduang et al., 2006). Defensive mechanisms of the intestine are also represented by the intraepithelial lymphocytes, which increase in number if a threat is detected.

The center of the mucosal fold is the lamina propria, which is in contact with both the epithelium and the submucosa. The lamina propria comprises vascularized connective tissue, nerves and more lymphocytes (Løkka et al., 2013) (Figure 4).

7.1.2 Digestion and absorption of macronutrients

The main role of the intestine is to digest food and absorb nutrients.

The first part of mechanical and enzymatic digestion starts inside the stomach, where the oxynticopeptic cells of the gastric glands secrete hydrochloric acid. The hydrochloric acid maintains an acidic environment, denatures the ingested proteins and activates pepsinogen into the powerful peptidase, pepsin.

When the chyme, the product of the initial digestion, enters the intestine, it activates a cascade of signals that stimulate secretion from the intestine and annexed accessory organs, i.e., mucosa, pancreas and gall bladder. The pH of chyme in the PI is higher than that of the stomach, mainly because of the presence of the bicarbonate provided by bile and pancreatic secretions (Marine et al., 2013). This alkaline environment is fundamental for the digestive enzymes and bile salts to further digest the chyme. The enzymes secreted by the pancreas are proteases (e.g. trypsin, chymotrypsin and elastase), lipolytic enzymes (e.g., lipase and phospholipase), α-amylase, DNase, and RNase. At the same time, the liver produces bile salts, which are stored in the gall bladder. Bile salts emulsify the lipids and fat-soluble components present in the chyme to facilitate the action of the lipases. The concentration of the bile salts is particularly high in the PI and it decreases gradually along the intestinal tract (Chikwati et al., 2013; Kortner et al., 2016).

After the enzymatic digestion has taken place, the obtained intermediate products are broken down and absorbed into the brush border membrane and the enterocytes. The brush border contains enzymes such as alkaline phosphatase, leucine-aminopeptidase, disaccharidases, and monoglyceride lipases, participating in the final digestion of nutrients.

Nutrient absorption across the brush border membrane and into the enterocytes occurs by different mechanisms, such as pinocytosis, simple diffusion, ion exchange

or active transport via transport protein complexes (Bakke et al., 2010). As previously described, nutrient absorption in Atlantic salmon happens primarily in the PI and annexed pyloric caeca. However, since the brush border membrane extends along the whole intestine, nutrient absorption partially occurs in the MI and DI (Bakke-McKellep et al., 2000).

The knowledge on nutrient-specific absorption in fish is limited. Based on current knowledge, most proteins are absorbed as di- or tripeptides at the brush border membrane of the first portion of the PI. This absorption occurs via two transport peptides: low-affinity/high-capacity H+ - dependent (PetT1) and high-affinity/low-capacity (PetT2) (Bakke et al., 2010). There is also evidence of various intestinal amino acid transporters in fish (Chen et al., 2022). Larger proteins that are not absorbed in the PI may enter the enterocytes of the DI via pinocytosis. In the enterocytes, peptides are further hydrolyzed into free amino acids before entering the circulatory system across the basolateral membrane.

Concerning glucose absorption, studies conducted on salmon have demonstrated the presence of the apical-located Na+/glucose symporter (SGLT1) (Bakke-McKellep et al., 2008; Sahlmann et al., 2015), integral membrane proteins which transport D-glucose and D-galactose into the enterocytes (Bakke-McKellep et al., 2000).

Lipid absorption and transport are not well understood in fish, although they are presumably similar to what happens in mammals. A detailed description of these processes will be provided in the sections below.

7.1.3 Barrier function

The barrier function of the intestinal tract is executed first by the mucus layer covering the intestinal mucosa. This layer acts as physical and chemical barrier against pathogens, allergens, commensal and opportunistic bacteria, toxins, and other adventitious components, and maintains intestinal homeostasis. In fish, the commensal bacteria produce bactericidal compounds such as peptide bacteriocins, hydrogen peroxide and lactic acid, representing an important source of defense against pathogens (Jutfelt & Sundh, 2023; Sundh & Sundell, 2015). The mucus, produced by goblet cells, contains protective components, such as mucins, lysozymes, lectins, antimicrobial peptides and immunoglobulins. The mucins have the main role to bind harmful agents and physically remove them from the epithelium by exploiting

the flow of mucus from the epithelium to the lumen (Jutfelt & Sundh, 2023). In rainbow trout (*Oncorhynchus mykiss*), it has been shown that mucus secretion may be stimulated by the presence of lipopolysaccharides, components of the outer membrane of bacteria (Sharba et al., 2022).

The cells of the intestinal mucosa constitute a further barrier, as these are connected together at their apical ends by tight junction proteins (Reuss, 2011; Sundh & Sundell, 2015), which in Atlantic salmon includes the claudin family, the occludin family and the zonula occludens family. The claudins, based on their isoforms are expressed in kidney and intestine (Kølbæk et al., 2002). The occludin family regulates transportation of water and macromolecules in Atlantic salmon's gill and intestine (Kølbæk et al., 2002). The zonula occludens are involved in paracellular signaling pathways (Otani & Furuse, 2020) (Figure 5). In addition, the brush border membrane presents specific kinds of intrinsic proteins, which control the intestinal osmoregulation by regulating water permeability. These are the aquaporins, main water channel proteins of the intestine. In Atlantic salmon *aqp8ab* has been identified as the most important intestinal gene regulating water uptake during the transfer of the fish from freshwater to seawater (Laverty & Skadhauge, 2012).

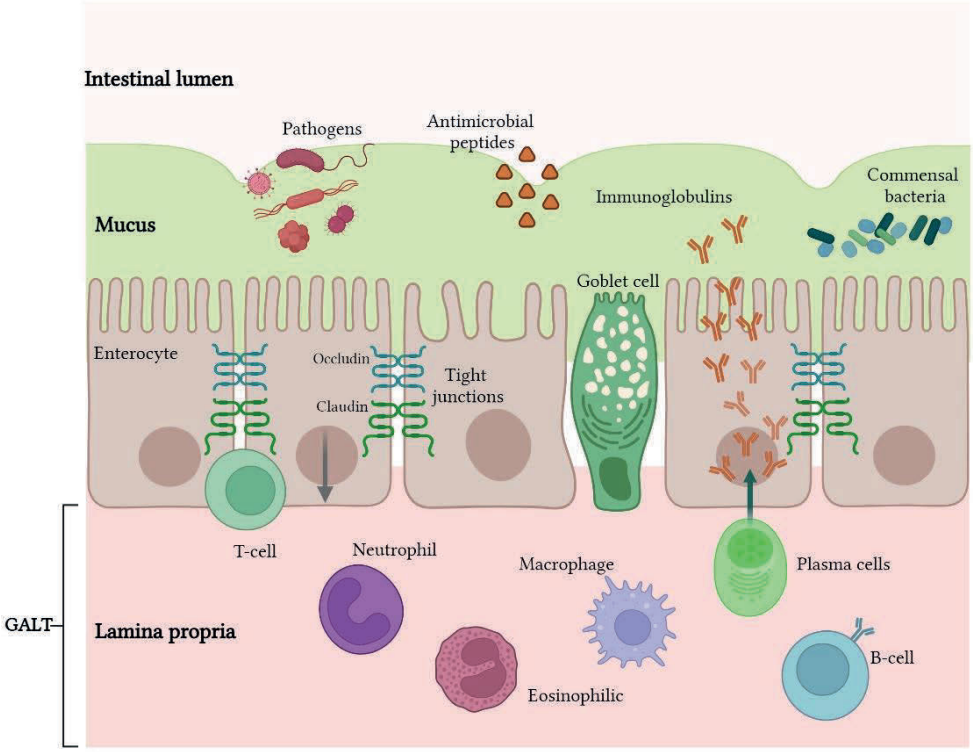

Figure 5. *Graphic representation of the intestinal barrier, composed of a layer of columnar epithelial cells connected by the tight junctions. In the epithelium the goblet cells produce the mucus. In the lamina propria the immunological barrier is represented by the T-cells, neutrophils, macrophages, eosinophilic granular cell, plasma cells and B-cells. GALT=gut associated lymphoid tissue. Modified with BioRender from Jutfelt and Sundh 2023.*

7.1.4 Immune function

Other than being a physical and chemical barrier, the intestinal mucosa plays an important role in the immune response, protecting the organism from those threats which have bypassed the first layers of defense. The fish intestinal immune repertoire is governed by the gut associated lymphoid tissue (GALT). GALT is less organized in teleost fishes than in mammals, lacking compartments such as the Peyer's patches, the isolated lymphoid follicles and the mesenteric lymph nodes. Therefore, protective and antimicrobial molecules, together with leukocytes (macrophages, granulocytes, lymphocytes and plasma cell) and intraepithelial lymphocytes (B and T cells) are

distributed among the lamina propria, submucosa and epithelium (Gomez et al., 2013; Lazado & Caipang, 2014; Løkka & Koppang, 2016).

As in other animals, the teleost immune system is subdivided into innate and adaptative. The innate system is composed of humoral and cellular defenses. The humoral defense is represented by many, highly diverse macromolecules present in extracellular fluids such as complement proteins and antimicrobial peptides (Silphaduang et al., 2006), while the cellular defense is carried out by mast cells, macrophages and granulocytes (Gomez et al., 2013).

The GALT and the intestinal epithelial cells in fish possess the so-called pattern recognition receptors (PRRs) which identify the presence of pathogens via pathogen-associated molecular patterns (PAMPs), small molecules present on the cell walls of certain microbes. When the PRRs identify and bind the PAMPs, a cascade of intracellular signaling is induced, leading to the release of cytokines and the consequent immune response (Boltaña et al., 2011). Four different types of PRRs have been identified in teleost fishes i.e., toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and peptidoglycan recognition proteins (PGRPs) (Boltaña et al., 2011).

Concerning the adaptative immune system, actions are mediated by two types of lymphocytes: B and T. In the intestine of teleost fish, the B cells are involved in the production of the immunoglobulins (Igs), which represent the humoral defenses of the adaptative immune system. In Atlantic salmon intestine, three kinds of Igs have been identified i.e., IgM, IgT and IgD (Bjørgen et al., 2023; Jutfelt and Sundh, 2023). The role of immunoglobulins is to identify and bind the antigens, molecules such as proteins, peptides (amino acid chains), polysaccharides (chains of simple sugars), lipids, or nucleic acids present in the pathogens (Grayfer et al., 2018). T-cell functions are not as well characterized in teleost fish. However, several studies conducted on Atlantic salmon have demonstrated that these cells are particularly abundant in the intestinal tissue and that their action is regulated by a set of genes such as cluster of differentiation 3γδ (*cd3γδ*), 4α (*cd34α*) and 8β (*cd8β*) (Bakke-McKellep et al., 2007; Marjara et al., 2012). Concerning the role of IgDs in mammals, they promote protective humoral responses in the presence of food allergens. A recent study

suggests that the same function may be also expressed in teleost fish as well (Bjørgen et al., 2023).

7.1.5 Microbiota

An additional element playing a pivotal role in the gut's immune function and health is the microbiota, which comprises a huge variety of microorganisms such as bacteria, fungi and archaea. These microorganisms are located both in the mucus and in the digesta, representing respectively the autochthonous and the allochthonous microbiota (Egerton et al., 2018). Several studies conducted on germ-free animal models have demonstrated that microbiota is involved in a broad range of functions, such as development of the intestinal structure of the host (Bates et al., 2006; Rawls et al., 2004), food digestion and absorption (Ray et al., 2012), energy production, lipid metabolism (Falcinelli et al., 2015; Semova et al., 2012) and prevention of the growth of pathogenic microorganisms (Jutfelt & Sundh, 2023; Sundh & Sundell, 2015). Most importantly, the microbiota is strictly linked to intestinal function and health (Mazmanian et al., 2008). For instance, intestinal microbiota can interact with the cell population of the immune system regulating their differentiation and maintaining homeostasis (Ivanov et al., 2009; Nies et al., 2005). In light of these findings, the intestinal microbiota is nowadays used as a therapeutic target for human intestinal diseases (Hryckowian et al., 2018; Narula et al., 2017).

Concerning the role of the microbiota in the aquaculture feed industry applications, supplementation of prebiotics, probiotics or microbial-derived products to fish feed has demonstrated several modulatory effects on intestinal disorders, such as soybean-meal induced enteritis (Kiron, 2012; Merrifield et al., 2010; Romarheim et al., 2011, 2013). For instance, feed supplementation of two lactic acid bacteria (*Lactococcus lactis* and *Carnobacterium maltaromaticum*) was reported to diminish the severity of enteritis induced by a soybean-meal inclusion of 38% (Navarrete et al., 2013).

Concerning the role of the microbiota on lipid metabolism in fish, a study conducted by Semova et al. (2012) on zebrafish (*Danio rerio*) showed that microbial colonization improved epithelial absorption of FAs, leading to an increased number of lipoproteins in the enterocytes and accumulation of FAs in the extraintestinal tissue.

Although the mechanisms of action of microbial products are still not well established, the latest sequencing technologies have allowed a deeper understanding of the organization of intestinal microbiota in Atlantic salmon. During the first developmental stages, the intestinal microbiota of Atlantic salmon comprises mainly Phyla Proteobacteria, Bacteroidetes, Firmicutes and Tenericutes. With the development and the passage from freshwater to seawater the abundance of Bacteroidetes and Firmicutes decreases, while Tenericutes increases (Llewellyn et al., 2016; Lokesh et al., 2019; Uren Webster et al., 2018).

The microbial diversity in Atlantic salmon is generally low at later life stages and it includes genera *Aliivibrio* (Proteobacteria), *Photobacterium* (Proteobacteria), *Mycoplasma* (Tenericutes), and *Brevinema* (Spirochetes) (Gupta et al., 2019; Llewellyn et al., 2016; Lokesh et al., 2019; Gajardo et al., 2016). However, it is challenging to find a specific reason for this variation, since the factors influencing the variety of the intestinal microbiota are numerous, such as the environmental conditions, fish behavior and diet. Diet formulation is one of those factors which influences gut microbiota the most, both in the long and short term. For instance, dietary supplementation with plant-based ingredients such as soybean has been demonstrated to increase the amount of lactic acid bacteria in salmon's intestine (Gajardo et al., 2017; Reveco et al., 2014; Schmidt et al., 2016). On the other hand, the use of insect (*Hermetia illucens)* meal can increase the amount of *Actinomyces*, *Bacillus*, *Brevibacterium*, *Corynebacterium* and *Enterococcus* both in salmon and in rainbow trout (Li et al., 2020).

7.2 Lipid classification

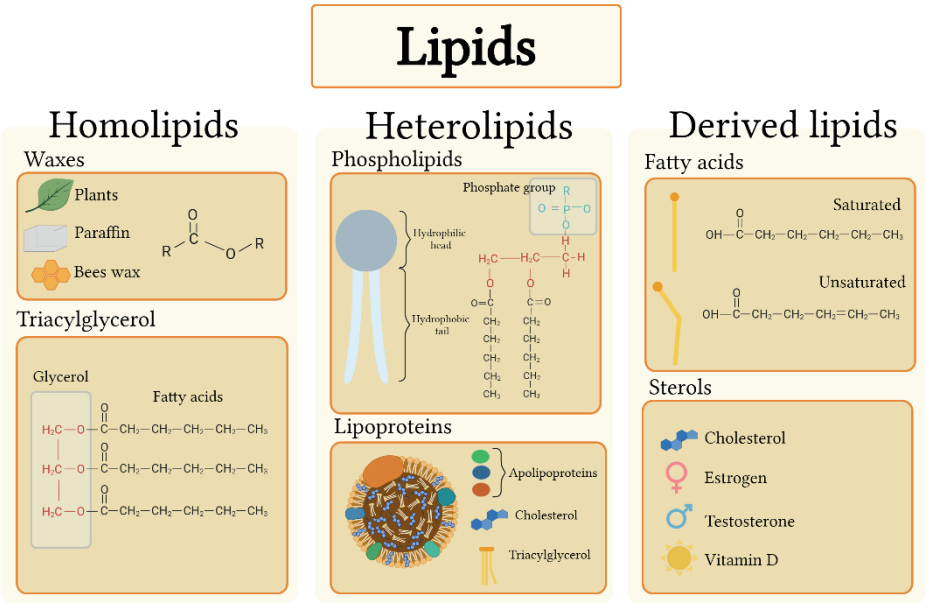

Figure 6. *Summary diagram showing the main above-described lipid classes with their structure and source. Modified on BioRender from www.vectormine.it.*

Lipids are essential components present in every microorganism, plant and animal as part of cell structure, to provide and store metabolic fuel, and to participate in many biological processes (Sargent et al., 2003). At present, lipids are the main source of digestible energy in farmed Atlantic salmon feed. Lipids are generally classified based on their chemical composition as simple lipids (or homolipids), compound lipids (or heterolipids) and derived lipids (Figure 6). Simple lipids are sub-divided in waxes and triacylglycerols (TAG), which provide FAs for energy production (Sargent et al., 2003). Compound lipids are esters of FAs conjugated with compounds other than glycerol. Depending on the compound involved they are sub-classified as glycosphingolipids, phospholipids (PL), amino lipids, sulfolipids and more complex aggregates such as lipoproteins (Tocher, 2003). Derived lipids, obtained by hydrolysis of simple and compound lipids, include FA, alcohols, monoglycerides, diglycerides (DAG), carotenoids and sterols. Among sterols, the most abundant in

animal tissues is cholesterol, which has an important structural function in cell membranes and lipid transport vehicles, and is the substrate for the synthesis of steroid hormones and bile acids (Baez, 2013; Gurr et al., 2002; Vance & Vance, 2008).

Concerning their nomenclature, FAs can be identified according to the IUPAC (International Union of Pure and Applied Chemistry, http:// www.iupac.org/) nomenclature or by their trivial names. However, at present, the most-used is the shorthand nomenclature of FAs, which foresees the naming of FAs by the abbreviation "FA" followed by the number of carbon atoms and double bonds, separated by a colon (e.g., FA 18:0 to represent stearic acid or octadecanoic acid, which has an 18-carbon chain with zero double bonds). In addition, the shorthand nomenclature describes the positioning of the first double bond from the omega end (methyl end) of the fatty acid chain (e.g., FA 18:1n-9). In a variation of the shorthand nomenclature, the word FA is substituted by a C (e.g., C 18:1n-9). In the present work this latest version of the shorthand nomenclature will be used to identify the encountered FAs classes (Eggers & Schwudke, 2020; Köfeler, 2016).

7.3 Digestion and absorption of dietary fat

In general terms, lipid digestion, absorption and transport in fishes are considered similar to those in mammals (Sargent et al., 1999), with the main dietary lipid sources being characterized by high levels of unsaturated FAs (Tocher, 2003).
In most terrestrial animals, dietary lipid digestion starts in the mouth with the lingual lipases, and it proceeds through the esophagus and stomach with the esophageal and gastric lipases. Although lipases have not been thoroughly studied in fishes, lingual, esophageal, and gastric lipases have been reported by several authors. For instance, the primitive Pacific hagfish (*Eptatretus stoutii*) showed the activity of high neutral lipases in its buccal cavity and pharyngocutaneous duct (Weinrauch et al., 2019). In the freshwater milkfish, instead, the activity of neutral lipase without bile salts has been reported in the esophagus and stomach.
Concerning marine fish, limited pre-gastric lipase activity has been observed in juvenile turbot (*Scophthalmus maximus*) (Koven et al., 1997) and winter flounder (*Pseudopleuronectes americanus*) (Murray et al., 2003).

After leaving the stomach and entering the pyloric intestine, lipids are emulsified by bile. Bile, stored in the gallbladder, contains water, cholesterol, phospholipids, electrolytes and bile salts. Bile salts are produced from bile acids by the hepatocytes in the liver. In salmon, the majority of bile acids are taurine-conjugated, with taurocholic acid being the predominant individual bile salt (Kortner et al., 2014). Bile also contains waste products such as bilirubin and biliverdin, which provide the characteristic yellow-green color (Løkka et al., 2022).

The entrance of the chyme in the proximal intestine stimulates bile secretion. The result of bile action is an emulsion, which aggregates with bile salts and phospholipids to create the primary micelles. Whereas no information is available about primary micelles size in fish, in mammals they have a diameter of around 1μm. The FAs contained in the primary micelles are then hydrolysed by the pancreatic lipases to form mixed micelles, multimolecular aggregates typically 4±6nm in diameter (Gurr et al., 2002). Pancreatic lipases are produced by the acinar cells of the exocrine pancreas and carry out their activity mainly in the proximal intestine and annexed pyloric caeca (Grosell et al., 2010).

In fish, three types of pancreatic lipases have been identified: pancreatic triacylglycerol lipase (PTL), bile-activated triacylglycerol lipase (BAL) and phospholipase A2 (PLA2s). PTL acts on TAG and DAG, and it is activated by the water/oil interface. The presence of PTL in fish has not been confirmed in many species, as Olsen and Ringø (1997) discuss in their review. However, PTL or its colipase's existence has been indicated in primitive species such as hagfish (*Myxine glutinosa*) and cartilaginous fishes like ratfish (*Chimera monstrosa*), skates (*Raja erinacea*) and sharks (*Somniosus microcephalus*) (Brockerhoff & Hoyle, 1965; Patton & Quinn, 1973; Sternby et al., 1983).

BAL is activated by bile salts and can hydrolyze a broad range of lipids, including wax esters and FAs produced by intestinal bacteria (Khan et al., 2017; Romano et al., 2020). BAL is probably the most abundant lipase in all marine fish species, showing amino acid sequences similar to those of mammals in winter flounder (*Pseudopleuronectes americanus)* (Murray et al., 2003), chinook salmon

(*Oncorhynchus tshawytscha*), New Zealand hoki (*Macruronus novaezelandiae*) (Kurtovic et al., 2010) and red drum (*Sciaenops ocellatus*) (Lazo et al., 2007).

Phospholipase A2's role has not yet been completely investigated in fish, however, this enzyme may be involved in phospholipids metabolism (Cahu & Zambonino Infante, 2001).

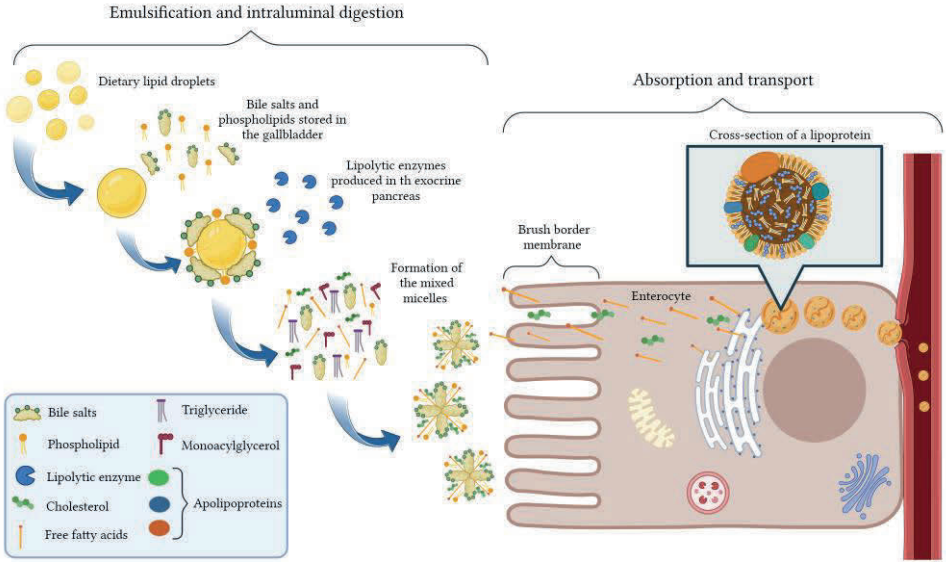

Figure 7. *Graphic representation of lipid emulsification, intraluminal digestion, and absorption in the enterocyte. Modified on BioRender by Daphne Siciliani from Penn et al. (2011).*

To be transported from the intestinal lumen into the enterocytes, the digestion products encounter two barriers. The first barrier is the mucus layer which covers the epithelial cell layer and coats the surface of the microvilli. The acidic nature of this layer and the membrane's highly convoluted structure promotes a fast and efficient translocation of the lipids. The second barrier is the brush border membrane. When the mixed micelles approach the mucus layer on the brush border membrane, its acidic nature dissolves them, and the released water-insoluble amphipathic FAs can enter the enterocytes by simple passive diffusion or via transporter-mediated mechanisms.

Most of the proteins involved in FA transport within the enterocytes are conserved across species. The main intestinal fatty acid transport proteins are scavenger receptors class B, SR-B1 and SR-B2 (also called cluster of differentiation CD36) and fatty acid transport proteins (FATP1-6). SR-B2 is involved in a broad range of processes, including immune response, and is able to bind several different ligands, such as FAs, lipoproteins and collagen (Cifarelli & Abumrad, 2018). On the other hand, SR-B1 shares most of the functions of SR-B2, but it appears to be specific for cholesterol transport (Cifarelli & Abumrad, 2018). SR-B1 is highly expressed in Atlantic salmon intestines (Kleveland et al., 2006; Sundvold et al., 2011).
After SR-B1 and SR-B2 promoted the transport of FAs through the lipid bilayer, FAs are bound to fatty acid binding proteins (FABPs), which participate in the mobilization of the FAs within the cytoplasm (Besnard et al., 2002; Doege & Stah, 2006). FABPs have been observed in several fish species, with species-specific locations and different functions in cells (Hardy & Kaushik, 2022). FABP2 is the main intracellular FABP in the Atlantic salmon intestinal epithelium (Venold et al., 2013).

7.4 Fatty acid synthesis

Both the intestine and liver play a key role in lipid metabolism, being more or less involved in FA synthesis and circulation, depending on the species (Nguyen et al., 2008).

Once long-chain FAs are absorbed by the enterocytes, they will, unless metabolized, be re-esterified into TAGs and PLs, among which phosphatidylcholine is the principal. Short- and medium-chain FAs do not need to be esterified to be exported, diffusing directly from the enterocytes to the portal blood.
TAG and phosphatidylcholine may be re-synthesized via two different pathways: the MAG acyltransferase (MGAT) and the glycerol-3-phosphate acyltransferase (GPAT). In mammals MGAT pathway uses 2-MAG to produce either TAG or phosphatidylcholine. If MAG is not available, then TAG may be re-synthesized from glycerol-3-phosphate. Most fish species appear to have all the enzymes involved in both pathways, however, the contribution of each pathway, and the species-specific

differences are still unclear (Hardy & Kaushik, 2022). For instance, Bogevik et al. (2008) suggested that GPAT may be the most efficient pathway in fish, while Oxley et al. (2005a) observed that in Atlantic salmon, TAG and PC synthesis proceeds faster through the MGAT than the GPAT pathway. Concerning the biosynthesis of TAG and phosphatidylcholine, several studies have been conducted to investigate the efficiency of FA esterification in different fish tissues. For instance, Atlantic salmon hepatocytes exposed to 20:5n-3 showed higher lipid accumulation than when exposed to oleic acid and 22:6n-3 (Vegusdal et al., 2005), which were converted respectively into TAG and phosphatidylcholine. Similarly, a study conducted on grass carp (*Ctenopharyngodon idella*) hepatocytes demonstrated higher TAG synthesis from 18:3n-3 than from 18:2n-6. Conversely, Nile tilapia (*Oreochromis niloticus*) hepatocytes efficiently esterified both 18:3n-3 and 18:2n-6. Such results suggest that the efficiency of esterification of specific FA is species-specific (Jiao et al., 2020). Moreover, farmed fish are exposed to mixtures of FAs, and their fate after being absorbed is still unclear and should be addressed in future.

Most of the FAs present in fish lipid are obtained from the diet. However, saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) can also be synthetized from carbohydrates or proteins (Liu et al., 2020). The pathways for FA production from nonlipid precursors are generally similar in mammals and fish (Henderson, 1996). Firstly, carbohydrates and proteins get catabolized in the mitochondria to produce citrate. Citrate is then transported to the cytosol to be converted to malonyl-CoA, which will be used by the enzyme fatty acid synthase (FAS) to produce palmitic acid, stearic acid, palmitoleic acid and oleic acid. The production of FAS is regulated by the gene fatty acid synthase (*fas*), which has shown the highest expression in the liver, and whose nucleotide sequence is conserved in several species (Leng et al., 2012; Tang et al., 2013; Wang et al., 2010). Another fundamental gene involved in FA synthesis is stearoyl-CoA desaturase (*scd*). Two isoforms of *scd* have been identified in fish, both being strongly expressed in the liver of grass carp (Chang et al., 2001; Evans et al., 2008; Polley et al., 2003) and large yellow croaker (*Larimichthys crocea*) (Xu et al., 2015).

Steroids, including cholesterol, bile acids and steroid hormones may be synthetized by almost every cell, however the main synthetic site is the liver. The pathway

foresees a series of reactions where acetyl CoA is converted to lanosterol by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), and then to cholesterol via the Bloch or Kandutsch pathway (Ikonen, 2008; Lee et al., 2013).
Also, sphingolipids and wax esters are mainly synthetized in the liver, following pathways which are very similar between fish and mammals (Cheng & Russell, 2004; Park et al., 2019).

7.5 Fatty acid transport

After dietary TAGs have been resynthesized within the enterocytes, they combine with phosphatidylcholine, creating structures with a membrane of phosphatidylcholine and a TAG core. Such structures can then be stored intracellularly as lipid droplets, or they can be incorporated into lipoproteins for further transport to other body compartments.
Lipid droplets (LD) are intracellular lipid stores which will be incorporated into lipoproteins in case of fasting. Lipoproteins can be classified depending on their origins, destination, composition and physical properties. The primary criterion used to classify lipoproteins is their density, which relies on the inner ratio between lipid and protein components. Based on this classification, high density lipoproteins (HDL) are the densest, followed by low-density lipoproteins (LDL), very low-density lipoproteins (VLDL), and chylomicrons. Blood lipoprotein composition in fish can be affected by many factors, and it is highly species-specific. For instance, in Atlantic salmon both HDL and LDL increased when the animals are fed highly lipidic diets (Torstensen et al., 2001). Additionally, it has been shown that replacing fish oil with plant-based oil may reduce HDL, VLDL and LDL (Jordal et al., 2007).
In mammals, chylomicrons are the main intestinal lipoproteins and are produced from apolipoprotein B48 assembled to cholesterol, phospholipids and TAGs into the endoplasmic reticulum. However, since fish cannot synthetize apolipoprotein B48 (Hardy & Kaushik, 2022), the term chylomicron may not be the most accurate for these lipoproteins.
Additionally, while in mammals chylomicrons convey lipids to the peripheral circulation and tissues via the lymphatic system, it has long been a debate if lymphatic vessels in fish exist or not (Vogel, 2010). The work of Denstadli et al. (2011) suggests

that the portal vein is an important transport route for lipids in Atlantic salmon, but that also other routes are possible. The work of Hellberg et al. (2013) on common wolffish (*Anarhichas lupus*), shows structures in the intestinal tissue which suggest presence of a lymphatic system, but their importance for lipid transport has not been described. In light of the fact that some fish species store fat in the liver, while others in more peripheral tissues, lipid transport routes may well differ between fish species.

Concerning lipoprotein formation in fishes, it is likely that this process is similar to that of mammals, as supported by emerging studies conducted on zebrafish (Morishita et al., 2019; Sæle et al., 2018).

The core of the intestinal lipoproteins is composed of TAG and esterified cholesterol.

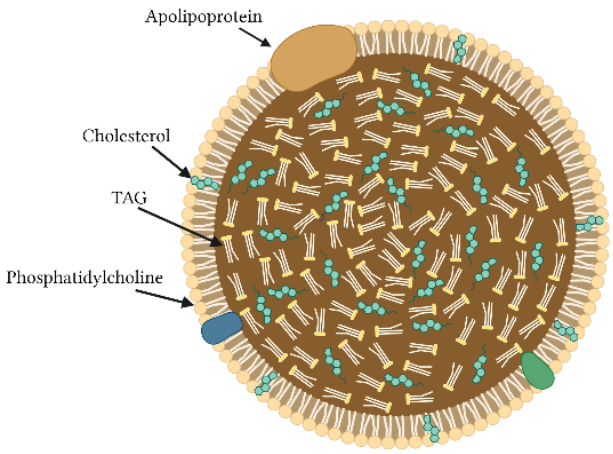

Figure 8. *Intestinal lipoprotein created with BioRender.*

The surface consists of apolipoproteins, phosphatidylcholine and free cholesterol (Figure 8) (Hardy & Kaushik, 2022). Apolipoproteins have the fundamental role to stabilize lipoproteins in the cytosol and in the bloodstream, provide affinity to cell-membrane receptors and regulate the activity of several enzymes involved in lipoprotein formation (Gurr et al., 2002).

The lipoproteins synthetized in the intestine are taken up by the liver, where they are dismantled and assembled into VLDL (van der Veen et al., 2017) to allow further lipid metabolism and transport to the peripheral tissues. What remains of VLDL after TAG and cholesterol are released is LDL. LDL can be re-taken up by the liver. The surplus of cholesterol in LDL and tissues is absorbed by HDL and transported back to liver for reuse (Lie et al., 1993).

7.6 Choline

Choline is a water-soluble, saturated quaternary amine, present in all cells and tissues (Gibellini & Smith, 2010). A large part of circulating choline is integrated in the structure of phospholipids, particularly in phosphatidylcholine molecules. As previously stated, phosphatidylcholine, and consequently choline, is a major component of lipoproteins and therefore essential in lipid transport (Gibellini & Smith, 2010; Z. Li & Vance, 2008). Besides being part of phosphatidylcholine, choline has a broad range of important roles, being fundamental in the synthesis of acetylcholine (a neurotransmitter found in the nervous system), providing support to cell membranes and ensuring cellular functions, and contributing to the water balance in the kidneys through its metabolite betaine (Sarter & Parikh, 2005). Additionally, choline has been discovered to have an important role as indirect methyl group donor during DNA and histone methylation processes (Ueland, 2011; Zeisel, 2017).

Choline, although the pathways for its de novo synthesis are present in most animals, has been established as essential for some mammals, birds and fish, which require dietary supply, at least at certain developmental stages and conditions, to secure normal functions of tissues and organs (Gibellini & Smith, 2010). Choline is widely distributed in several foods and feeds, although its content varies among products. For instance, fishmeal contains about 3000 mg/kg of choline whereas plant raw materials contain much less. Soy protein concentrate, as an example, contains about 600 mg/kg of choline (Aquilina et al., 2011). Choline may be supplemented in the diet as choline chloride, available from a broad range of feed suppliers and largely used in agriculture and aquaculture (Aquilina et al., 2011). Insufficient dietary choline supply may lead to several metabolic dysfunctions. In fish studies, choline-deficiency symptoms comprise high lipid concentration in the liver, low growth performances, early death and poor feed efficiency, and have been reported in several fish species such as carp (*Cyprinus carpio*) (Wu et al., 2011), lake trout (*Salvelinus namaycush*)(Ketola, 1976), rainbow trout (*Oncorhynchus mykiss*)(Rumsey, 1991), Yellow perch (*Perca flavescens*)(Twibell & Brown, 2000), channel catfish (*Ictalurus*

punctatus)(Zhang & Wilson, 1999), cobia (*Rachycentron canadum*)(Benetti et al., 2021) and yellowtail kingfish (*Seriola lalandi*) (Liu et al., 2019). Recent studies in Atlantic salmon have demonstrated clear choline deficiency symptoms related to intestinal lipid metabolism, such as vacuolation and excessive accumulation of dietary lipid (steatosis) in the pyloric caeca (Hansen et al. 2020a; Hansen et al. 2020b).

7.7 Overview of choline metabolism

Choline metabolism, as described in mammals, mainly follows three pathways, i.e. in the synthesis of the neurotransmitter acetylcholine, betaine and phosphatidylcholine. Acetylcholine is synthetized by the enzyme choline acyltransferase from acetyl coenzyme A and choline, derived from endogenous or dietary sources (Sarter & Parikh, 2005). The synthesis takes place in the cytosol of pre-synaptic cholinergic neurons, in the neural tissue, although several studies have reported that it may also take place in muscles, intestine, and lymphocytes. After the acetylcholine synthesis, a specific low-affinity acetylcholine transporter packs the neurotransmitter into vesicles and releases it at the synaptic cleft, where it binds to the receptors of the post-synaptic neurons in the central and peripheral nervous systems (Everitt & Robbins, 1997; Fujii et al., 2008; Lè Ne Varoqui & Erickson, 1998; Zeisel & Blusztajn, 1994). Concerning the oxidation of choline to betaine, a 2-step reaction takes place in both liver and kidney, and are respectively catalyzed by choline dehydrogenase (ChD) and betaine aldehyde dehydrogenase (BAD) (Buchman et al., 1993; Muñoz-Clares et al., 2010). Other than being an important osmolyte, betaine takes part in DNA methylation, being a direct methyl group donor (Lever & Slow, 2010; Ueland, 2011). The role of choline and betaine in epigenetic modifications will be further discussed later in this thesis.

Among choline's roles, the most relevant to the aim of this study is as a substrate for the synthesis of phosphatidylcholine. Dietary phosphatidylcholine is firstly hydrolyzed to lysophosphatidylcholine in the intestine before being taken up by the enterocytes, and then re-esterified to phosphatidylcholine (Li & Vance, 2008). The transport of dietary choline into the enterocytes is mediated by three specific

transporters: the high affinity transporter (CHT1), the intermediate affinity transporters (CTL) and the low-affinity organic cation transporter (OCT) (van der Veen et al., 2017). After choline has been transported into the enterocytes, it is integrated into phosphatidylcholine via the CDP-choline pathway, also known as the Kennedy pathway (Figure 9). In this pathway, choline is first phosphorylated to phosphocholine by choline kinase (ChK), encoded by the *chk* gene. The phosphocholine is then converted to CDP-choline by phosphocholine cytidyltransferase, encoded by *pcyt1α*. In the last step of the pathway, CDP-choline is finally converted to phosphatidylcholine by CDP-choline:1,2-diacylglycerol choline phosphotransferase (CPT), encoded by *chpy1* (Li & Vance, 2008; Pritchard & Vance, 1981). Although this pathway has not been extensively investigated in fish, studies conducted on fugu (*Takifugu rubripes*) and zebrafish (*Danio rerio*) (Lykidis, 2007; Tocher et al., 2008) demonstrated the presence of the same biosynthetic enzymes and protein families involved in phosphatidylcholine synthesis in mammals. Additionally, the activity of CPT in microsomes from intestinal mucosa of Atlantic salmon has been demonstrated to be comparable to that of mammals (Oxley et al., 2005b). Most likely, the CDP-pathway is therefore the main route for phosphatidylcholine production in fish, as it is in mammals (Hazel, 1990).

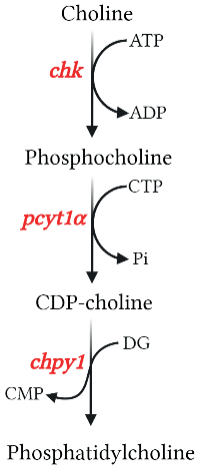

Figure 9. *Summary diagram of the CDP-choline pathway (Kennedy pathway). Created with BioRender.*

7.8 Estimation of nutrient requirements

General guidelines concerning the estimation of nutrient requirements for different fish species are given by the book "Nutrient requirements of fish and shrimp" (Jobling, 2011). However, at present there is no precise consensus on which biomarker(s) to use when estimating a specific nutrient requirement. According to the National Research Council (NRC, 2011), biomarkers should have a functional relationship with the nutrient in focus.

The most common research strategy when estimating nutrient requirements of animals is to design and conduct dose-response feeding trials, covering levels lower as well as higher than predicted optimal level of the selected nutrient, and thereafter observing different response variables (biomarkers).

Concerning estimation of vitamin requirements in fish, most studies have been conducted on fry and fingerlings fed diets containing graded levels of the examined vitamin. In these cases, the examined biomarkers were mostly related to growth rate, feed conversion ratios and data related to the level of the tested nutrient in a tissue, generally the liver (Craig & Gatlin, 1997; Griffin et al., 1994; Halver & Hardy, 2022; Ketola, 1976; Shiau & Cho, 2002; Wilson & Poe, 1988b). Most of these requirement values were obtained from small fish raised in controlled, optimal environments using highly digestible diets, and the obtained data were assumed to be representative for fish raised in different conditions and at different developmental stages (Duan et al., 2012; Halver & Hardy, 2002; Kennedy et al., 2007; NRC, 2011; Qin et al., 2017; Shiau & Cho, 2002). However, it is important to take into account that vitamin requirements may be influenced by feed intake, and other factors, such as fish size, age, growth rate, stage of sexual maturity, and production and environmental factors, such as disease pressure, water temperature and water quality (Rørvik et al., 2003). More importantly, it should be kept in mind that optimal supply for other important functions than growth (e.g. disease resistance) may differ from that of growth and feed utilization. Observations of health biomarkers should therefore be included in the studies (Pan et al., 2017).

It is important to consider that many nutrients for which specific requirements have not been established yet can interact with the metabolism of others. For instance, B vitamins such as folate, cobalamine, riboflavine, niacin, pyridoxine and zinc can interfere with choline production and functions. Therefore, it will be necessary to update the information regarding choline requirements as more knowledge about these vitamins is acquired.

The discussion regarding which statistical method is the most suitable when defining nutrient requirements in fish has been addressed by several studies (Baker, 1986; Cowey, 1992; Mercer et al., 1993, 1978; Robbins ,1989). According to the review

paper published by Shearer (2020), the ANOVA and the broken line models are the most frequently used to estimate dietary nutrient requirement from dose-response data. However, a re-evaluation of both these methods showed that they may significantly underestimate the requirement. The main flaw of applying the ANOVA method to dose-response studies is that nutrient levels are treated as discrete, rather than continuous. Consequently, the estimated optimal requirement is not precise, but it is stated as a range between input levels (Shearer, 2000).

The broken line method involves the production of two straight lines to represent the dose-response relationship. The ascending line shows the increase in response to the increasing nutrient intake, while the horizontal line represents nutrient abundance. The breaking point will indicate the estimated nutrient requirement. The main criticism of the broken line is that it describes adequately dose-response studies applied to individuals, but it is not reliable when applied to populations (Hernandez-Llamas, 2009).

The broken line method and ANOVA may be used in combination, although no significant difference in the estimation is produced (Fox et al., 1995; He & Lawrence, 1993; Koshio et al., 1993). Shearer suggested that the quadratic model, or 5-SKM, is the most reliable to apply to dose-response studies (Shearer, 2000). The quadratic model uses the least square to estimate the requirement level forming a symmetric parabola. The least square describes the variance in prediction of the dependent variable (response) as a function of the independent variable (nutrient level) and the deviations from the fitted curve. The statistical analyses will reveal the nutrient level able to cover the requirement for 50% of the population. To find the level which covers the requirement of 95% of the population, 2 $*$ standard error of the mean (sem) should be added to the average requirement level.

New advancements in modern computation power have led to increasing interest in the use of different statistical methods to be applied to dose-response studies. Among those methods, the Bayesian approach has lately been used to evaluate dose-response data obtained from human medicine studies (Muehlemann et al., 2023).

The following section provides background knowledge regarding the Bayesian approach, which has been used for the evaluation of a group of data described in this thesis.

7.9 Bayesian approach to dose-response studies

The Bayesian approach has, until now, seldom been applied to fish feeding trials. It is, however, commonly used in trials within human medicine, e.g. for the evaluation of results of human clinical trials, especially those related to drugs dosage (Bittl & He, 2017).
The Bayesian method provides an intuitive and safe quantitative and methodologically robust approach to incorporate existing data into the design of new dose-response trials, while reflecting and examining the inherent assumptions and uncertainties. Consequently, the number of patients, or experimental animals, may be reduced while maintaining the overall strength of evidence to demonstrate efficacy of a new treatment/diet formulation. For instance, in the dose-response study presented in this thesis, which evaluated effects of increasing level of dietary rapeseed oil, the application of the Bayesian method allowed use of observations from individual fish in a net pen, only one net pen per level and fish size, and to compare effects in tissues and organs without data transformations.

The are two main distinctions between the frequentist and the Bayesian statistical approaches. Firstly, frequentists base their inferences only on a single experiment, while the Bayesians make inferences by synthesizing information across old experiments or knowledge (Ruberg et al., 2023). In addition, Bayesians and frequentists have two different ways to provide evidence to answer research questions. Frequentists validate hypotheses against observed data by using the *p* value. The *p* value is a value which describes the probability of having a test statistic of interest at least as extreme as what we observe assuming the null hypothesis were true. The result of testing the hypothesis is an indirect answer since the hypothesis will be assumed as true until the data refute it. On the other hand, Bayesian statistics can directly answer the research question by determining the probabilities across the hypothesis given the newly obtained data (Ruberg et al., 2023).

To design and interpret Bayesian trials, it is necessary to understand the key elements of the Bayesian analysis, i.e., prior, likelihood, posterior distribution, and predictive probability (Muehlemann et al., 2023). The prior is the first step while planning Bayesian analysis, since the future inferences will be formulated based on it. Defining the prior requires careful consideration of information sources, such as previously conducted trials and information about the treatment taken into exam or the population of interest. There are several ways to select which priors are more reliable

than others, for instance the source and quality of the data, how contemporaneous those data are with the current experiment, how the data were collected, as well as information related to the considered population (Ruberg et al., 2023). Once the prior has been defined, it is also fundamental to assign a weight to that prior. For instance, if the prior has minimal, or inconsistent data, then that prior may have less weight than the data obtained from the new study. On the other hand, if the prior is very informative and closely related to the new experiment, then the prior will have a strong influence on the final analysis of the trial (Muehlemann et al., 2023; Ruberg et al., 2023). The prior used to analyse part of the results described in this thesis, was obtained based on earlier studies of relationship between dietary FA profile and the various observations of somatic indices and FA profile in the tissues. Additionally, effects of fish size were expected.
In our study, five different hypotheses with an equal a priori likelihood were formulated:

m1: no effect of rape oil level, no effect of fish size
m2: effect of rape oil, no effect of fish size
m3: no effect of rape oil, effect of fish size
m4: effect of rape oil, effect of fish size, different intercepts, similar slopes
m5: effect of rape oil, effect of fish size, different intercepts, different slopes

Another important element of the Bayesian method is the likelihood, i.e., the probability of observing the data given the parameters of a model.
After the data from the trial have been obtained, the priors and the likelihood can be mathematically combined by applying the Bayes formula to obtain the posterior probability.

$$P(A|B) = \frac{P(B|A)P(A)}{P(B)}$$

Where A and B are events, P(A|B) is the posterior probability, P(B|A) is the likelihood and P(A) and P(B) are the priors probability.

Conceptually, the posterior probability distribution is the weighted average between the knowledge available before the experiment and that attained from the new

experiment. This weight depends on how informative the priors are, the sample size and the variability in the experiment (Ruberg et al., 2023).

Once the posterior distribution has been defined, predictive probability statements can be formulated based on the effects of the treatment. In the study described by the current thesis, the statements were obtained by comparing the effects of rapeseed oil level on absorbed FAs, and FAs in the PI, mesenteric tissue, and the liver, based on visual examination of the estimated functions presented with 95% credible interval. A credible interval is an interval within the domain of a posterior distribution, within which an unknown parameter falls with a given probability, in this case 95%.
To interpret the obtained results the Bayesian factor, BF12, is used. According to Kass and Raftery (Kass & Raftery, 1995), the values of Bayesian factors indicate evidence of relationship between priors and posterior distribution as follows:

BF12	1 to 3.2	Not worth more than a bare mention
BF12	3.2 to 10	Substantial
BF12	10 to 100	Strong
BF12	> 100	Decisive

p.
The result of the approach indicates the probability of each of the suggested functions to be the correct function of the relationship.

Advice and support for the employment of the Bayesian approach was given by Aliaksandr Hubin, associate professor at NMBU's Bioinformatics and Applied Statistics Research Group.

7.10 Choline requirement

Although choline is defined as an essential nutrient for some mammals throughout their development (Gibellini & Smith, 2010; Jobling, 2011), it has long been considered essential for fish only during their early life stages. Concerning pacific salmon and rainbow trout larvae, choline requirement has been estimated to be around 800 mg/kg feed (Jobling, 2011). However, knowledge regarding Atlantic salmon along its developmental stages is still very limited. According to available studies, choline deficiency primarily leads to poor growth performances, high mortality, fatty liver, and hemorrhagic kidney and intestine (Duan et al., 2012; Halver & Hardy, 2002; Ketola, 1976). Symptoms more related to lipid accumulation, such as whitish appearance of the intestinal mucosa, are less observed (Arai, S., Nose, T., 1972). For this reason, most of the works addressing choline function and metabolism in fish are focused on observing the effects of choline deficiency on biomarkers such as growth performances and liver lipid level (Craig & Gatlin, 1997; Griffin et al., 1994; Halver & Hardy, 2002; Jobling, 2011; Ketola, 1976; Rumsey, 1991).

A series of recent studies documents choline's essentiality and requirement in post-smolt Atlantic salmon and indicates that biomarkers of lipid metabolism in the pyloric intestine may be more sensitive than corresponding markers in the liver (Hansen et al., 2020a; Hansen et al., 2020b; Krogdahl et al., 2020a). In the dose-response study included in the series, the observed biomarkers were closely related to intestinal lipid transport: the organosomatic index of the pyloric intestine, the histologically observed degree of vacuolation of enterocytes, and the macroscopically observed appearance of whiteness in the pyloric caeca (Hansen et al., 2020a). Additional biomarkers were the expression of four genes strictly involved in lipid transport and metabolism, apolipoprotein-AIV (*apoAIV), apolipoprotein AI (apoAI)*, *phosphate cytidylyltransferase 1A* (*pcyt1α) and perilipin 2* (*plin2)*.

The results of the study showed a clear dose-response relationship between dietary choline level and the severity of the selected steatosis symptoms. The data suggested a requirement of at least 3.4 g choline/kg (Hansen et al., 2020a). However, considering the role of choline in lipid metabolism, it is likely that the requirement may vary with dietary lipid level and quality. Additionally, other production-related

factors such as feed intake and growth performance, environmental conditions and fish developmental stage may also affect the requirement, and should, therefore, be investigated.

7.11 Choline's role as methyl group donor in DNA methylation processes

All somatic cells in an organism carry the same DNA information, however cell types and functions vary according to differences in the expression of their genes. As a result, the control of gene expression is of fundamental importance for cell differentiation and development (Meehan et al., 2005). The pattern of gene expression is maintained through mitosis and meiosis. Therefore, the cells inherit and propagate specific instructions not caused by changes in the nucleotide sequence of the DNA. These transmitted instructions are defined as epigenetic information. Epigenetic mechanisms such as DNA methylation, histone modification and gene expression by non-coding RNAs have important roles in many biological processes and their functioning can be influenced by several factors, including environmental exposure, aging, disease development and nutrition (Waddington, 1942.).
The methylation of the DNA and post-translational histone modifications are the most studied and characterized epigenetic modifications, whereas non-coding RNAs are relatively less known, and their roles have not been well established (Roundtree & He, 2016). DNA methylation and histone modification impact gene expression by performing shifts in nucleosome positioning and therefore controlling chromatin structure (Rothbart & Strahl, 2014). These processes are not only inheritable both in mammals and in fish (Podgorniak et al., 2022), but they are also potentially reversible, a characteristic which makes them promising targets in pharmacological studies (T. Chen & Dent, 2014).

DNA methylation is the most stable epigenetic modification and at present the most mechanistically comprehended (Smith & Meissner, 2013). From a chemical perspective, DNA methylation involves transfer of a methyl group (CH_3) from S-adenosyl-L-methionine (SAM) to the fifth carbon of the cytosine comprised within the cytosine-phosphate-guanine dinucleotides, to produce 5-methylcytosine

(5mC)(Moore et al., 2013). The direct consequence of DNA methylation is repression of the gene expression (Jones, 2012). The methylation mechanism is regulated by a group of enzymes known as DNA methyltransferases (DNMTs), which include DNMT1, DNMT3A, DNMT3B and DNMT3L. DNMT1 methylates hemi-methylated cytosines, maintaining the methylation process. DNMT3A and DNMT3B, in contrast to DNMT1, perform de novo methylation of unmethylated cytosines. Finally, DNMT3L assists DNMT3A and B by increasing their ability to bind to SAM (Duymich et al., 2016; He et al., 2011; Jin et al., 2011).

DNA methylation normally occurs in the so-called CpG sites (CpGs), sections of the DNA sequence where a cytosine is directly followed by a guanine. CpGs are distributed all along the genome (Klose & Bird, 2006). Once methylation has occurred in the CpGs, the methyl-CpG binding proteins (MBPs) identify them and recall chromatin remodeling corepressor to the sites, to suppress the gene expression and form strongly compacted chromatin structures which are transcriptionally inactive and known as heterochromatin. Several studies have shown that nutrients and bioactive components can affect epigenetic mechanism in different ways and at different steps, providing a wide range of possible outcomes (Gardiner-Garden & Frommer, 1987; Reddington et al., 2013). Most of the knowledge attained concerning nutrient participation in epigenetic regulation refers to one-carbon nutrients and metabolites which act as methyl donors. Choline, with its role as indirect methyl group donor during DNA methylations, is among the nutrients which have gained particular interest in the last decades for their role in epigenetic modification (Konstantinova et al., 2008). During methylation processes, choline's oxidation product, betaine, promotes re-methylation of S-adenosylhomocysteine to S-adenosylmethionine by betaine-homocysteine S-methyltransferase (BHMT)(Mahmoud & Ali, 2019) (Figure 10).

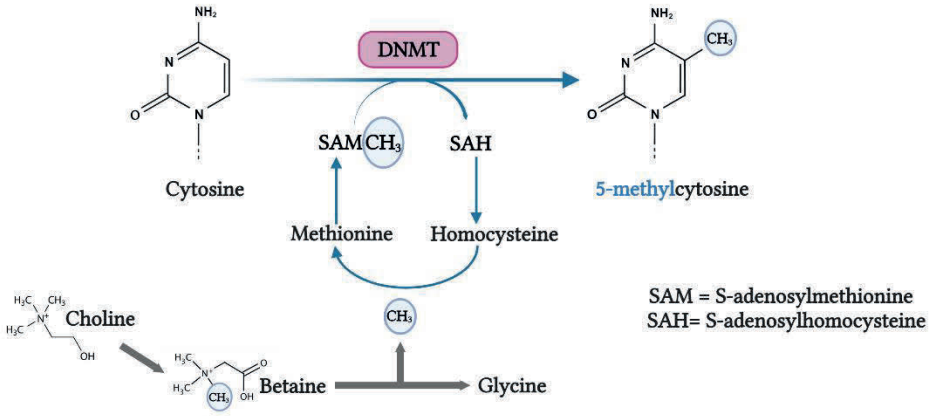

Figure 10. *Overview of the DNA methylation mechanism. Choline is oxidized into betaine, which participates to the methylation of homocysteine to methionine, which is then converted to SAM, the universal methyl group donor.*

Studies have shown that alterations in choline supply may lead to changes in global and gene-specific DNA methylation. For instance, the work conducted on mouse models in gestation by Niculescu et al. (2006) demonstrated that choline deficiency strongly influenced the phenotype of the offspring, showing clear signs of hypermethylation of specific genes related to brain development. In addition, the experiment performed by Skjærven et al. (2004), which tested a diet containing a mixture of large amount of methyl group donors (i.e., choline, betaine, vitamin B_{12}, and folic acid) on zebrafish, showed that inclusion of methyl donors in the diet of parental fish influenced the expression of genes involved in lipid transport and metabolism in the offspring. However, the knowledge achieved so far over choline's role in DNA methylation is still limited and no study has been conducted to estimate whether dietary choline deficiency can influence Atlantic salmon's methylome.

7.12 In vitro approaches

In vitro models of the fish intestine are gaining attraction across different research fields, such as nutrition, pharmacology, immunology, pathology and microbiology (Prabhu et al., 2018; Geppert et al, 2016; Holen et al., 2021; Minghetti et al., 2017;

Oldham et al., 2023; Pasquariello et al., 2023; Wang et al., 2019). In fish nutrition research, such an approach addresses ethical concerns by reducing the number of experimental animals used and reducing the reliance on costly and time-consuming feeding trials (Moyano et al., 2015).

The growing demand for high-quality plant-based ingredients in fish feeds has underscored the necessity for a better understanding of the impact of such compounds on fish intestinal health and nutrient metabolism (Boyd et al., 2020; Turchini et al., 2019). For instance, as previously described in this thesis, the shift from the proportion of fish meal towards plant ingredients in the diet of farmed Atlantic salmon revealed a knowledge gap regarding lipid metabolism. Within this framework, cell-based models of the intestinal epithelium show great potential as screening tools for examining how alternative fat sources impact the physiological processes occurring in the fish intestine during lipid transport and metabolism. In addition, intestinal in vitro models have a great potential as study tools to increase our understanding of uptake and transport mechanisms through e.g. genome editing approaches.

Currently, the rainbow trout intestinal epithelial cell line, RTgutGC, is the most studied immortal cell line derived from the fish gut (Kawano, 2009). Two additional rainbow trout cell lines were very recently derived from the proximal (RTpi-MI) and the distal (RTdi-MI) intestine (Pasquariello et al., 2021). These three models have shown characteristics typical of the in vivo intestinal tissue. For instance, when grown on a permeable membrane, RTgutGC, RTpi-MI and RTdi-MI can form a selectively permeable polarized barrier which resembles the intestinal epithelial layer. Additionally, they can form tight junctions and desmosome cell-to cell adhesions (A. Kawano et al. 2011; Kawano 2009; Minghetti et al. 2017; Oldham, Dudefoi, and Minghetti 2023). High seeding density induces all the cell lines to show clear signs of maturation, represented by the upregulation of alkaline phosphatase gene (*ialp*), while those genes related to the stem-cell niche, such as *sox9, hopx* and *lgr5*, are suppressed (A. Kawano et al., 2011; Pasquariello et al., 2021). Fish cell lines also show great advantages compared to mammalian ones, since they are easier to maintain for

long periods and can better tolerate a broad range of temperatures and hypoxia (Goswami et al., 2022).

However, these models have only recently been applied in nutrition research and have yet to be established for routine analysis. Moreover, studying lipid metabolism in cell models is particularly challenging due to the low water solubility of lipids. To date, the only feasible approach to studying FA metabolism in cell systems is to complex them with albumins (Alsabeeh et al., 2018). Consequently, studies on dietary fat absorption are typically conducted using human cell lines (Minekus et al., 2014) or animal models. For instance, Nauli and Whittimore (Nauli & Whittimore, 2015) exposed Caco-2 cells, a cell line derived from human epithelial colorectal adenocarcinoma (Hidalgo et al., 1989), grown on a permeable membrane system to a lipid mixture containing oleic acid at varying concentrations. The results highlighted the efficient formation of lipoproteins in the basolateral compartment of the system, offering the development of an in vitro model for investigating dietary fat absorption and intestinal metabolism of drugs, vitamins, and other liposoluble substances. In parallel, Gomez-Lechon et al. (2007) investigated lipid metabolism in the liver by using HepG2, a human hepatocyte-derived cell line, to establish an experimental model of hepatocellular steatosis. Their study demonstrated that HepG2 cells exposed to high concentrations of oleic and palmitic acids exhibited behavior similar to that of liver tissue in humans afflicted by fatty liver syndrome.

To the best of our knowledge, only one study has been conducted to explore lipid metabolism in fish intestinal cell lines. This study, conducted by Selvam et al. (2022), investigated the metabolism of three classes of FAs (oleic acid, arachidonic acid, and palmitic acid) in the RTgutGC. Selvam et al. (2022) demonstrated that RTgutGC can undergo lipid absorption and transport processes similar to those found in vivo mammalian and fish models. This includes synthesizing TAG and FFAs, producing lipid droplets, and expressing a set of genes involved in lipoprotein and lipid droplet formation.

Considering the findings obtained by Selvam et al. (2022), the question arises as to whether it is possible to develop a fish in vitro model capable of replicating the steatosis condition observed in vivo, facilitating the investigation of the cellular, molecular, and biochemical mechanisms underlying lipid accumulation. This thesis

represents a first attempt to address such a question, with the goal to create the first in vitro fish steatosis model.

7.13 Hypothesis

The following hypotheses were the basis for the thesis:

- Production related factors such as dietary lipid level and source, water temperature and fish size can influence steatosis severity and therefore choline requirement.

- Choline is an indirect methyl group donor during DNA methylation, hence dietary choline level can influence Atlantic salmon's intestinal epigenome.

- Fish intestinal cell lines may represent a useful tool to investigate molecular and cellular mechanisms underlying lipid transport and metabolism.

8 Aims of the study

The overall goal of this PhD thesis was to describe and understand interactions occurring between dietary choline level, lipid metabolism, production-related conditions, and epigenetic regulation in Atlantic salmon.

To achieve this, the following subgoals were formulated:

- To define whether steatosis symptoms and therefore choline requirement may be affected by dietary lipid level and water temperature **(Paper I)**.

- To define whether steatosis symptoms and therefore choline requirement may be affected by dietary lipid source and fish size **(Paper II)**.

- To characterize the epigenetic changes occurring in the pyloric cpaeca of Atlantic salmon exhibiting varying degrees of steatosis symptoms after being fed diets containing increasing levels of lipids and choline **(Paper III)**.

- To profile the molecular and cellular mechanisms underlying lipid accumulation and metabolism in a fish intestinal cell line by establishing an in vitro model able to mimic intestinal steatosis **(Paper IV)**.

9 Strategies

The strategies chosen for achieving the goals of this PhD thesis were the following:

- Atlantic salmon parr raised in fresh water at two different temperatures, 8 and 15 °C, were fed to diets containing increasing dietary lipid level: ranging from 16 to 25%, and a deficient choline supply. This was done with the aim of investigating whether water temperature and dietary lipid level may influence the severity of steatosis and, therefore, affect choline requirements.

- Atlantic salmon raised in seawater and having different initial body sizes, 1.5 kg and 4.5 kg, were fed six choline-deficient diets containing an increasing amount of rapeseed oil and a decreasing amount of fish oil. The aim was to investigate whether fish size/background and lipid source may influence steatosis severity, and therefore choline requirement.

- Atlantic salmon parr were fed four diets containing increasing dietary lipid level, from 16 to 28%, and low choline supply (1830–2310 mg/kg). Pairwise analyses were conducted in order to define whether varying dietary lipid level and dietary choline supply may influence Atlantic salmon's intestinal epigenome.

- A newly developed epithelial cell line derived from the distal intestine of the rainbow trout (RTdi-MI) was exposed to two concentrations of oleic acid, 250μM and 31.25μM, to investigate molecular and cellular mechanisms underlying lipid metabolism in in vitro models.

10 Summary of the results

Paper I provides an estimation of effects of increasing dietary lipid level and water temperature on steatosis indicators such as altered organosomatic index of the pyloric intestine (OSIPI), enterocytes hyper-vacuolation and expression of genes involved in lipid metabolism.
Four choline-deficient plant-based diets were formulated differing in lipid level of 16, 20, 25 and 28% and fed to salmon of 25 g initial weight in duplicate tanks per diet at two different environmental temperatures: 8 and 15°C. After 8 weeks of feeding, samples of blood, pyloric caeca tissue and gut content from six fish per tank were collected, for analyses of histomorphological, biochemical and molecular biomarkers of steatosis and choline requirement.

Feeding activity and consequently growth performance was significantly higher at 15°C than at 8˚C. On the other hand, no clear effect on growth was caused by the increasing lipid level. Among the biomarkers selected for evaluation of state of choline supply, the most sensitive indicators were observed in the pyloric caeca, i.e., OSIPI and histological observation of cell vacuolation of enterocytes/epithelial cells. These indicators increased in severity with the increasing dietary lipid content and with the increasing water temperature, supposedly as a consequence of the higher feed intake. These findings support the importance of choline's role in lipid transport, since the higher dietary lipid content in a choline-deficient condition increased severity of the steatosis symptoms. The molecular analyses of the pyloric caeca tissue revealed a dose-response relationship between dietary lipid level and the expression of genes involved in lipid metabolism and transport. Among the assessed genes, *plin2, apoAI, apoAIV* and *pcyt1α* showed the greatest effects of lipid level and temperature and seemed to be the most sensitive gene expression biomarkers for choline requirement in this study. The expression of *plin2, apoAI* and *apoAIV,* genes involved in lipoprotein synthesis, showed a clear dose-response effect given by the increasing lipid level which mirrored the histology results at 8°C. At 15°C the picture was less clear, since the expression of the same genes peaked at 20% of dietary lipid inclusion

and decreased with higher percentages. On the contrary, *pcyt1α* was strongly downregulated by the increasing lipid level at both water temperatures. Further discussion of these results is limited by several complicating factors. First, the Kennedy Pathway, which regulates choline's phosphorylation into phosphatidylcholine, is itself a highly complex system and not thoroughly understood. Second, *pcyt1α*, a key gene involved in this pathway, is regulated by several rate-limiting enzymes.

Most of the assessed genes, all involved in lipid transport and metabolism, showed the same pattern as *plin2*, especially at 8°C. The FA composition of the pyloric caeca mirrored that of the diet, while no clear effect was observed in the FA composition of the liver. In contrast to the results obtained for the pyloric caeca, the increasing lipid level and the water temperature did not impact the liver regarding organosomatic index, the histological lipid accumulation or the FA content of the liver, underlining the different roles of the two organs.

In **Paper II**, the work focused on testing production-related conditions (dietary lipid source and fish size) to estimate their possible effects on steatosis symptoms. Six choline-deficient diets were formulated varying in ratios of rapeseed oil to fish oil. The diets were fed to two groups of Atlantic salmon differing in weight (1.5 and 4.5 kg), smoltification (autumn (S0) and spring (S1)), and breed (Aquagen AS and Salmobreed AS), called small and large fish, respectively. The fish were kept in seawater. The feeding trial lasted 8 weeks and, when concluded, the same sampling procedures and laboratory techniques as in **Paper I** were applied. In light of the regression design of this experiment, the statistical evaluation of the obtained data was conducted using a Bayesian approach, described in the introductory chapter.

The results showed no effects of growth performances given by the lipid source. On the other hand, fish size influenced the thermal growth coefficient (TGC), which was higher in small than in large fish. Concerning fat digestibility, the higher level of dietary rapeseed oil increased fat digestibility, in accordance with other studies. Higher rapeseed oil levels increased lipid supply to the fish, resulting in a dose-response relationship between the dietary rapeseed oil level and the magnitude of its

effects on steatosis biomarkers (i.e., OSIPI and histological observation of cell vacuolation). Concerning the effects of the fish size on enterocyte vacuolation, the number of severely affected fish was higher among smaller than larger fish, likely a consequence of the relatively higher feed intake.

The OSIPI results did not mirror this histological condition, probably underlying the high flexibility of the intestine, which may adapt to increasing workloads by increasing the absorbative capacity per unit of tissue.

The molecular analyses of the pyloric caeca were carried out on a pool of four genes considered as the most sensitive biomarkers for choline requirement: *plin2, apoAI, apoAIV* and *pcyt1α.* However, no significant effect of fish background or lipid source was observed on the other genes investigated in the same study. Taking into account that our previous studies showed opposite results, we consider the knowledge achieved so far as not sufficient to further discuss the absence of the gene expression response.

In order to estimate the magnitude of impact of the treatments on choline requirement, we selected the histological scores of steatosis in the PI and the OSIPI as biomarkers. The increase in rapeseed oil from 0 to 24% led to an increase in choline requirement of about 650 mg/kg in large fish and 450 mg/kg feed in small fish. The difference in choline requirement between the two fish sizes, when fed the lowest rapeseed oil level, was 250 mg/kg. At the highest rapeseed oil level, the difference was 100 mg/kg.

In this study we also conducted analyses on FA content and composition in the feed, PI tissue, mesenteric fat and liver. Although the FA composition of all the observed tissues reflected that of the absorbed lipid, the FA composition of the mesenteric tissue resembled that of the absorbed lipid more closely than the composition of the pyloric intestine. On the other hand, the composition in the liver showed greater differences, in accordance with its role as key organ for lipid metabolism in an animal body. This information strengthens the basis for designing further experiments addressing choline requirement and it highlights the role of tissue-specific analyses in garnering results.

In light of the role of choline as methyl group donor in DNA methylation, the aim of **Paper III** was to describe epigenetic changes occurring in the pyloric caeca of Atlantic salmon fed to diets containing increasing lipid and choline level. The pyloric caeca samples processed in this study belonged to the fish fed four diets containing respectively 16% (L16), 25% (L25) and 28% (L28) of lipid level. The L16-L25 diets were all choline deficient, while the L28 diet contained a higher but suboptimal choline level.

To evaluate the presence of differentially methylated cytosines (DMCs) we applied the reduce representation bisulfite sequencing (RRBS) protocol and performed library sequencing. Thereafter, we used different bioinformatic tools such as Trim Galore, Bismark aligner, methylkit package and HOMER to analyse the obtained data and compare the number of DMCs between the different dietary treatments. The presented results focus on the observed differences between the diet L25 and L16, having the highest difference in lipid percentage but similar choline level; and the differences between the diet L28 and L25, containing similarly high lipid level but two different choline contents (about 22% difference).

The results showed that those genes associated with DMCs consequently to higher dietary lipid level, code for membrane components and transporters and microRNAs important for lipid homeostasis. The variation in choline level affected methylation in genes involved in FA biosynthesis and transport, lipolysis and lipogenesis, and genes related to immune functions, including genes involved in differentiation of immune cells. More specifically, the comparison between L25 and L16 showed that changes in lipid level alone only moderately affected the methylome. Among the genes associated with DMCs the most relevant were *tjap1,* and *mir-212*. Encoding for a peripheral tight junction protein, *tjap1* was epigenetically modulated in L25 compared to L16, possibly contributing to alterations in FA transport because of the excessive lipid accumulation. Fish fed L25 also showed hypermethylation of *mir-212*, a key regulator of the expression of target genes involved in hepatic lipid homeostasis, including *tjap1*.

Concerning the comparison between L28 and L25, the difference in choline level between the two diets had higher effects on the pyloric caeca methylome.

In fish fed L28, *tjap1* was hypomethylated. According to what was described previously, this result showed that hypo- and hypermethylation of *tjap1* were respectively associated to high and low dietary choline levels, suggesting this gene as a possible epigenetic biomarker. The higher choline level also led to hypomethylation of *slc22a15*, an important gene involved in the regulation of tissue levels of carnitine and betaine, both involved in the control of FA levels in the intestinal mucosa. Another gene hypomethylated by the higher choline level was *scd*, which is involved in the catalysis of the biosynthesis of monounsaturated FAs. Such a result may be related to the decrease in severity of choline deficiency symptoms with increasing choline level. Among the affected immune genes, *irf4a*, is a key regulator of differentiation of immune cells including T and B lymphocytes, macrophages, and dendritic cells. The gene showed hypomethylation in the intron region with increasing dietary choline level, indicating that dietary choline level may play a role in salmon mucosal immune function.

The experiment described in **Paper IV** represents the first attempt to establish an in vitro model able to mimic the steatosis conditions observed in the in vivo Atlantic salmon intestine. In order to generate an in vitro steatosis model, a newly developed cell line derived from the distal intestine of the rainbow trout (RTdi-MI) was exposed to three (including control) different concentrations of oleic acid: 0μM, 31.25 μM and 250 μM dissolved in L-15 containing 1% of fetal bovine serum. The cells were sampled 24, 72 and 168 hours (1 week) after exposure. Thereafter, cell viability, lipid droplet accumulation, FA profiles, and the expression of genes involved in lipid droplet formation and lipid synthesis and metabolism were assessed.

The results showed that oleic acid did not influence cell viability to a significant extent. Only after 168h of exposure did cell metabolism slightly decrease at the highest lipid concentrations compared to the control. The morphological assessment of the cells revealed a gradual accumulation of granules around the cells' nuclei, in parallel to the increasing oleic acid level in the medium and the exposure time. Such granules, absent in the control, have been identified as lipid droplets and stress granules. The high oleic acid concentration may have triggered a stress response in

the cells, leading to the shutdown of translation patterns and further accumulation of translation initiation complexes into stress granules. The formation of stress granules is often associated with lipid droplet synthesis. In the present study the increasing amount of lipid droplets was probably related not only to the stress response itself, but also to the high oleic acid concentrations. The increasing presence of lipid droplets was confirmed by the BODIPY assay results, which allowed a live visualization of the lipid droplets accumulating within the cytosol. Accordingly, the assay showed that the surface area of the cells increased with the increasing oleic acid concentration in the medium. On the other hand, the intensity of the fluorescence remained similar between the treatments and the control. The quantification of the lipids retained within the cells was performed by thin layer chromatography. The results confirmed that, among the FA classes, oleic acid (18:1n-9) was the most abundant, especially in the PL and the TAG fraction, in cells exposed to oleic acid at both 31.25 and 250 μM.

The molecular analyses were carried out on a pool of six genes involved in lipid droplet formation (*plin2),* FA synthesis and metabolism *(fas, elovl5 and fads2d5)* and transcription factors related to lipid metabolism *(srebp1 and pparg).* First and foremost, all the genes were similarly expressed in both cells and rainbow trout intestinal tissue. Such finding confirmed the presence, in the RTdi-MI, of metabolic compartments involved in lipid droplet accumulation and lipid metabolism similarly to those present in vivo. Concerning the effects of oleic acid concentration and exposure time, the expression of all the genes was significantly influenced by both factors and by their interaction. The main biomarker for intestinal steatosis, *plin2*, was significantly upregulated by the increasing oleic acid level 24h after exposure. Such an outcome may indicate an attempt by the cells to store the increasingly high oleic acid into lipid droplets. A similar scenario was observed for *pparg.* The expression of most of the other genes, such as *fas, fads2d5 and srebp1,* showed a positive dose-response relationship with the concentration of oleic acid in the exposure medium. At the same time the increasing exposure period increased the expression of all the genes. Taken together, these results confirm that RTdi-MI show characteristics similar to those of in vivo intestinal tissues of fish subjected to highly

lipidic feeds. Such an outcome highlights that RTdi-MI may represent a valid alternative to conduct further studies over the physiological and molecular mechanisms underlying lipid accumulation and steatosis in fish intestine.

11 Methodological considerations

For the experiments presented in **Papers I and II**, sample collection and subsequent analyses including gene expression profiling, histomorphological evaluation, blood plasma biochemistry and lipid class analyses were performed following well-established methods. The most challenging aspects and less-standardized techniques addressed in these works will be further discussed below. Specifically, the feed formulation of **Paper I** and the experimental animals used for **Paper II** are discussed. Concerning the methods applied, the following chapter will focus on the application of the RRBS technique and challenges faced during the downstream analyses described in **Paper III**, and the use of newly developed in vitro approaches (**Paper IV**).

11.1 Materials

11.1.1 Fish

The feeding trials for the first **(Paper I and III)** and the second experiment **(Paper II)** were conducted in two different research facilities, respectively at Nofima's Research Station in Sunndalsøra (NO) and at the LetSea research facility at Dønna (NO). Both facilities are approved by the Norwegian Animal Research Authority (NARA) operating following Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations).

In view of the further discussion of the results, some details concerning the experimental animals used in the second feeding trial warrant attention.
The initial aim of the second experiment **(Paper II)** was to establish whether fish size and dietary fat quality might influence choline requirement in Atlantic salmon.
Salmon of two sizes to be compared under the same environmental conditions unavoidably differ in production background (as these are piscivorous species). In the present study, the strategy to reach different sizes, was to use fish differing in

smoltification procedure, i.e., autumn (S0/Small) and spring smolt (S1/Large). At the time of the experiment, due to delivery problems, it was not possible to get fish differing in size from the same breed. The small fish were delivered by AquaGen As, smoltified and put to sea in the autumn of 2020, while the larger fish were delivered by Salmobreed As, smoltified and put to sea in the Spring 2020. Consequently, the two populations of fish differed in size and smoltification procedure, as well as in genetic background. However, as the experiment was one of a series of screening experiments conducted to find factors affecting choline requirement, the difference in genetic background, was considered acceptable. Such differences are taken into consideration and discussed in **Paper II**.

11.1.2 Diets

To perform the feeding trial for the first experiment **(Paper I and III)** four similar diets with high content of plant ingredients, were formulated. These diets had similar choline-deficient levels, and varying lipid levels of 16%, 20%, 25% and 28%. Upon arrival of the diets at the experimental site, fat leakage from the diet with 28% fat was discovered. A new batch was made with higher content of soy lecithin for better emulsification. The resulting increase in dietary choline from the added soy lecithin content was not realized until after the feeding period was concluded. However, this unexpected event was found not to significantly disturb the aims of the experiment. The results obtained for the diets containing 16, 20 and 25% of lipid level were considered sufficient to address the aims of the study (**Paper I**). The results for the 28% diets are included **(Paper I),** as the results shed light on and confirm outcomes of previous studies. The same fish used for the experiment described in **Paper I** were used also for the epigenetic analysis of **Paper III**. By exploiting the differences in dietary lipid level and the accidentally higher choline supply in one of the diets, it was decided to run pairwise analyses to investigate whether these two factors could influence DNA methylation in the pyloric caeca.

11.2 Methods

11.2.1 Reduced Representation Bisulfite Sequencing (RRBS)

Several technologies are nowadays available to measure the number of methylated cytosines in DNA. The most commonly used are based on specific treatments which make it possible to distinguish between methylated and unmethylated cytosines. These techniques can be divided into chemical strategies followed by DNA sequencing (bisulfite sequencing) and affinity enrichments methods. Although the affinity enrichment methods are less expensive than the bisulfite sequencing, they need a higher amount of input DNA while offering a lower quality of the results.

On the other hand, bisulfite sequencing analyses represent the current gold standard. To apply the bisulfite sequencing, DNA is treated with sodium bisulfite, which converts the unmethylated cytosines to uracil, while keeping the methylated ones unchanged. The sequencing of the obtained DNA strand will show the level of methylation present within the sample. The main bisulfite sequencing methods are whole genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS). Through the WGBS method it is possible to analyze the methylation levels of the whole genome, the so-called methylome. This protocol involves a library preparation step with bisulfite treatment, followed by the whole genome sequencing of the sample. The result is an efficient identification of all the methylated cytosines on a genome-wide scale, with a single nucleotide resolution. A big advantage of WGBS is that the resulting methylome allows the identification of all the differentially methylated sites and regions associated with experimental treatments. In contrast, the RRBS uses methylation-intensive restriction enzymes, which select defined genome fractions containing CpG rich DNA regions (CpG islands), avoiding the whole genome sequencing. By reducing the complexity of the genome and the number of reads the RRBS results are more cost-efficient than the WGBS. However, since RRBS is a more targeted approach which focuses the enrichments only on the CpG islands, it is possible that those areas of the genome with a low GC content will be excluded from the analyses.

When comparing the two approaches, we decided to select for this study the most cost-efficient one. Indeed, even though the WGBS provides a genome wide methylation profile, it requires a high number of reads to achieve adequate

sequencing depth, and it also needs a high amount of input DNA. On the other hand, RRBS requires less input DNA and fewer reads. Although using the RRBS, it is possible to miss some areas of the genome, we considered this matter inconsequential, since the majority of methylation normally occurs in the areas where a high GC content is present.

11.2.2 Mapping efficiency

After library preparation via RRBS technique and following sequencing, we analyzed the resulting data by the use of bioinformatic tools. During processing, the bisulfite treated sequencing reads are aligned (or mapped) to the genome of interest, to perform the so-called methylation calls. At the end of the alignment, we uniquely determine the position of bisulfite reads in the strand.

In our study, we had an average of 27.5 million reads per sample. Of these, only 10 million reads were uniquely mapped to the Atlantic salmon genome, showing a mapping efficiency of 39%. The unique mapping efficiency we obtained was low, but not surprising. Low unique mapping efficiency is a common factor in RRBS studies, and it has already been observed in other fish species, such as zebrafish (Dhanasiri et al., 2020) and tilapia (Podgorniak et al., 2022). Supposedly, this outcome is caused by the characteristic of RRBS to only offer specific areas of the DNA to sequence, instead of the whole genome. Other possible explanations may be biases of the aligning software or samples contamination.

11.2.3 Annotation of the genes associated with DMCs

The DMCs were associated to their corresponding genes, which were then annotated. In our study, only 50% of the genes associated to DMCs found correspondence to a gene name/symbol. This outcome is caused by the poor annotation of the genome of the Atlantic salmon compared to the genome of model fish (e.g. zebrafish).

11.2.4 Formulation of an in vitro steatosis model

Recently, the study of intestinal disorders by the use of cell models has allowed the investigation over complex in vivo phenomena in more controlled, simplified and repeatable conditions. However, if on one side the use of in vitro models represents a

huge improvement for research, on the other hand it presents a number of challenges and contingencies related to their relatively new application.

The goal of the in vitro experiment described in this thesis was to produce a cellular steatosis model able to simplify the study of lipid accumulation and metabolism. The challenges of this plan were related not only to the use of cell models but also to the choice of nutrient supplemented. Indeed, the creation of a so-called in vitro steatosis model foresees the exposure of the intestinal cells to an excessive lipid supply. The biggest issue when producing a medium supplemented with lipids is lipids' limited solubility. Such details/properties make it particularly challenging for lipids to disperse in the aqueous medium and be taken up by the cells. There are three possible ways to overcome this issue: (1) bind the lipids to a soluble carrier molecule (such as albumin), (2) develop a formulation which drives the lipid to assembly to the required particle size and, (3) reduce the lipids to a size which is transient and stable. As already done for several other studies conducted on the mammalian Caco-2 intestinal cells (Nano et al., 2003; Trotter, Ying Ho & StorchZ, 1996), it was decided, for the work described in this thesis, to buy a mixture of oleic acid conjugated to albumin. Albumin is the best lipid carrier protein, and it has several hydrophobic moieties which make it an efficient vehicle for FAs (van der Vusse, 2009).

11.2.5 Development of a lipid extraction and quantification protocol

Although the development of a lipid extraction protocol is of fundamental importance in lipidomic analysis, it is still unclear which method is the most efficient to apply to cell cultures. Moreover, no validated or standardized protocol has ever been designed and applied to cell cultures derived from fish intestine. A weak protocol may lead to a lack of reproducibility, accuracy and precision of further quantitative and qualitative analyses. The solvent to be chosen for the extraction should efficiently solvate polar, neutral and non-polar lipids. Additionally, the procedure should include a step to eliminate the particulate and deliver the target compounds at a concentration adequate to run further analyses.

At present several lipid extraction techniques are available, however, the most used are the Folch method and Bligh and Dyer method (Kumar et al., 2015).

The Folch method, which represents the gold standard for lipid extractions techniques from animal tissues, was developed by Folch et al. and uses chloroform

and methanol in a ratio 1:2 together with water. The result is a biphasic system consisting in upper phase containing non-lipidic material and a lower phase containing the lipids conjugated to the chloroform. The Bligh and Dyer method is an alternative version of the Folch protocol, and it foresees the production of a monophasic solvent containing chloroform and methanol (1:2) homogenized with the tissue samples and water. The result is then diluted again with water and chloroform in order to obtain a biphasic system. Although the aim of the Bligh and Dyer technique was to improve the Folch method, it has been observed that it ensures the sole extraction of phospholipid, but not neutral lipids. Therefore, for our experiment, we decided to use the original Folch method to develop a lipid extraction protocol from fish intestinal cells.

At the selected time points after exposure, the cell pellets were harvested, centrifuged to remove the aqueous phase and frozen in glass vials, to avoid any attachment of the lipids to a plastic surface. After thawing, chloroform, methanol and water mixture were added to the pellet.

Although the Folch method is standardized and reliable, it has a significant weakness i.e., the location of the lipids in the lower layer of the biphasic system. As a consequence, when collecting the lipids, it is necessary to remove or pass through the upper layer, a technically complicated procedure which can lead to errors, especially when dealing with big sample sets.

If the layer is successfully collected, the obtained sample is a mixture of lipids dissolved in chloroform. However, many lipid quantification kits do not work with chloroform-based samples. Therefore, we had to evaporate the samples and resuspend them in DMSO. Unfortunately, as a consequence of the technical complexity of the Folch method, it was impossible to obtain an efficient resuspension of our lipids in the DMSO, which remained undissolved at the bottom of our glass vials. Therefore, to attain a quantitative assessment of the FAs present in our cells, we finally decided to perform a thin layer chromatography (TLC) paired to gas chromatography flame ionization detector (GC FID). Thin layer chromatography is a simple, relatively cheap and reliable technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing (silica plate). In our study TLC was conducted to perform a first separation of the total FA retained in

the cells into three fractions: FFA, PL and TAG. Every lipid fraction has a different affinity for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined separated spots. An example of how TLC products appeared in our study is showed in Figure 11.

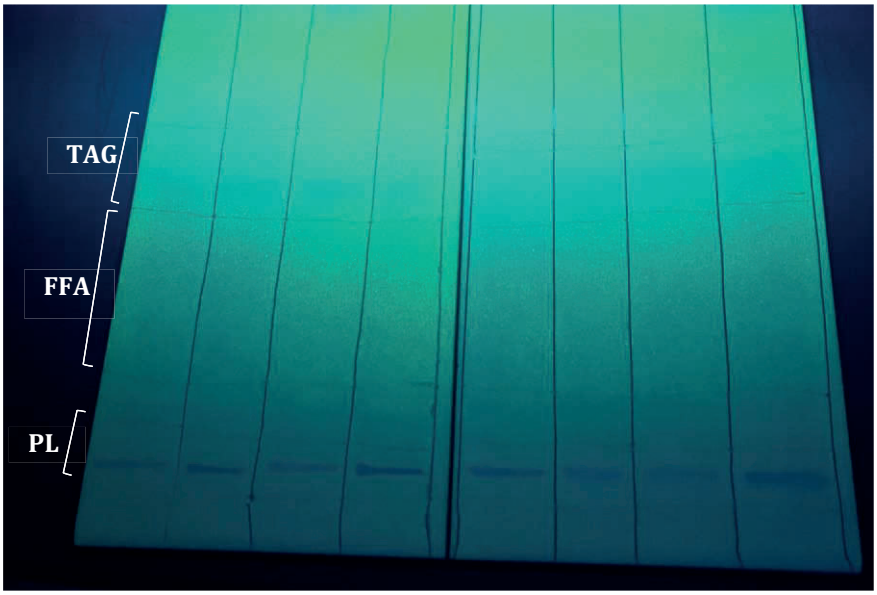

Figure 11. *Silica plate sprayed with a fluorescent solution showed, under the UV light, the three separated fractions present in the tested samples (TAG, triglycerides, FFA, free fatty acid, PL, phospholipids).*

After TLC was performed, the three FA fractions were scraped off the silica plates to perform the quantitative analysis of the single FAs via GC FID. The flame ionization detector is the most widely used detector for GC, often employed to detect and quantify organic compounds such as fatty acids (Misra et al., 2019). An alternative to the FID is the GC mass spectrometry. While the FID analysis offers a better overall quantitative characterization of the compounds, the MS provides a more qualitative representation of the samples (Misra et al., 2019). While using the FID, the analysis of cell-derived materials may represent a challenge to the sensitivity of the technique, because of the scarce amount of compound. Such issue may cause an overestimation of the quantity of some single FAs. A possible, still expensive and time consuming,

way to overcome such challenge in future studies may be to apply both FID and MS, in order to obtain quantitative and qualitative characterization of the samples.

12 Discussion of the results

Based on the summary of results presented in section 5.3, the following topics deserved further discussion than described in the papers.

1. Effects of production-related factors on steatosis symptoms and choline requirement

2. Effects of choline and lipid level on the pyloric caeca methylome of the Atlantic salmon

3. Establishment of an in vitro steatosis model

12.1 Effects of production-related factors on steatosis symptoms and choline requirement

Previous studies conducted to find causative factors of intestinal steatosis in Atlantic salmon highlighted the fundamental role of choline in intestinal lipid transport (Hansen et al., 2020b; Hansen et al., 2020a, Krogdahl et al., 2020). Further investigations estimated choline requirement in post-smolt Atlantic salmon fed a plant-based diet to be around 3.4 g/kg feed with a dietary lipid level of 29% (Hansen et al., 2020a). The primary hypothesis raised after those studies was that, in light of the importance of choline in lipid transport and metabolism, choline requirement may depend on dietary lipid intake. Additionally, other biological and environmental conditions, such as temperature, lipid source, and fish size may affect the requirement.

The lipid level+water temperature and the lipid source+fish size experiments were planned in order to investigate whether lipid level and water temperature (**Paper I**), and fish size and lipid source (**Paper II**) could influence steatosis symptoms and thereby choline requirement. The investigations were conducted by observing the

effects of the varying production-related conditions on the biomarkers which Hansen et al. (2020a) identified as closely related to lipid transport in the intestinal mucosa. Such biomarkers are: (1) organosomatic index of the pyloric intestine (OSIPI), (2) the histological evaluation of lipid vacuolation and macroscopically observed appearance of whiteness in the pyloric caeca, and (3) the expression of four genes involved in lipid transport and metabolism: *plin2*, *apoAIV, apoAI* and *pcyt1α.* The observations obtained from these two studies (**Paper I and II**) led to the overall conclusion that the main factor influencing choline requirement was the dietary lipid intake, which Hansen et al. defined as lipid load (Hansen et al., 2020a). According to the lipid level+environmental temperature and lipid source+fish size experiments, all these variable factors affected the lipid load, resulting in increased lipid accumulation in the pyloric intestine and annexed pyloric caeca and to a higher expression of the selected biomarker genes.

12.1.1 Effects of lipid level and water temperature

In fish and other poikilothermic animals, the rate of metabolic processes increases with increasing environmental temperature (Kieffer et al. 1998; Veilleux et al., 2015). Accordingly, feed intake and growth performance are highly influenced by water temperature (Liu et al., 2021; Qiang et al., 2017; Mellery et al., 2016). This pattern was also observed in the study reported in **Paper I**, where fish raised at 15 °C grew significantly faster than those at 8 °C. The accompanying higher lipid intake increased the severity of the steatosis symptoms in the fish raised at 15 °C, as indicated by all the biomarkers (the OSIPI, the histological evaluation, and the expression of *plin2*, *apoAIV, apoAI* and *pcyt1α).* Other than the biomarker genes, the expression of several other genes involved in fatty acid transport (*apolipoprotein-b, microsomal triglyceride transfer protein, fatty acid binding protein 2a2)*, cholesterol metabolism (*3-hydroxy-3-methylglutaryl coenzyme a reductase, acyl-coa cholesterol acyltransferase, atp binding cassette a1, isopentenyl diphosphate delta isomerase 1*) and phosphatidylcholine synthesis (*choline kinase, choline transporter and phosphatisylethanolamine n-methyltransferase*) was quantified. The results showed that some of these genes were more highly expressed in fish raised at 8 °C compared to those raised at 15 °C. Such an outcome contrasts with what observed in the other biomarkers and may be the

effect of the interaction between lipid levels and water temperature. In parallel to the effects given by the water temperature, the increasing dietary lipid level increased the severity of steatosis symptoms, resulting in a dose-response relationship at both water temperatures. The dose-response relationship between lipid level and steatosis was particularly clear for the histology results and the OSIPI.

Concerning the gene expression results, most of the genes showed a dose-dependent increase by the dietary lipid level at 8 °C. On the other hand, no clear relationship was observed at 15 °C, probably because of the interaction previously mentioned.

The discrepancies between the gene expression results and the other biomarkers may be caused by the complicated pathways, poorly described in fish, in which the assessed genes are involved. Choline kinase (encoded by *chk*) is the first enzyme involved in the Kennedy pathway (Figure 9), and it regulates the phosphorylation of choline to phosphocholine. In this study the expression of *chk* was upregulated by the increasing dietary lipid level, possibly indicating a higher choline requirement. The second enzymatic step involves the conversion of phosphocholine into the high energy donor CDP-choline, a process regulated by *pcyt1α*. In this study the expression of *pcyt1α* was the opposite of *chk*, being downregulated by the increasing lipid level (Figure 12). One could question why lipid level affected *chk* and *pcyt1α* in opposite directions, as both are involved in the same pathway, and somehow dependent on the same dietary choline supply. There are two likely explanations for this phenomenon. Firstly, higher expression of *chk* may have been induced by the insufficient supply of choline in fish fed the higher lipid level, in order to ensure that all the available choline is fully utilized. At the same time, since *chk* expression increased phosphocholine synthesis, it is likely that phosphocholine inhibited its own conversion into CDP-choline through a negative feed-back mechanism. Such a response has been described in a study conducted on human breast cancer cells (Zhu et al., 2008).

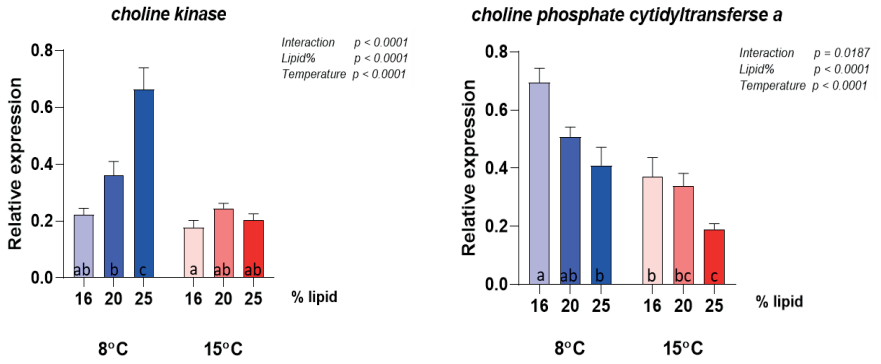

***Figure 12**. Expression patterns of choline kinase (chk) and choline phosphate cytidyltransferase a (pcyt1a). Data are mean normalised expression levels + SEM. Different letters denote statistically significant differences among diet groups.*

A second explanation may be related to the fact that there are two branches of the Kennedy pathway; one involved in the synthesis of phosphatidylcholine from choline (CDP-choline), the other involved in phosphatidylethanolamine production from ethanolamine (CDP-ethanolamine) (Gibellini & Smith, 2010). The enzymes involved in both pathways can overlap significantly. For instance, studies conducted in mammals demonstrated that the enzyme choline kinase (encoded by *chk*) can phosphorylate ethanolamine, in addition to choline, in the first step of the pathway (Kent, 1995). Similarly, in the last step of the pathway, choline phosphotransferase can use both CDP-choline and CDP-ethanolamine to synthetize phosphatidylcholine and phosphatidylethanolamine (Henneberry, Wistowe, & McMaster, 2000; Henneberry & McMaster, 1999; Hjelmstad et al., 1994). The only enzyme specific for phosphatidylcholine production is CDP-choline, regulated by *pcyt1α* (Hjelmstad et al., 1994; Kent, 1995). It is therefore possible that the higher expression of *chk* is related to its role in the phosphorylation of ethanolamine, and not choline.

The results obtained from the *lipid level+water temperature* study offered additional confirmation of the importance of dietary choline supplementation. In the materials and methods chapter of this thesis, it was discussed why the diet containing the

highest lipid level was supplemented with soy lecithin. Soy lecithin contains several types of phospholipids, including phosphatidylcholine. As reported by Krogdahl et al. (Krogdahl et al., 2020), phosphatidylcholine supplementation in adequate amounts will, similarly to choline supplementation, improve lipid transport and eliminate steatosis symptoms. The diet with 28% lipid contained 2.3 g/kg of choline, circa 0.4 g/kg more than the other diets. Such a difference slightly affected the severity of steatosis, lowering the expression of most of the biomarkers and confirming how choline supplementation is fundamental for lipid transport. However, although choline level was different among the diets, it was always too low to ensure adequate lipid transport. Therefore, no fish showed a normal intestinal condition. This indicated, consistent with Hansen et al., that 2.3 g/kg of choline is still not sufficient to eliminate steatosis symptoms (Hansen et al. 2020a).

12.1.2 Effects of lipid source and fish size

The importance of dietary fat level was confirmed by the observations of the lipid source+fish size study (**Paper II**).

First and foremost, fish size had significant effects on growth, with the small fish showing higher growth performance than larger fish. Nevertheless, the feed conversion ratio (FCR) was the same for the two groups. Generally, higher growth performance in smaller fish, especially for migratory species, is explained by a strong biological pressure to reach maturation and a body weight suitable to perform migration and reproduction. As observed in the lipid level+water temperature study, higher growth performances imply higher feed intake and therefore higher lipid intake, which increased the severity of the steatosis symptoms.

In parallel, the varying dietary lipid source (rapeseed oil to fish oil ratio) and the FA composition of the feed influenced lipid digestibility and consequently lipid supply to the fish. Rapeseed oil, characterized by a low melting point, is more digestible than fish oil, which has high levels of saturated and polyunsaturated fats and high melting point. Consistent with this, the results showed that increasing level of rapeseed oil to fish oil ratio increased fat digestibility.

The increased lipid digestibility led to a higher lipid supply to the fish, contributing to the dose-response effect observed from the histological assessment. However, to which extent the higher digestibility was involved in worsening steatosis symptoms cannot be estimated based on the present study. Another characteristic of fats with low melting point is that their digestibility is less influenced by environmental temperature. For instance, in the lipid level+water temperature study, where the primary lipid source was rapeseed oil, no differences in fat digestibility were observed between the two water temperatures groups. The impact of lipid digestibility on steatosis symptoms and therefore choline requirement is a matter to be taken into account when formulating further diets containing both fish oil and plant-based oil.

If the histological assessment offered a clear picture, the gene expression results showed an unexpected scenario, with the majority of the biomarker genes being unaffected by fish size or lipid source. A visual examination of the raw data might indicate a slight effect of fish size and rapeseed oil level in the examined genes, however, follow-up studies with a higher number of observations are needed to get more solid information on this aspect. The only gene which showed an effect of fish size was *apoAIV*, significantly more expressed in larger fish. This outcome contrasts with the histology results, which showed that smaller fish were the most affected by steatosis. A likely explanation for this outcome may be related to other unknown factors regulating *apoAIV* expression. In addition, large fish may have generally higher basal levels of *apoAIV* compared to small fish.

According to Hansen et al., the histologically observed degree of vacuolation and the macroscopically observed whiteness of the pyloric intestine were the indicators most closely related to steatosis (Hansen et al., 2020a). Therefore, they can be considered more reliable indicators of overall lipid transport processes. Additionally, it is possible that, despite the dietary choline deficiency in this study, the difference in lipid load among the diets was not large enough to result in differential expression of the transcriptome.

Nevertheless, a general lack of strong transcriptional response has been observed in previous studies addressing choline requirement. The works conducted by Hansen et al. (2020a; 2020b) and Krogdahl et al. (2020) highlighted that, even in those cases

where the histology results showed a significantly high severity of lipid vacuolization, the number of differentially expressed genes was lower than expected when comparing a normal vs a steatotic morphology.

12.1.3 Estimation of choline requirement

At the present state of knowledge, it is not possible to precisely estimate choline requirement for Atlantic salmon raised in conditions different from those described by Hansen et al. (2020a). However, the results obtained from the lipid level+water temperature and lipid source+fish size studies form the basis for discussion to obtain information on the magnitude of impact of these production-related factors on choline requirement. The estimation was conducted by taking into consideration those biomarkers that, throughout the studies, have demonstrated more sensitivity to steatosis symptoms i.e., the OSIPI and the histology scores of steatosis in the pyloric caeca. However, since the pyloric intestine is characterized by high adaptability to dietary changes, the OSIPI may not be considered as reliable as the histology scores when evaluating steatosis severity. The results obtained by Hansen et al.'s dose-response study (2020a) are used as the basis of our evaluation. In that study, the fish were fed a set of diets containing the same lipid percentage (29%) and increasing choline levels. A choline level of 1.9 g/kg corresponded to an OSIPI of 2.0, while when the choline level was 1.2 g/kg the OSIPI was 2.4. In our lipid level+water temperature study, the lipid level, raising from 16% to 25%, increased the OSIPI by 0.8 units, while the higher water temperature increased the OSIPI of 0.5 units. Considering that the average choline level for the diets was 1.9 g/kg, the combination of the effects of the increasing lipid level and water temperature may result in an increase of choline requirement of 600 mg/kg.

Although we did not observe direct effects of the increasing rapeseed oil level in the lipid source+fish size experiment, we demonstrated that the shift in FA composition influenced lipid digestibility and therefore lipid supply, worsening steatosis symptoms. The shift from fish oil to rapeseed oil caused a 5% increase of lipid digestibility, corresponding to an increase in the histological steatosis score of about 0.7 points. Considering that in the lipid source+fish size study the average choline

level for the diets was 1.5 g/kg, this variation suggests a shift in choline requirement of 600 mg/kg.

The higher lipid intake observed in the small fish affected the histology score by 0.5 points, corresponding to an increase in choline requirement of about 200 mg/kg. Based on the OSIPI, instead, the difference in lipid intake raised choline requirement of about 400 mg/kg in the small fish compared to the large ones. The combination of the effects of the different lipid sources and fish size may result in a shift of choline requirement of 1.0 g/kg.

12.2 Effects of choline and lipid level on the pyloric caeca methylome of the Atlantic salmon

Although the difference in choline supply among the tested diets was not large (1.9 vs 2.3 g/kg), the study identified the presence of differentially methylated cytosines (DMCs) in a set of genes involved in lipid transport (Figure 5). The effects given by the lipid percentage were only minor, but not absent.

A notable finding was the influence of both lipid level and choline supply on the expression of *tjap1*. Comparing the two diets with the same insufficient choline supply and the highest and the lowest lipid level, we observed that the promoter region of *tjap1* was hypermethylated in the fish fed the highest lipid percentage. On the other hand, when the choline supply among the diets was different, the promoter region of *tjap1* was hypomethylated in the higher-choline condition. These results suggest that *tjap1* may represent an epigenetic biomarker to assess the effects of dietary choline level on DNA methylation in the pyloric caeca. The role of *tjap1* is to encode tight junction associated protein 1, a fundamental component of all the tight junctions. Tight junctions are connections between epithelial cells able to maintain the mucosal integrity, control paracellular permeability and movement of ions, macromolecules and immune cells (Jutfelt & Sundh, 2023) (Figure 13). In teleost fishes, such connections comprise occludin and claudins. When tight junctions' strands are lost consequently to external interventions, the intestinal permeability is lost, leading to a condition known as "leaky gut"(Camilleri, 2019; Otani & Furuse, 2020).

It is likely that the hypomethylation given by the higher choline level upregulated *tjap1*. Although *tjap1* gene expression was not measured, the hypomethylation could represent an attempt to improve membrane integrity; a result which would be consistent with the important role of choline in the production of phospholipids, key components of cell membranes. Solute carrier family 22 member 15 (*slc22a15)* was another gene hypomethylated by the increasing choline level. This gene regulates the transport of betaine's subproduct, carnitine, across cells membranes. Carnitine controls the transport of FAs from the cytosol to the mitochondria, to be oxidized for energy production (Sreedharan et al., 2011; Yee et al., 2020). Betaine, as previously introduced, is the result of oxidation of choline and a direct methyl group donor. The study conducted by Krogdahl et al. (2023) on Atlantic salmon showed that dietary choline supplementation corresponded to higher levels of betaine in plasma, confirming that choline and betaine levels are correlated. It is therefore possible that the higher choline level in the diet induce a higher production of betaine, which induced hypomethylation of *slc22a15*, enhancing carnitine transport within the intestinal enterocytes, to improve FA transport and energy production.

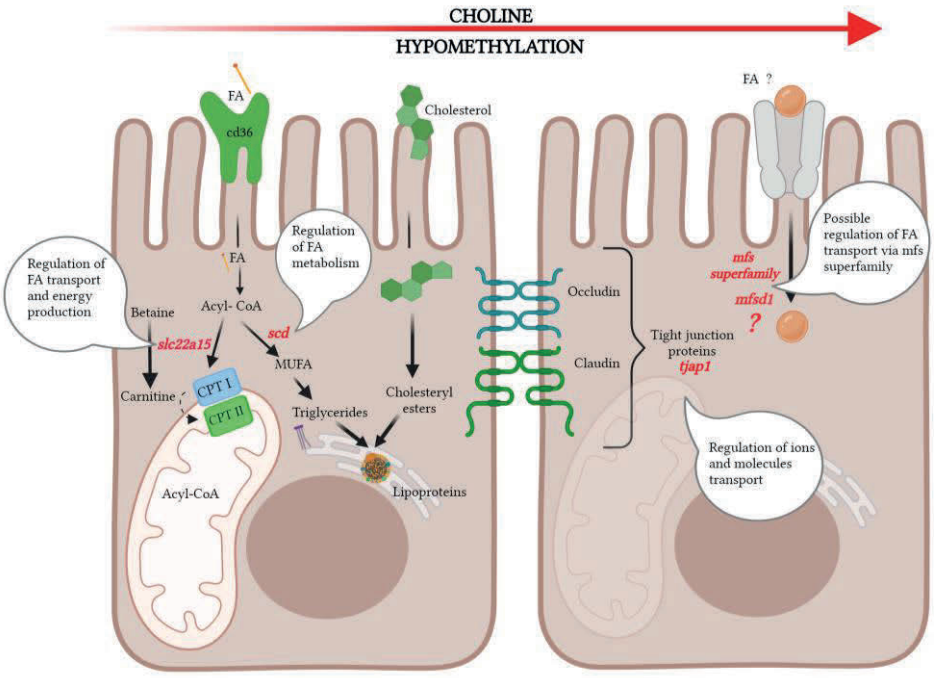

Figure 13. *Systematic representation of the epigenetic regulation in the pyloric caeca of farmed Atlantic salmon given by the increasing dietary choline supply. The higher choline inclusion in one of the experimental diets induced hypomethylation in the promoter region of slc22a15, scd, tjap1 and mfsd1, which are involved in lipid transport and metabolism.*
Image created with BioRender.

The slightly lower severity of steatosis symptoms may also have led to the hypomethylation of *scd. Stearoyl-CoA Desaturase* (*scd*) encodes an enzyme involved in the conversion of FFA in MUFA, primarily oleic acid (Paton & Ntambi, 2009). Monounsaturated fatty acids are then converted into TAG to be integrated into lipoproteins and transported into the bloodstream. Another gene whose promoter region was hypomethylated by the higher choline level is *mfsd1*. This gene is a member of the major facilitator superfamily (mfs) of transporters, which are involved in the transport of small molecules and nutrients across cell membrane.
The role of *mfsd1* has been mainly investigated in the mouse liver, where it regulates homeostasis and health (Massa López et al., 2019). The absence of information concerning its function in the intestinal tissue makes it challenging for us to speculate further. However, according to the results observed for the other genes, it is possible that its upregulation, given by the hypomethylation, improved FA transport (Figure 12). The effects given by the different lipid levels were less significant than those given by the choline level. However, it was interesting to notice that the gene *mir-212*, a short non-coding RNA molecule, was hypermethylated in the fish fed the highest lipid level. This gene regulates the expression levels of other genes through mRNA cleavage or repression of their translation (Burek et al., 2019).

12.3 Consequences of choline deficiency on growth performance, health, and welfare

According to the dose-response study conducted by Hansen et al. (2020a), steatosis symptoms may occur without clear effects on growth performance. Such a finding is confirmed by other studies conducted on Atlantic salmon (Espe et al., 2017; Krogdahl et al. 2020), fingerling channel catfish (Wilson & Poe, 1988a) and juvenile giant

grouper (Yeh et al., 2015). On the other hand, several experiments demonstrated beneficial effects of choline supplementation on the growth performance of juvenile Atlantic salmon (Hansen et al., 2020b; Hung et al., 1997; Poston 1989) and carp (Duan et al., 2012; Wu et al., 2011). Different outcomes of studies addressing choline deficiency may be related to the duration of the feeding trials. It is likely that fish subjected to insufficient dietary choline supply for long periods would show worse growth performance compared to fish exposed to the same diet for relatively short time. On the other hand, fish may find a way to overcome insufficient growth performance by increasing their feed intake; a strategy which would probably affect the FCR. It would be necessary, therefore, to evaluate the effects of choline deficiency on growth performance and fish development on a whole length production trial.

Choline deficiency may have implications on fish health not only related to growth performance. According to the Fish Nutrition book (Halver & Hardy, 2022), inadequate dietary choline supply may cause pathological changes and death in diverse fish species. For instance, Kitamura et al. (1967) described that a dietary choline level of 0.8g/kg leads to anemia, projected eyes, light colored body and extended abdomen in rainbow trout. The same condition caused hemorrhagic kidney and intestine in chinook salmon (Halver et al., 1957). Additionally, studies conducted on rainbow trout (Poston, 1989), hybrid tilapia (Shiau et al., 2000) and cobia (Mai et al., 2009) demonstrated that severe choline deficiency may strongly reduce fish survival rates. According to these studies, choline-deficient diets may be considered harmful to farmed Atlantic salmon welfare, especially if combined to extremely high dietary lipid level and if continued for long farming periods. In the lipid level+water temperature study, all the analyzed dietary groups showed steatosis symptoms, and to the highest lipid level (25%) corresponded a substantial number of fish severely affected. This outcome is unacceptable from a fish health and welfare perspective, especially considering that dietary lipid level fed to farmed Atlantic salmon may reach 40% in extreme cases. Additionally, such fat inclusion levels may be fed over a longer time period than that experienced by experimental animals. As we demonstrated that steatosis severity increases with increasing lipid level in farmed Atlantic salmon, the question arises whether prolonged exposure to highly lipidic diets would lead to

health issues, such as steatorrhea and other pathological changes. These types of health issues may make the fish more susceptible to factors like stressful management procedures (such as de-licing), infectious agents and parasites.

Another trait to be considered when addressing fish health is disease resistance, which represents at present the biggest cause of economic loss in the aquaculture industry (Mzula et al., 2021; Naylor et al., 2021). In this context, DNA methylation may correlate with the regulation of those genes potentially involved in disease resistance or susceptibility. For instance, hypermethylation of the promoter regions of immune genes can lead to their silencing, and therefore repress immune response to a specific pathogen. A study conducted on grass carp observed that targeted hypermethylation of a CpG island dedicated to resistance against grass carp reovirus (GCRV), inside the retinoic acid-inducible gene I (RIG-I), led to the downregulation of the gene and to a significant increase in susceptibility to the virus (Shang et al., 2016). Additionally, an experiment conducted on brine shrimp (*Artemia franciscana*) observed that treatments with a plant-based phenolic compound caused hypomethylation of a set of immune genes related to resistance against *Vibrio parahaemolyticus* (Roy et al., 2019). In the epigenetic study described in **Paper III**, one of the genes affected by the increasing choline level was transcription factor *irf4a*, whose role is of fundamental importance in regulating immune cell differentiation, including T and B lymphocytes, macrophages and dendritic cells (Nam & Lim, 2016). Studies conducted on mice reported that *irf4* regulates inflammation in diet-induced obesity, as well as lipolysis and lipogenesis (Eguchi et al., 2011; 2013).

The results obtained in our work showed that higher choline level hypomethylated *irf4a* in its intron region, suggesting a possible involvement of choline in disease resistance. Another gene influenced by higher choline level was dominant somatostatin receptor, *sstr2a*, which was hypermethylated in its promoter region. Somatostatin receptors mediate the action of somatostatin, which regulates secretory and proliferative responses of target cells (Patel, 1999).

Taken together these results support the hypothesis of a possible involvement of choline in disease resistance, important information to address in follow-up choline requirement studies.

12.4 Establishment of an in vitro steatosis model

The results discussed in **Paper IV** confirmed the hypothesis that high OA concentration in the exposure medium can lead intestinal cell lines to display the same lipid accumulation signs characteristic of the enterocytes of the Atlantic salmon affected by steatosis.
Such a result was supported by the formation of a high number of intracellular LDs. The LDs increased the surface area of the cells, changed their shape from a cubic to more rounded, and compressed the nuclei towards the cell membrane. These morphological features not only correspond to those of the in vivo enterocytes affected by steatosis, but they also resemble the appearance of preadipocytes cultured in vitro (Bou et al., 2020; Todorčević et al., 2008; Ytteborg et al., 2015). Further studies will therefore be necessary to assess the possibility of a maturation of intestinal cell lines into adipocytes. At present no immortalized cell line derived from fish adipocytes is available to conduct studies on lipid metabolism in adipose tissue.

The gene expression results not only confirmed the scenario of the lipid accumulation, but they also highlighted, for the first time, that these cells possess the same compartments responsible for production of proteins involved in lipid metabolism in vivo. The intracellular FA quantification confirmed that OA was accumulating in the cells with the increasing exposure concentration and time. In addition, OA distributed differently within the lipid fractions, being more concentrated in PL and in the TAG compared to the FFA fraction, a tendency which suggests an attempt of the cells to begin lipoprotein production (Bou et al., 2020).

An interesting result related to the FA quantification was a low retrieval of the different FAs in the TAG and FFA fractions compared to the PL. One of the most challenging aspects of extracting and quantifying lipids from cell samples is the low amount of material to extract from. Consequently, the amount of some FA classes may be so scarce that even GC will be unable to retrieve them. This was the case for the TAG and the FFA fractions, while the PL fraction was the most abundant as well as the

one showing the highest amount of retrieved FA. Therefore, it was our understanding that most of the classes were present in all the fractions, but they could not be retrieved because their amount was too low. On the other hand, one of the most abundant FAs retrieved in all the fractions was C15:1, a so-called odd-chain FA. Odd-chain FAs are not usually reported in lipid quantification studies, because their amount is often too low. When observing the raw data, we wondered if the high quantity of C15:1 could derive from the fetal bovine serum (FBS) used in the growth media for the cells. Odd-chain FAs are indeed largely abundant in cow's milk (Poppitt, 2020). However, we discovered that the quantification of FA classes is not performed on the commercially available FBS (Merck, personal comm.).

Another possible explanation for the high amount of retrieved C15:1 is, once again, related to the low quantity of available cell pellet. When analysing FAs from extremely limited samples, the output may be slightly "noisy", showing peaks derived from the plastic materials and possibly added antioxidants. Although most of the experiments were conducted on glass materials, the presence of plastic equipment could not always be avoided. An overall solution to both issues related to limited sample quantity may be to seed and expose the cells in plates with wells of big areas, or even in cell culture flasks. Although such an approach was considered while running the laboratory work, it was assumed detrimental for the repeatability and standardization of the experiment.

After the results obtained from **Paper IV** confirmed the possibility of the production of an in vitro steatosis model, the question arises whether the supplementation of growth media with specific nutrients (e.g., bile components) could initiate lipoprotein formation. Although studies of lipid metabolism in mammalian and fish intestinal cell lines seeded on 3D systems confirmed the ability of the cells to synthetize lipoproteins (Ponce de León-Rodríguez et al., 2019; Selvam et al., 2022), in no such experiments have the effects of other nutrients on lipid transport been investigated. In light of the results obtained from Hansen et al. (2020a) and discussed throughout in this thesis, dietary choline supplementation prevents diet-induced lipid accumulation in vivo. It is thus likely that the supplementation of a highly lipidic medium with choline may enhance lipid transport and lipoprotein formation also in

the RTdi-MI. The use of in vitro models to investigate the cellular mechanisms underlying lipid transport would represent a key achievement in the study of lipid metabolism in fish.

In vitro models are characterized by high versatility, a feature which makes them a particularly precious tools to study molecular mechanisms via genome editing. For instance, future approaches may foresee the use of CRISPR-Cas technology to support investigations of the role of specific genes involved in FA synthesis and transport (Qiu et al., 2022).

13 Main conclusions

The overall conclusion of the present study is that the main factor influencing choline requirement in Atlantic salmon is dietary lipid load.

Regarding **Paper I**

- Higher dietary lipid supply corresponded to a higher severity of steatosis symptoms, implying a higher choline requirement.

- Water temperature influenced fish metabolism and therefore feed intake and dietary lipid load. In consideration of the impact of lipid load on steatosis severity, it is possible to conclude that a higher water temperature corresponds to a higher choline requirement.

Regarding **Paper II**

- Dietary lipid source did not directly influence steatosis symptoms. However, the increasing rapeseed oil/fish oil ratio increased lipid digestibility, which increased lipid supply to the fish and therefore choline requirement.

- Fish size influenced feed intake and thereby lipid load, which were both higher in smaller fish. The increased lipid load influenced steatosis symptoms, implying a higher choline requirement in small fish.

Regarding **Paper III**

- Varying degrees of steatosis symptoms, caused by different dietary choline levels, induced hyper and hypomethylation of genes involved in FA biosynthesis and transport, lipolysis, lipogenesis and immune functions, confirming the role of choline in epigenetic regulation in Atlantic salmon.

- Different dietary lipid levels induced hyper and hypomethylation of genes involved in membrane transport, signaling pathways and microRNAs regulating lipid homeostasis.

Regarding **Paper IV**

- RTdi-MI exposed to high concentrations of oleic acid showed signs of lipid accumulation and lipid metabolism from cellular, biochemical, and molecular perspectives. Additionally, the morphology of RTdi-MI resembled that of in vivo enterocytes affected by steatosis.

14 Future perspectives

The present thesis re-confirmed the vital role of choline in lipid transport, highlighting the effects of production related conditions such as dietary lipid level and source, water temperature and fish size/background on the severity of steatosis symptoms. Additionally, this study has, for the first time, elucidated choline's function as an indirect methyl group donor in Atlantic salmon's epigenetic regulation.
However, many questions remain unanswered regarding choline requirement, FA metabolism and epigenetic regulation in Atlantic salmon.
The following matters should be addressed in future studies:

- What would choline requirement for Atlantic salmon raised at the most demanding conditions be?

- What are the transport routes of lipids from the intestine to the peripheral tissues and internal organs in Atlantic salmon?

- What are the pathways behind lipid metabolism and choline requirement influenced by different production-related conditions?

- Can we confirm *tjap1* as an epigenetic biomarker for dietary choline level? Are there other available biomarkers?

- Does choline have a role in disease resistance?

- Can RTdi-MI seeded on two-chambered systems produce lipoproteins?

- Can choline support lipoprotein formation when supplemented to RTdi-MI exposed to high concentrations of oleic acid?

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16 Papers I-IV

Paper I

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RESEARCH ARTICLE

Effects of dietary lipid level and environmental temperature on lipid metabolism in the intestine and liver, and choline requirement in Atlantic salmon (*Salmo salar* L) parr

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Abstract

Choline was recently established as an essential nutrient for Atlantic salmon at all life stages. Choline deficiency is manifested as an excessive accumulation of dietary fat within the intestinal enterocytes, a condition known as steatosis. Most of today's plant-based salmon feeds will be choline-deficient unless choline is supplemented. Choline's role in lipid transport suggests that choline requirement may depend on factors such as dietary lipid level and environmental temperature. The present study was therefore conducted to investigate whether lipid level and water temperature can affect steatosis symptoms, and thereby choline requirement in Atlantic salmon. Four choline-deficient plant-based diets were formulated differing in lipid level of 16, 20, 25 and 28 % and fed to salmon of 25 g initial weight in duplicate tanks per diet at two different environmental temperatures: 8 and 15 °C. After 8 weeks of feeding, samples of blood, tissue and gut content from six fish per tank were collected, for analyses of histomorphological, biochemical and molecular biomarkers of steatosis and choline requirement. Increasing lipid level did not affect growth rate but increased relative weight and lipid content of the pyloric caeca and histological symptoms of intestinal steatosis and decreased fish yield. Elevation of the water temperature from 8 to 15 °C, increased growth rate, relative weight of the pyloric caeca, and the histological symptoms of steatosis seemed to become more severe. We conclude that dietary lipid level, as well as environmental temperature, affect choline requirement to a magnitude of importance for fish biology and health, and for fish yield.

Keywords: **Choline requirement: Fish nutrition: Gut health: Lipid accumulation: Plant feed**

Introduction

Increasing use of plant ingredients in diets for Atlantic salmon, which inevitably changed content of micronutrients as well as antinutrients, have been suggested to be a possible cause of increased gut health challenges observed in farmed Atlantic salmon[1,2]. Steatosis in the pyloric caeca is one of the frequent symptoms[3], characterised by a whitish and swollen appearance of the intestine and by the presence of lipid droplets accumulating in the enterocytes. In severe cases, the condition is known as lipid malabsorption syndrome (LMS), and lipidic digesta is present throughout the intestinal tract, with lipid loss and pollution of the environment as concequenses[4]. The steatosis may be the result of various suboptimal conditions in the complex process of lipid digestion and absorption. Deficient supply of long-chain fatty acids may alter fatty acid metabolism in enterocytes and result in intracellular lipid accumulation, as shown by Bou *et al.*[5]. The latter study also showed that the fatty acid composition of the diet, intestinal mucosa, and liver differed, indicative of fatty acid metabolism in both body compartments. A study of intracellular trafficking

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of fatty acids employing the rainbow trout enterocyte cell line RTgutGC, showed differences between fatty acids regarding the accumulation of lipid droplets in the cytosol, being higher for oleic acid than palmitic acid[6]. A study conducted by Bogevik *et al.*[7] on enterocytes isolated from Atlantic salmon highlighted that elongation and desaturation of fatty acids is limited in these cells. It is, therefore, possible that variation in dietary fatty acid composition might affect lipid metabolism and turnover, and thereby the degree of lipid accumulation in the pyloric caeca. Additionally, it has been observed by Ballester-Lozano *et al.*[8], that gilthead sea bream (*Sparus aurata*) fed to a diet poor in *n*-3 LC-PUFA showed signs of lipid accumulation in the proximal intestine. However, the more likely explanation for the excessive lipid accumulation in enterocytes is deficient supply of choline, which will limit the capacity for production of lipoproteins for export of fat from enterocytes to the internal compartments of the fish[9,10]. In a series of studies in Atlantic salmon, an inverse relationship between symptoms of steatosis and dietary choline level has been demonstrated[9,11,12]. Choline is a water-soluble organic compound involved in a broad range of critical physiological mechanisms across all life cycle stages. Among its several functions choline plays a pivotal role in forming the hydrophilic head of the phosphatidylcholine molecule, a major component of the very low-density lipoprotein (VLDL) complex[13], which carries triglycerides synthesised in the intestine and liver to adipose tissue, muscles and other organs. Several signs of choline deficiency, such as high hepatic lipid concentration, reduced growth performance, early death and poor feed efficiency, have been reported in fish species such as carp (*Cyprinus carpio*)[14], lake trout (*Salvelinus namaycush*)[15], rainbow trout (*Oncorhynchus mykiss*)[16], yellow perch (*Perca flavescens*)[17], channel catfish (*Ictalurus punctatus*)[18], cobia (*Rachycentron canadum*)[19] and yellowtail kingfish (*Seriola lalandi*)[20]. However, only a few other studies with Japanese eel (*Anguilla japonica*)[21] and faba grass carp (*Ctenopharyngodon Idella*)[22] have addressed the lipid transport capacity in the intestine and described the characteristic whitish appearance of the intestine, as a result of a deficiency of choline.

The National Research Council[23] has recognised choline as an essential nutrient for several fish species, but for some, including the Atlantic salmon, the conclusion has so far been that choline is essential only at the very young stages. Choline is present in numerous raw materials, but the content differs greatly, in particular between marine and plant ingredients. Therefore, supplementation with choline is necessary for many fish species fed diets based on plant ingredients. Choline requirement of fish seems to vary between species and between experiments within species. The review of Mai *et al.*[24] reports estimates of choline requirement ranging from 500 mg/kg diet in a study of hybrid striped bass (*Morone saxatilis* _ *Morone chrysops*), to 4000 mg/kg diet[23] in a rainbow trout study. However, estimates of requirement in rainbow trout differ greatly between studies. One indicates a requirement of 800 mg/kg and suggests that many factors, biotic as well as abiotic, and not at least choice of deficiency biomarker, may be of importance for the requirement. Regarding Atlantic salmon, only one published study, by Hansen *et al.*[10], addresses choline requirement at seawater stages. The results showed that choline is essential for salmon also for fish in seawater. The obtained data made Hansen *et al.*[10] suggest that a dietary choline inclusion of 3350 mg/kg is necessary in order to avoid choline deficiency signs in the pyloric caeca of post-smolt Atlantic salmon[10] weighing around 450 g and fed a diet with 29 % lipid. The results call for further studies to define requirement under conditions which might be more demanding than those employed in the study. In most of previous studies of choline requirement in fish, the main biomarkers have been weight gain and liver lipid content[18,25–28]. However, in Hansen *et al.*'s studies, growth, liver index and lipid content were rather insensitive markers of variation in choline supply. The characteristics of the mucosa of the pyloric intestine (PI), on the other hand, such as organosomatic index, level of enterocyte vacuolation and expression of several genes involved in lipid assembly in the enterocytes, storage and transport were among the response variables which showed a clear dose-response relationship with dietary choline inclusion.

The experiment described in the present report is the first of two experiments following up the study of Hansen *et al.*[10]. These studies aim to identify conditions which might affect choline requirement in Atlantic salmon importantly. Choline's role in lipid transport suggests that dietary lipid level, lipid quality, feed intake, size, developmental stage, environmental temperature and day length may affect the requirement, and that such conditions interact in their effects on choline requirement, as further elaborated on in the discussion. The conditions selected for investigation in the present experiment were dietary lipid level and environmental temperature. The following addresses effects of lipid quality and fish size. The results of the present experiment, will, together with a second serve as basis for planning a final dose-response study with choline fed to the fish under the most choline-demanding conditions.

Materials and methods

To limit the use of fish for welfare reasons, and of experimental and laboratory facilities for cost and time reasons, a screening strategy was chosen for the experiments, i.e. observing effects in fish fed choline-deficient diets, containing choline in a range sensitive for variation in choline supply, i.e. less than 50 % of the level indicated as sufficient in the study of Hansen *et al.*[10]. To get an indication of the quantitative aspects of the observed variation, the observed variation in the biomarkers were compared to the corresponding variation in biomarkers in Hansen *et al.*'s study induced by variation in choline level and taken as indication of change in choline requirement. In Hansen *et al.*'s study choline was supplemented as choline chloride, a condition which might raise questions regarding variation in digestibility between sources. In a recent study, choline in plant-based diets, without and with choline chloride supplementation, showed digestibility above 90 % for all diets[29], highest for the supplemented diets. As the condition in the present and previous experiment differed to some extent, the indicated change in choline requirement

Table 1a. Diet receipts and results and nutrient content as formulated and analysed

	Diets			
	16	20	25	28
Ingredients, %				
Fish meal NA Con-Kix 72 %	20·0	20·0	20·0	20·0
Soya SPC >62 %, non gmo	24·2	16·1	8·1	0·0
Wheat gluten 80	0·0	5·8	11·6	17·4
Maize gluten 60 (min. 58 %)	10·0	9·6	9·3	8·9
Pea protein 65 %	13·3	13·4	13·6	13·7
Wheat (Milling quality)	14·8	11·6	8·4	5·2
Tapioca starch	0·0	0·7	1·3	2·0
Fish oil	3·2	4·9	6·7	8·5
Rapeseed oil, crude	7·4	10·3	13·3	14·9
Premix	0·5	0·5	1·1	1·1
Lecithin (Soy)	0·8	0·8	0·8	2·0
Formulated nutrient composition				
Formulated lipid (%)	16·0	21·0	26·0	31·0
Crude protein (%)	42·0	42·0	42·0	42·0
Gross energy (MJ/kg)	17·3	18·8	20·5	22·3
Total choline (mg/kg)	1619	1590	1560	1542
Analysed nutrients				
Dry matter (%)	93·0	92·3	92·0	92·9
Crude protein (%)	47·9	46·0	44·3	44·7
Lipid (%)	15·5	20·4	25·1	27·6
Choline (mg/kg)*	1930	1830	1940	2310
Nitrogen free extracts (%)	22·4	19·8	15·4	13·4
Ash (%)	8·6	7·4	6·8	7·5
Energy (MJ/kg)	20·6	22·3	23·4	24·0
Yttrium (%)	0·00044	0·00056	0·00049	0·00103

* See materials and methods for explanation and considerations regarding the high choline level in the D28 diet.

Table 1b. Content of fatty acids in the diets, % of sum of fatty acids*

	Diets			
Fatty acid	16	20	25	28
C14:0	2·05	2·10	2·05	2·12
C16:0	9·81	9·54	9·15	9·58
C16:1*n*7	2·76	2·98	2·97	3·03
C18:0	2·14	2·15	2·89	3·01
C18:1*n*9c	31·1	31·9	33·5	33·5
C18:2*n*6c	19·3	18·5	18·4	18·4
C20:0	0·36	0·37	0·50	0·48
C20:1*n*11	3·02	3·14	3·16	2·94
C20:2*n*6	0·16	0·17	0·18	0·18
C20:3*n*6	0·03	0·03	0·03	0·03
C20:4*n*6	0·15	0·15	0·14	0·17
C22:1*n*11	3·45	3·76	3·79	3·40
C18:3*n*3	5·20	5·18	5·16	5·21
C20:5*n*3	3·42	3·42	3·22	3·17
C22:5*n*3	0·22	0·22	0·21	0·22
C22:6*n*3	3·64	3·65	3·43	3·34
∑ Identified acids	88·0	88·7	90·8	90·9
∑ *n*-3	12·5	12·5	12·1	12·0
∑ *n*-6	19·8	18·9	18·8	18·9
∑ MUFA	40·6	42·2	43·7	43·2
∑ Saturated	15·1	15·0	16·2	16·8

MUFA, monousaturated fatty acids.
* The results show the area of peak for the fatty acid in the chromatogram given as % of sum of the fatty acids areas of which some were not identified.

should be considered only indicative, and not to represent an exact estimate of effects on the requirement.

Diets

Four similar diets with high content of plant ingredients, deficient choline level, and varying in lipid level from 16, 20, 25 and 28 % were formulated. The diets were formulated to be iso-nitrogenous, as often recommended for nutritional studies. This means that with increasing lipid content in the diet, ingredients with low protein content had to be replaced with ingredients with a higher protein level. For each step up of lipid in the diet, the alteration in these plant ingredients were changed proportionally, i.e. increasing level of maize gluten (80 % protein) and decreasing a mixture of SPC and wheat, all of high quality. The variations were expected not to change nutrient digestibility or passage rate. The formulations and macronutrient compositions are shown in Tables 1a and 1b. The diets were supplemented with standard vitamin and mineral premixes in accordance with NRC guidelines (2011)[23]. Yttrium oxide (0·50 g/kg) was added as an inert marker for estimation of apparent nutrient digestibility. The diets were formulated to contain level of long-chain ω3 fatty acids well above requirements to avoid other causes of steatosis than choline deficiency. The experimental diets were produced by extrusion (feed pellet size 6 mm) using a BC 45 twin screw extruder (Clextral, France). Upon arrival of the diets at the experimental site, fat leakage from the diet with 28 % fat was discovered. A new diet was made with higher content of soy lecithin for better emulsification. The consequences of the intervention, i.e. a dietary choline level higher than planned, was not realised until after the feeding period was completed. However, this unexpected event was found not to significantly disturb the aims of the experiment. Even though the results obtained for the diets containing 16, 20 and 25 % of lipid level were sufficient to draw conclusions regarding the aims of the study, the results for the 28 % diets are included in this presentation. In fact, the higher choline level in the 28 % lipid diet supplied useful additional information, confirming not only that the severity of the steatosis in the pyloric caeca varies with choline supply, but also the important role of choline for sufficient lipid transport and metabolism in the pyloric caeca. Additionally, to include the results for the diet with 28 % lipid, also strengthens the statistical power of the experiment.

Experimental animals and conditions

The feeding trial was conducted at Nofima's Research Station in Sunndalsøra (NO), which is 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 experiment. As no harmful procedures were forced upon the fish before euthanisation, a specific permission was not needed for this experiment. Atlantic salmon with an average initial weight of 24 g were assigned to $0{\cdot}6 \times 0{\cdot}6$ m^2 (125 L) flow through tanks, 140 fish per tank. Each diet was fed to fish in duplicate tanks

for each dietary treatment, and each water temperature: 8 and 15 °C, i.e. a total of sixteen tanks. The temperature range is considered to cover the optimal range for Atlantic salmon[30]. A 24 h light regime was employed. Water temperature was measured daily, and dissolved oxygen weekly to secure 80–100 % of saturation. To secure feeding to satiation, the fish were fed 15 % in surplus of anticipated requirement, according to feeding tables and expected growth rate, using belt feeders.

Sampling

After 5 weeks of feeding, the biomass in each tank was reduced to keep biomass at a lower level to ensure sufficient oxygen supply. A total of fifty fish were randomly removed from each tank at the highest temperature, and twenty fish per tank at the lower temperature, to similar biomasses in all tanks. After another 3 weeks of feeding, six fish in fed state from each tank were sampled randomly, anaesthetised with tricaine methane-sulfonate (MS-222) and killed by a sharp blow to the head, in accordance with the Norwegian Animal Welfare act. Weight and length of each sampled fish were recorded. Fish remaining in the tanks at the end of the sampling were weighed in bulk. Total weight gain was calculated by adding up the weights of the sampled and the remaining fish. Blood was sampled from the caudal vein into vacutainers with lithium heparin and kept on ice. After centrifugation, plasma was collected in 2 ml aliquots, frozen in liquid nitrogen and kept at 80 °C. Following blood sampling, the fish were opened ventrally, and the abdominal organ package was removed from the abdominal cavity. The liver was separated from the package and weighed. Thereafter, the intestine, freed of external fat was sectioned as follows: PI, the section from the pyloric sphincter to the most distal pyloric caecum; mid intestine (MI), from the latter pyloric caecum to the earliest area with higher diameter and darker pigmentation, distal intestine (DI), from the latter end of the MI to the anus. The sections were opened and digesta from the section were collected, snap-frozen in liquid N_2 and stored at −80 °C. Those fish found with empty intestine were excluded from the sampling. Each intestinal section was then weighed before tissue samples were collected for histological and gene expression analyses. The samples for histological examination were immediately fixed in 10 % neutral-buffered formalin (4 % formaldehyde) and kept at room temperature. Whereas the samples for gene expression analyses were rinsed in sterile saline water, stored in RNA later® at 4 °C, and moved to −40 °C after 24 h. The fish that were removed for reduction of biomass at 5 weeks were stripped for faeces, while the remaining fish in the tanks were stripped for faeces at termination of the experiment, method as described by Austreng[31]. The faecal samples were pooled for each tanks, frozen in liquid N_2 and stored at −20 °C.

Chemical analyses of feed, gut contents, plasma, PI and liver including fatty acid analyses (FAME)

Samples of the feed and faeces were analysed for dry matter (DM), ash, crude protein (CP) and crude fat (CF) and energy at Nofima, Sunndalsøra. Fatty acid content was analysed at the Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway. DM was determined by drying the samples to a constant weight at 103 °C. Determination of ash content, samples were combusted at 550 °C for 10 h. Total nitrogen was analysed by Kjeldahl auto analyzer, and energy by bomb calorimetry (Parr 1271 adiabatic bomb calorimeter). Fatty acid composition was quantified by the FAME method described by O'fallon[32]. Choline level in the diets was analysed by Eurofins. The method involves extraction by methanol and water, alkaline hydrolysis to free choline from phosphatidyl choline, and quantification by isotope dilution LC-MSMS. The choline results show total choline, free and bound.

Analyses of yttrium content in feed and faeces were carried out by pre-digestion with concentrated ultrapure HNO_3 at 250 °C using a Milestone microwave UltraClave III (Milestone Srl, Sorisole, Italy). Samples were then diluted (to 10 % HNO_3 concentration), and yttrium was determined by inductively coupled plasma optical emission spectrometry (ICP-OES analysis) with a PerkinElmer Optima 5300 DV (PerkinElmer Inc., Shelton, CT, USA). The plasma nutrient and cholesterol analyses were carried out at the Central Lab of the Faculty of Veterinary Medicine, by standard hospital methods.

Histology

Pyloric caeca samples were processed at the Norwegian University of Life Sciences (NMBU) using standard histological techniques: dehydration in graded ethanol, clarification in xylene, embedding in paraffin and sectioning of 5 µm thick sections. The sections were then dewaxed, re-hydrated and stained with haematoxylin and eosin to perform the histological evaluation. The samples were randomised, and the main signs of vacuolation were assessed using a light microscope. According to the proportion of tissue affected by the presence of lipid-like vacuoles, swollen and irregular cells and condensed nuclei, the severity of the syndrome was scored as Normal (≤10 %), Mild (10–25 %), Moderate (25–50 %), Marked (≥50 %) and Severe (≥75 %) (Fig. 1).

RNA extraction, cDNA synthesis and gene expression analyses

Gene expression analysis was performed from ninety-six samples collected from the pyloric caeca of the same six fish per tank used for the preceding analyses. Gene expression profiling was conducted by Quantitative Real-Time PCR (qPCR) following the MIQE guidelines[33]. Total RNA was extracted from pyloric caeca samples (around 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). RNA purity and yield was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and RNA integrity was assessed by 2100

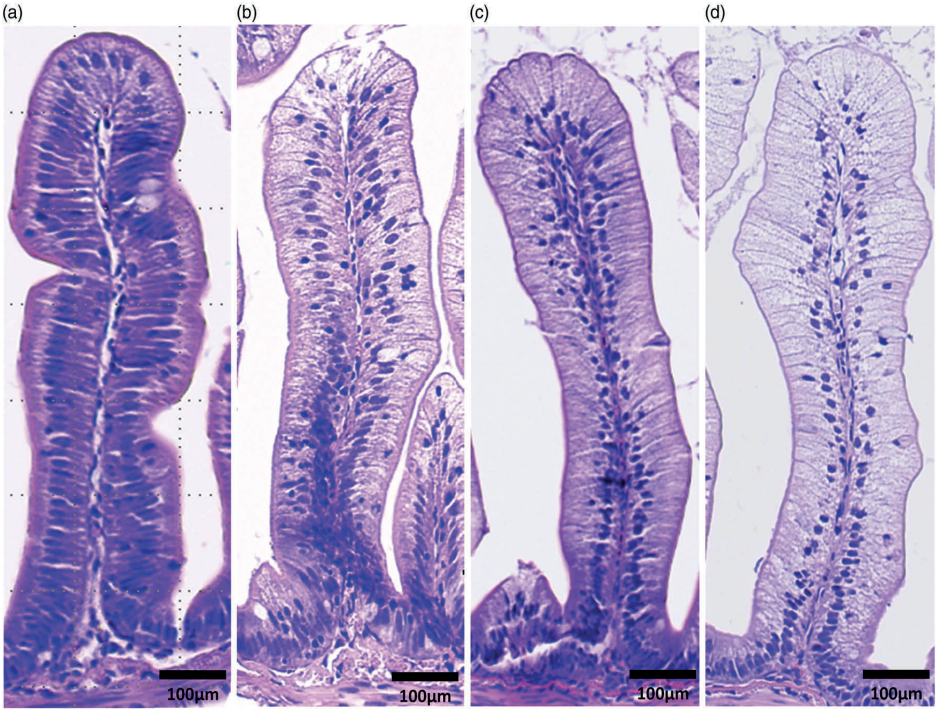

Figure 1. Histological severity of vacuolation of the pyloric caeca tissue (steatosis) representative for (a) normal, (b) moderate, (c) marked and (d) severe.

Bioanalyzer by the use of a RNA Nano Chip (Agilent Technologies). Total RNA was stored at −80 °C for upcoming analyses. Before proceeding with the synthesis of the first-strand cDNA, the RNA from the fish of each tank was pooled two by two. Afterward, 0·8 µg of the total pooled RNA, oligo $(dT)_{20}$ primers and Superscript III in 20 µl reactions (Invitrogen) were used to conduct the synthesis. To achieve negative controls, the same process was performed, omitting RNA and enzyme. cDNA was then diluted 1:10 and stored at −20 °C before the following qPCR procedure. The gene expression profiling was performed on a pool target genes involved in lipid metabolism, lipid uptake and transport and phosphatidylcholine synthesis. The primers to be used in the qPCR reaction were obtained from literature and from previous works conducted in this group[9,12]. Additional information concerning genes name or primers source, efficiency and size, is shown in Supplementary Table S1. For new assays, primer optimisation was carried out by PCR gradient assays, followed by assessment of PCR reaction efficiency (E) using serial dilutions of a pool of randomly selected cDNA samples. A LightCycler 96 (Roche Diagnostic) was used to perform DNA amplification and gene expression analyses. Each reaction mix contained 2 µl PCR-graded water, 5 µl of LightCycler 480 SYBR Green I Master mix (Roche Diagnostics) and 0·5 µl of both forward and reverse primer. Every sample was analysed in duplicate alongside a no template control. The three-step qPCR program included an enzyme activation step at 95 degrees for 5 min, followed by 40–45 cycles of 95 degrees (10 s), 55, 58, 60 or 63 °C (10 s), and 72 °C (15 s). Quantification cycle (Cq) values were calculated using the second derivative method. The products obtained from the qPCR were assessed analysing the melting curve. To perform relative normalisation of the qPCR assay, a pool of reference genes was selected as suggested by Kortner *et al.*[34]. Considering the absence of a universally stable reference gene, we tested the stability of a selected pool already used by our group for several previous studies conducted on the pyloric caeca of Atlantic salmon[9,11]. The most stable reference genes, selected based on their overall coefficient of variation (CV) and their interspecific variance were RNA polymerase II (*rnapoli*), hypoxanthine phosphoribosyl transferase 1 (*hprt1*) and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). Mean normalised expression of the target genes was calculated from raw Cq values by relative quantification[35]. The panel of target genes was the same used by Hansen *et al.*[8–10] and it is represented by a set of genes involved in lipoprotein assembly, choline and phosphatidylcholine synthesis and cholesterol synthesis. As also done in the previously cited studies by Hansen *et al.*, among the whole pool of genes, four were selected as main molecular biomarkers because of their particular receptiveness to steatosis: *plin2*, *apoA-I*, *apoA-IV* and *pcyt1α*.

Calculations

Fish growth was calculated as specific growth rate (SGR, percentage growth per day)= ((ln FBWg/ln IBWg)/D) × 100. IBW and FBW represent the initial and final body weight as

tank means, and D represents the number of feeding days. The condition factor was calculated as: CF =((FBW × 100)/ fork length cm^3). The organosomatic indices (OSI) were calculated as: (organ weight g/body weight g) × 100. Apparent digestibility (AD) for each nutrient was determined by using yttrium oxide (Y_2O_3) as inactive marker and estimated as follows: $AD_n = 100 - (100 \times (M_{feed}/M_{faeces}) \times (N_{feed}/N_{faeces}))$, where M represents the percentage of Yttrium oxide in feed and faeces and N represents the percentage of a specific nutrient in feed and faeces.

Statistical analyses

In light of the fact that this experiment was a screening study and had a regression design with close relationship between the five treatments, duplicate observations were considered sufficient. The design gives 11 degrees of freedom for the error term, and a reasonably accurate estimate of the error. Tank mean was used as the statistical unit. The results were subject to two-way analyses of variance (ANOVA) with lipid level and temperature as class variables. The Shapiro–Wilk test was used to assess the normality of the variance. The Tukey's test was used rank treatments in the molecular analyses, while Duncan's test was used for the other results. The level of significance for all analyses was set at $P < 0{\cdot}05$, and P-values between 0·05 and 0·1 were considered to indicate a trend in effects as indicated in the text. The plan was to evaluate the results by regression analyses, but the event described above in the production of the D28 diet, made the result for the diet with the highest lipid content unsuitable for regression analyses. The two-way ANOVA was therefore chosen as the most suitable method.

Results

Organosomatic indices (OSI), histological evaluation and lipid content in pyloric and liver tissue

Relative organ weight was calculated for PI (OSIPI) and liver (HSI) (see Table 2). Increasing fat level in the diet increased OSIPI significantly, with a difference of 0·8 units between fish fed the diet containing the 16 and the diet containing the 25 % of dietary lipid inclusion at 8 °C, as well as at 15 °C. The effect of temperature was significant and averaged 0·5 units. The analyses of fatty acid composition of the PI showed that lipid accumulation was the main cause of the increase in OSIPI, increasing in sum of fatty acids by 8–9 %, independent of temperature (Fig. 2). The histological examination of the PI tissue (Fig. 3) gave results in line with observations of lipid content and showed severe increase in accumulation of lipid droplets in the cytosol of the enterocytes, i.e. steatosis, with increasing dietary lipid level at both temperatures (P=0·0003). The difference between the temperatures was not significant (P=0·3170).

Concerning HSI, no significant differences were observed between fish fed the 16 and 25 % lipid diets, but in the case of fish fed the diet with 28 % of lipid level, elevated the index significantly by 0·2 units at both temperatures, and an average difference between the temperatures of 0·4, lowest at the low temperature (Fig. 2).

Growth performances and apparent nutrient digestibility

The fish grew well and only two fish, from different treatments, died during the feeding period. Feeding activity and consequently growth performance (Table 2) was significantly

Table 2. Results for all fish in the tanks regarding growth (SGR and TGC), and for sampled fish regarding body weights (BW, g), body length (BL, cm), yield, and organosomatic index of pyloric intestine (OSIPI, %), sum of fatty acids in pyloric caeca (LipPI, %), liver index (HSI, %), sum fatty acids in liver (LipLi, %) given as means of lipid level and temperatures, and treatment means, and statistics from two-way ANOVA

		Performance, organ weights, lipid level								
Temperature	Lipid	SGR	TGC	BW	BL	Yield	OSIPI	LipPI	HSI	LipLi
Class means										
	16	1·98	1·62	67·8	17·0	87·0^{a}	3·3^{b}	8·1^{c}	1·2^{b}	4·4^{ab}
	20	1·92	1·62	70·7	17·2	87·0^{a}	3·6^{b}	13·9^{b}	1·2^{b}	5·0^{a}
	25	1·90	1·54	66·4	17·0	86·5^{b}	4·1^{a}	20·2^{a}	1·2^{b}	4·8^{a}
	28	1·96	1·57	68·3	17·1	86·4^{b}	4·1^{a}	16·6^{ab}	1·4^{a}	3·5^{b}
8		1·14^{b}	1·32^{b}	47·2^{b}	15·4^{b}	86·2^{b}	3·5^{b}	14·8	1·5^{a}	6·3^{a}
15		2·74^{a}	1·86^{a}	89·4^{a}	18·7^{a}	87·2^{a}	4·0^{a}	14·4	1·1^{b}	2·5^{b}
Tank means										
8	16	1·21	1·39	46·2	15·3	86·7	3·0	8·8	1·4	6·2
8	20	1·19	1·31	44·9	15·2	86·4	3·5	13·4	1·4	7·4
8	25	1·10	1·29	49·9	15·6	85·8	3·8	19·0	1·5	6·7
8	28	1·05	1·27	47·9	15·5	85·9	3·7	17·8	1·6	5·0
15	16	2·74	1·85	89·4	18·7	87·3	3·5	7·3	1·0	2·6
15	20	2·65	1·94	96·6	19·2	87·6	3·7	14·4	1·0	2·5
15	25	2·70	1·79	82·9	18·4	87·1	4·3	21·4	1·0	2·9
15	28	2·87	1·87	88·8	18·7	86·9	4·5	15·3	1·2	2·0
*Statistics (Two-way ANOVA)**										
p(model)		<0·0001	<0·0001	<0·0001	<0·0001	0·0003	0·0004	0·0004	<0·0001	<0·0001
p(temp)		<0·0001	<0·0001	<0·0001	<0·0001	<0·0001	0·0016	0·8638	<0·0001	<0·0001
p(lipid)		0·3285	0·4254	0·7969	0·9133	0·0248	0·0012	0·0002	0·0031	0·0892
Pooled SEM		0·051	0·057	4·4	0·32	0·22	0·17	1·7	0·048	0·44

* The results are based on a model without interaction as a model with interaction gave insignificant results for the interactions. Yield = 100*(gutted weight/body weight).

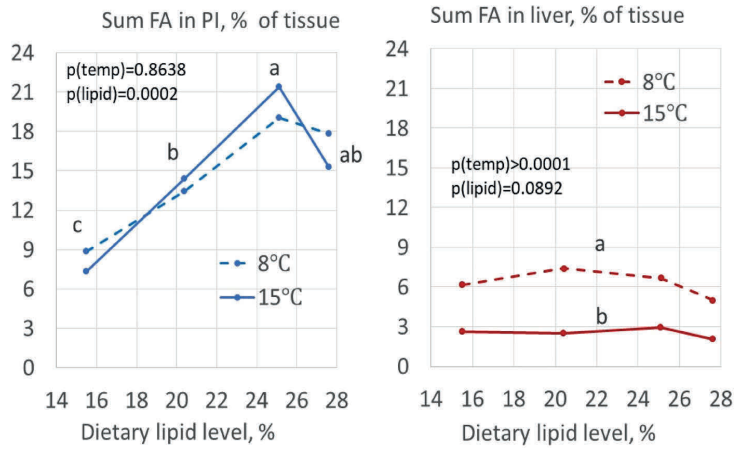

Figure 2. Effects of dietary lipid level on sum of fatty acids (Sum FA) in tissue from pyloric intestinal (left, PI) and liver (right).

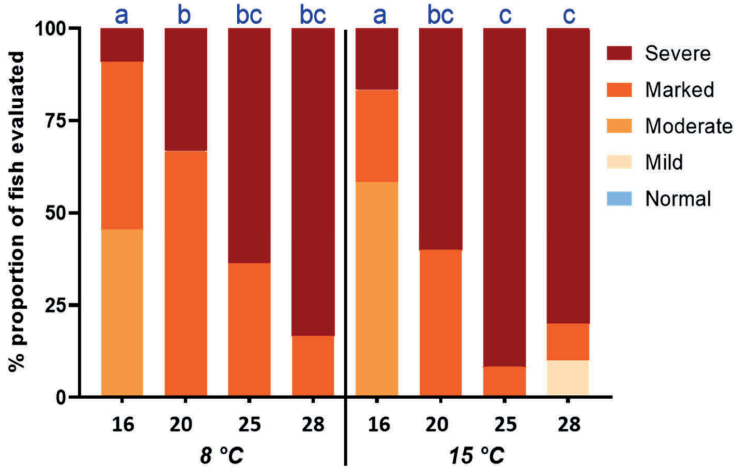

Figure 3. Number of pyloric caeca tissue scored for enterocyte steatosis. *X*-axis presents dietary lipid level at two rearing water temperatures of 8 and 15 °C. Superscript letters represent a significant statistical difference.

and positively affected by water temperature. Dietary lipid level did not affect final weight significantly. However, yield decreased with increasing lipid level from 16 to 25 %, whereas no further decrease was observed for the highest lipid level.

The results regarding nutrient digestibility are shown in Table 3. Lipid digestibility decreased slightly but significantly with increasing lipid level in the diet, whereas digestibility of CP, energy, ash and DM showed the opposite picture. Faecal DM was lower for the high lipid diet than for the other diets. Regarding effects of temperature, the only significant difference was observed for DM, for which digestibility was lower in fish raised at 15 compared to 8 °C.

Lipid content in pyloric and liver tissue

Tables 1b, 4 and 5 present results regarding content of individual fatty acids in the diets, and in the tissues of the pyloric caeca and liver, expressed as relative values, i.e. % of total fatty acid. The corresponding results, in absolute amounts, i.e. expressed as g/kg tissue, are illustrated in Fig. 4. The statistical evaluations of these results are presented in Table 6 for the relative values, and in Table 7 for the absolute amounts. The results for the proportion of saturated, monounsaturated and C18:2 fatty acids (Tables 4–6) show that the profile for these fatty acids in the pyloric tissue, to a great extent, mirrored the diet, while the effects of temperature were minor. The liver showed a somewhat different picture, less clearly mirroring the diet composition, e.g. with lower level of C18:2, and with greater effects of temperature which differed in direction depending on the fatty acid in focus, e.g. regarding C16:0 and C18:1, both showing significant temperature effect. The content of *n*3 fatty acids in the pyloric tissue showed a more dynamic picture. Temperature effects were minor also for these fatty acids. The liver, again, showed a different picture

Table 3. Results regarding apparent nutrient digestibility and faecal dry matter given as means of lipid level and temperatures, and treatment means, and statistics from two-way ANOVA

		Digestibility					
Temperature	Lipid level	Lipid	Protein	Ash	Energy	Dry matter	Faeces DM
Class means							
	16	95·8^{a}	90·0^{c}	34·6^{b}		75·1^{b}	12·5^{a}
	20	96·2^{a}	90·9^{b}	37·6^{ab}		79·0^{b}	12·3^{a}
	25	94·6^{b}	91·5^{a}	41·1^{b}		79·0^{b}	11·9^{a}
	28	94·4^{b}	91·8^{a}	50·6^{a}		83·0^{a}	10·9^{b}
8		95·2	90·9	42·8^{a}		80·0^{a}	12·0
15		95·2	91·2	39·2^{b}	85·8	79·2^{b}	11·8
Tank means							
8	16	95·8	90·0	37·0		76·0	12·7
8	20	96·3	90·8	40·3		79·6	12·6
8	25	94·5	91·3	42·7		81·4	12·2
8	28	94·4	91·4	51·1		83·1	10·7
15	16	95·8	90·0	32·2	81·9^{c}	74·2	12·4
15	20	96·0	90·9	34·8	86·1^{b}	78·4	12·0
15	25	94·8	91·7	39·6	87·1^{ab}	81·2	11·6
15	28	94·3	92·2	50·1	88·1^{a}	83·0	11·1
*Statistics (Two-way ANOVA)**							
p(model)		0·0046	0·0002	<0·0001	0·0006	<0·0001	0·0028
p(temp)		0·9892	0·1005	0·0011		0·0414	0·2827
p(lipid)		0·0023	0·0001	<0·0001	0·0006	<0·0001	0·0016

* The results are based on a two-way model without interaction as a model with interaction gave insignificant results for the interactions. For digestibility of energy, faecal samples from fish fed at low temperature was insufficient and prevented analyses for energy content. For DE, a one-way analysis was performed, as sample size from the fish raised at low temperature was insufficient for analyses.

with elevated levels of C22:6*n*3, and great, significant decreasing effect of increasing temperature.

Expressed as amount in the tissue (%, Tables 4, 5, and 7), the results show the same pattern in the pyloric caeca as well as in the liver, but with greater differences due to the pronounced differences in lipid content in the organs. The effect of the unintentional raise in choline level between diet containing 25 and 28 % lipid was particularly pronounced, with decrease in fatty acid accumulation in the pyloric caeca tissue for most of the fatty acids, an effect which was not seen in the liver.

The results for the *n*3 fatty acids (Fig. 5) appeared more dynamic, but with minor effect of temperature. The results for the liver indicate conversion of the C18:3*n*3 and C20:5*n*3 fatty acids to C22:6*n*3 this, and with clear temperature effects, in particular for C22:6*n*3. Table 7, which shows the quantitative aspects of these results, confirms the former results. They also illustrate the magnitude of the change in lipid metabolism, which was greater than indicated by the graphs which illustrate the results in g/kg tissue, whereas the tissue weight increased about 25 %. The drop in fatty acid level in the pyloric caeca between the diets containing 20 and 25 % of lipid level, most likely, was due to the higher choline content of this diet.

Intestinal gene expression

The gene expression analyses were conducted on the same panel of genes selected by Hansen *et al.*[10] to describe possible metabolic alterations induced by variation in choline supply and support discussion regarding choline requirement. The results for the highest lipid diet (28 %) did not give meaningful results in this context, due to the event in the feed production described above and are therefore not included in the interpretation of the molecular results. Selected genes showing clear effects of either dietary lipid level or temperature are illustrated in Fig. 6 and complete results are presented in Figs. 7–10. Overall, the expression of the genes were higher at 8 °C than at 15 °C, and there was a significant or close to significant interaction between temperature and dietary lipid level for all the genes. Whereas most of the genes at 8 °C showed increasing expression with increasing lipid level, the expression increased or was stable between the diet containing 16 and 20 % of fat and decreased, or tended to decrease, between the 20 and 25 % at 15 °C. The exception was *pcyt1α* which showed decreasing expression with increasing lipid level at both temperatures (Fig. 6). At 8 °C, the expression of *plin2* was significantly upregulated with increasing lipid level, while at 15 °C, the pattern was slightly different showing an upregulation up to 20 % of fat inclusion, for then to decrease at 25 % of dietary lipid level. Concerning both *apoA-I* and *apoA-IV*, at 8 °C their expression followed the same dose-response curve observed for *plin2*. However, at 15 °C, the picture was less clear. While *apoA-IV* showed the same pattern observed in *plin2*, with the expression peaking at 20 % dietary lipid and then decreasing with the highest lipid percentage, *apoA-I* showed an inverse relationship, being highly expressed with the lowest lipid inclusion. The curve describing the expression of *pcyt1α* followed at both temperatures a clear inverse relationship with the dietary lipid inclusion. The same trend was observed at 15 °C. The majority remainder of the assessed (Figs. 7–10) genes showed the same expression pattern already observed in the biomarker genes (Fig. 6). At 8 °C, the genes involved in fatty acid synthesis, like *srebp1*, *acat* and *hmgcr*, were upregulated with the increasing lipid level. On the

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Table 4. Content of saturated, monounsaturated, *n*6, *n*9 and *n*11 fatty acids, % of total fatty acids, in pyloric caeca and liver tissue with indicators of significance of effects of lipid level and temperature given as means of lipid level and temperatures, and treatment means*

Temperature	Lipid	C14:0	C16:0	C16:1*n*7	C18:0	C18:1*n*9c	C18:2*n*6c	C20:0	C20:1*n*11	C20:2*n*6	C20:3*n*6	C20:4*n*6	C22:1*n*11
Pyloric caeca													
	15·5	2·01^{b}	10·73^{a}	2·41^{c}	2·97^{a}	28·79^{c}	14·70^{b}	0·32	3·62^{b}	0·94^{a}	0·79^{a}	0·50^{a}	3·31
	20·4	2·10^{ab}	9·72^{a}	2·71^{b}	2·61^{b}	31·90^{b}	15·19^{b}	0·36	3·99^{a}	0·87^{b}	0·54^{b}	0·33^{b}	3·86
	25·1	2·14^{a}	9·07^{c}	2·86^{ab}	2·47^{c}	35·91^{a}	16·26^{a}	0·37	4·16^{a}	0·84^{bc}	0·43^{c}	0·24^{c}	3·86
	27·6	2·16^{a}	9·43^{bc}	2·91^{a}	2·55^{bc}	35·10^{a}	16·07^{a}	0·35	3·90^{ab}	0·80^{c}	0·41^{c}	0·30^{b}	3·51
8		2·09	9·60	2·68	2·59^{b}	32·17^{b}	14·93^{b}	0·36	3·96	0·81^{b}	0·55	0·36^{a}	3·82
15		2·11	9·87	2·76	2·71^{a}	33·68^{a}	16·19^{a}	0·34	3·88	0·92^{a}	0·54	0·32^{b}	3·46
8	15·5	2·03	10·46	2·38	2·88	27·99	13·82	0·34	3·80	0·90	0·80	0·52	3·59
8	20·4	2·07	9·58	2·67	2·50	31·00	14·76	0·35	3·91	0·84	0·55	0·36	3·84
8	25·1	2·18	9·16	2·87	2·43	35·37	15·78	0·36	4·14	0·78	0·45	0·26	3·87
8	27·6	2·11	9·21	2·81	2·53	34·34	15·35	0·38	3·98	0·74	0·39	0·30	3·96
15	15·5	1·99	10·99	2·44	3·05	29·60	15·57	0·31	3·44	0·98	0·77	0·48	3·03
15	20·4	2·14	9·87	2·75	2·72	32·80	15·63	0·37	4·08	0·91	0·53	0·29	3·88
15	25·1	2·10	8·99	2·84	2·51	36·46	16·75	0·37	4·17	0·91	0·42	0·21	3·86
15	27·6	2·21	9·64	3·00	2·57	35·86	16·80	0·32	3·83	0·86	0·42	0·30	3·06
Liver													
	16	1·26	11·61	2·14	6·28	28·20	8·30	0·16	3·55	1·55	2·23	1·63	0·76
	20	1·24	10·68	2·12	5·49	28·10	9·21	0·16	3·90	1·94	2·03	1·36	0·79
	25	1·24	9·43	2·05	4·29	28·64	10·20	0·16	4·27	2·10	1·66	1·22	0·90
	28	1·25	10·62	1·88	4·44	25·48	9·70	0·17	3·74	1·97	1·54	1·31	0·88
8		1·38	9·22	2·56	5·15	33·01	10·20	0·17	4·42	1·75	1·60	0·96	0·98
15		1·11	11·95	1·54	5·10	22·20	8·50	0·14	3·31	2·03	2·13	1·80	0·68
8	16	1·39	10·03	2·74	6·28	34·20	9·14	0·17	4·13	1·43	1·90	1·07	0·88
8	20	1·38	9·02	2·74	5·53	34·77	10·27	0·18	4·56	1·82	1·61	0·85	0·99
8	25	1·37	8·77	2·45	4·37	32·33	10·71	0·17	4·61	1·97	1·51	0·92	1·05
8	28	1·39	9·06	2·32	4·42	30·74	10·68	0·19	4·39	1·79	1·37	0·99	1·03
15	16	1·12	13·19	1·53	6·29	22·20	7·47	0·15	2·97	1·68	2·56	2·19	0·65
15	20	1·09	12·34	1·51	5·46	21·43	8·14	0·14	3·25	2·05	2·45	1·87	0·60
15	25	1·12	10·10	1·66	4·22	24·95	9·70	0·15	3·93	2·23	1·82	1·52	0·76
15	28	1·11	12·18	1·44	4·46	20·22	8·72	0·15	3·09	2·16	1·71	1·64	0·73

* *P*-values and pooled SEMs are given in Table 6.

Table 5. Sum of fatty acids (FA, % of diet) and content of *n*3 fatty acids (% of total FA) in pyloric caeca and liver tissue given as lipid level means, temperatures mean, and treatment means

Temperature	Diet lipid	C18:3*n*3	C20:5*n*3	C22:5*n*3	C22:6*n*3	Sum *n*3	Sum MUFA	Sum Sat
In the pyloric caeca								
	15·5	3·43^{c}	1·80^{a}	0·43^{a}	8·95^{a}	13·5	35·9	15·4
	20·4	3·66^{b}	1·50^{b}	0·34^{b}	6·03^{b}	18·4	68·6	24·7
	25·1	3·87^{a}	1·26^{c}	0·26^{c}	4·15^{c}	21·8	106·0	33·1
	27·6	3·94^{a}	1·53^{b}	0·31^{b}	5·03^{c}	19·8	85·2	27·8
8		3·67	1·68^{a}	0·36^{a}	6·26	19·6	73·9	25·6
15		3·79	1·36^{b}	0·31^{b}	5·82	17·0	73·2	24·8
8	15·5	3·32	1·93	0·46	9·01	15·2	39·4	16·8
8	20·4	3·60	1·73	0·37	6·45	19·2	65·2	23·8
8	25·1	3·80	1·43	0·29	4·52	21·8	99·4	31·6
8	27·6	3·94	1·64	0·33	5·04	22·3	91·7	30·1
15	15·5	3·54	1·67	0·41	8·88	11·8	32·4	14·0
15	20·4	3·72	1·28	0·32	5·61	17·6	72·0	25·6
15	25·1	3·94	1·08	0·23	3·77	21·8	113·2	34·8
15	27·6	3·95	1·41	0·29	5·02	17·4	78·7	25·6
In the liver								
	16	1·38	1·87	0·60	14·50	18·5	34·8	19·7
	20	1·65	1·97	0·53	15·12	19·5	35·1	18·0
	25	1·99	2·08	0·46	15·12	20·0	36·1	15·6
	28	1·97	2·40	0·57	17·62	22·9	32·2	17·0
8		1·93	1·62	0·38	10·53	14·7	41·2	16·4
15		1·56	2·53	0·70	20·65	25·7	27·9	18·8
8	16	1·55	1·39	0·39	9·35	12·8	42·1	18·3
8	20	1·88	1·43	0·35	9·03	12·9	43·2	16·5
8	25	2·11	1·78	0·37	11·48	16·0	40·6	15·1
8	28	2·19	1·90	0·41	12·26	17·0	38·7	15·5
15	16	1·21	2·35	0·81	19·65	24·2	27·5	21·2
15	20	1·42	2·52	0·72	21·21	26·1	27·0	19·5
15	25	1·88	2·38	0·55	18·77	23·9	31·5	16·0
15	28	1·75	2·89	0·74	22·98	28·7	25·7	18·4

other hand, the picture observed at 15 °C was less clear and no significant difference could be observed between the three different lipid levels. Concerning *srebp2*, another important gene involved in fatty acid synthesis, its expression showed a completely different picture, being only slightly influenced by water temperature and not by lipid level. Among the genes dedicated to the lipid transport, at 8 °C *mtp* showed the same dose-response effect already observed in other genes. Concerning the enzymes participating in phosphatidylcholine synthesis, the expression of *chk* was significantly enhanced by increasing lipid levels by the lower temperature and the higher lipid level, while it was downregulated at 15 °C and there was no difference between the three lipid levels.

Discussion

Effects on organosomatic index and cell vacuolation of the pyloric caeca

The results show that among the biomarkers selected for evaluation of state of choline supply, the most sensitive indicators were related to the pyloric ceaca, i.e. OSIPI, histological observation of cell vacuolisation and lipid accumulation, in line with the results showed by Hansen *et al.*[10]. All the indicators increased with increasing dietary lipid content, and partly with increased temperature, probably as a consequence of the higher feed intake given by the higher water temperature. The temperature effects observed in the present study are parallel to results observed in in rainbow trout by Ng *et al.*[36]. The mechanisms underlying these observations were, most likely, related to choline's role in lipid transport. Choline deficiency leads to insufficient transport of fat from the cytosol across the basolateral cell membrane of intestinal enterocytes. As discussed below, the cell metabolism is consequently altered towards temporary storage of the lipid in intracellular lipid vacuoles, resulting in excessive lipid accumulation[37–40].

Effects on growth performance and lipid digestibility

The results regarding effects of environmental temperature on growth performance are in accordance with findings of several other studies conducted on Atlantic salmon[41–43] and other fish species such as rainbow trout[44], darkbarbel catfish (*Pelteobagrus vachellii*)[45] and yellowtail kingfish (*Seriola lalandi*)[46]. As fish are poikilotherms, the observation that fish raised at 15 °C grew 40 % more than those raised at 8 °C was as expected. The overall mechanism underlying this effect is the impact of the higher temperature on the metabolism of the fish, which leads to a concomitant increase in feed intake and growth[47,48]. The marginal effects of variation in temperature on lipid digestibility are partly in line with the results of a study of Grisdale-Helland *et al.*[49]. Their work showed that with soybean oil as source of lipid, temperature, 5 *v.* 12 °C, did not affect lipid digestibility significantly. However, when fish oil was the source, lipid digestibility increased with temperature. The conclusion was that lipids

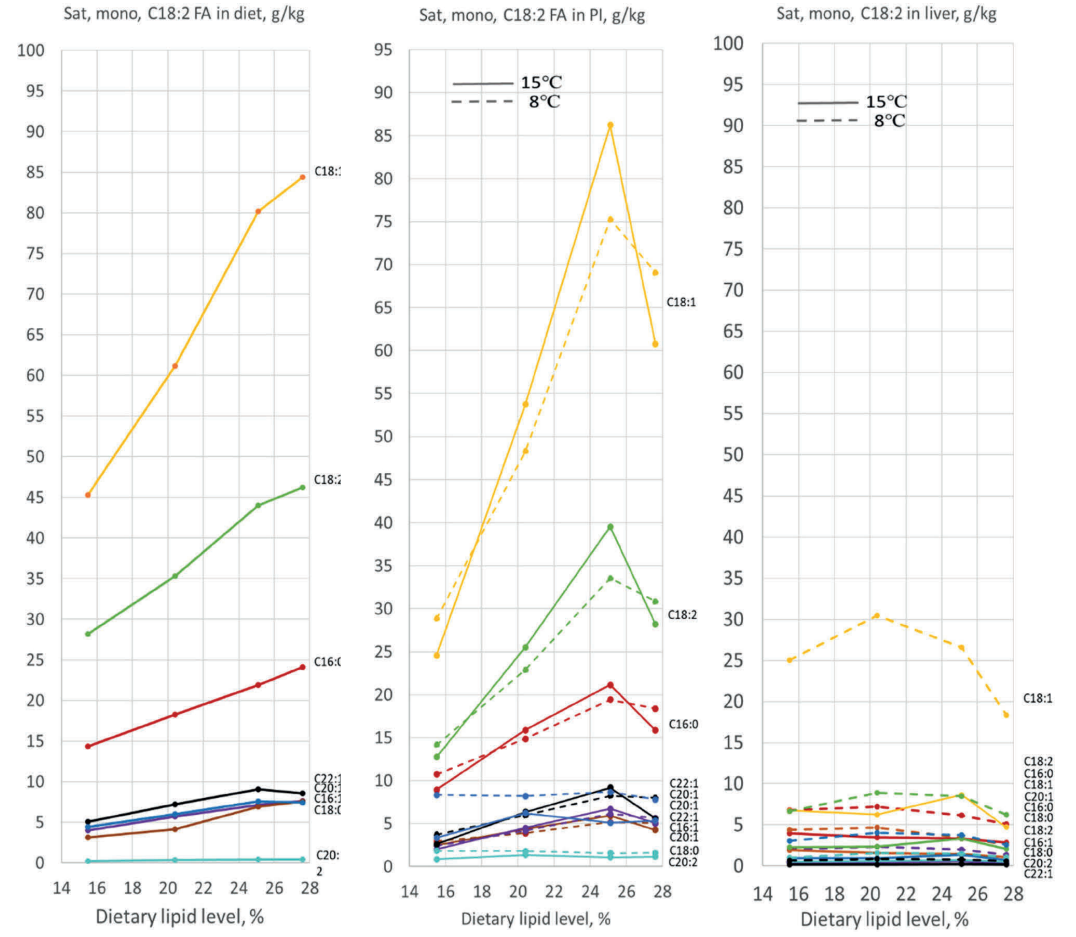

Figure 4. Level of saturated (Sat) and monounsaturated (MUFA) fatty acids in the diet (left), and in tissue from pyloric intestine (middle, PI) and liver (right), expressed as g per kg. The results of two-way ANOVA are shown in Tables 5 and 6.

Table 6. Results of two-way NOVA for content of fatty acids in tissue from the pyloric intestine (PI) and liver given as % of total fatty acids*

In the pyloric intestine							
	C16:0	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C20:1**	**C22:1**
p(model)	0·0002	0·0002	<0·0001	<0·0001	0·0009	0·0472	0·1834
p(temp)	0·1307	0·1744	0·0067	0·0019	0·0008	0·4886	0·1236
p(lipid)	0·0001	0·0001	<0·0001	<0·0001	0·0064	0·0289	0·2487
	C18:3*n*3	**C20:5****	**C22:5****	**C22:6**	**Sum *n*3****	**Sum MUFA**	**Sum Sat**
p(model)	0·0003	0·0002	<0·0001	<0·0001	<0·0001	0·0082	0·0097
p(temp)	0·0549	0·0005	0·0009	0·1711	0·0647	0·9300	0·8628
p(lipid)	0·0002	0·0011	<0·0001	<0·0001	<0·0001	0·0041	0·005
In the liver							
	C16:0	**C16:1**	**C18:0**	**C18:1****	**C18:2****	**C20:1****	**C22:1**
p(model)	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001
p(temp)	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001
p(lipid)	0·0088	0·0673	0·0077	0·0519	0·0316	0·0297	0·0972
	C18:3*n*3	**C20:5**	**C22:5**	**C22:6****	**Sum *n*3****	**Sum MUFA****	**Sum Sat**
p(model)	<0·0001	0·0002	0·0255	0·0191	<0·0001	<0·0001	0·0001
p(temp)	<0·0001	<0·0001	0·0117	0·007	<0·0001	<0·0001	0·0003
p(lipid)	0·0156	0·1142	0·1027	0·1074	0·0107	0·0756	0·0005

* The results are based on a two-way NOVA model without interaction as a model with interaction gave insignificant results for the interactions. For the pyloric intestine the analyses were conducted on Log_{10}-transformed data.
** The statistical evaluation showed significantly lower values for the highest lipid level (28 %) compared to the second highest (25 %).

Table 7. Results of two-way NOVA for content of fatty acids in pyloric and liver tissue given as g/kg tissue

In the pyloric intestine							
	C16:0**	**C16:1****	**C18:0****	**C18:1****	**C18:2****	**C20:1**	**C22:1**
p(model)*	0·0008	<0·0001	0·0041	0·0001	0·0002	0·0465	0·0041
p(temp)	0·7200	0·9194	0·9917	0·8601	0·6168	0·5046	0·4612
p(lipid)	0·0004	<0·0001	0·002	<0·0001	<0·0001	0·0282	0·0022
	C18:3**	**C20:5**	**C22:5**	**C22:6**	**Sum *n*3****	**Sum MUFA****	**Sum Sat****
p(model)*	0·0005	<0·0001	<0·0001	0·0464	<0·0001	<0·0001	<0·0001
p(temp)	0·9666	0·0002	0·0014	0·0081	0·0004	0·9987	0·5768
p(lipid)	0·0002	0·0003	<0·0001	0·3751	<0·0001	<0·0001	<0·0001
In the liver							
	C16:0	**C16:1**	**C18:0**	**C18:1****	**C18:2****	**C20:1****	**C22:1**
p(model)*	0·0001	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001
p(temp)	<0·0001	<0·0001	0·7768	<0·0001	<0·0001	<0·0001	<0·0001
p(lipid)	0·0166	0·0986	<0·0001	0·0637	0·0008	0·0039	0·0342
	C18:3*n*3**	**C20:5**	**C22:5**	**C22:6**	**Sum *n*3****	**Sum MUFA****	**Sum Sat**
p(model)*	<0·0001	<0·0001	0·0001	<0·0001	<0·0001	<0·0001	<0·0001
p(temp)	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001	<0·0001
p(lipid)	<0·0001	0·0308	0·1547	0·039	0·0253	0·0513	0·0105

* The results are based on a model without interaction as a model with interaction gave insignificant results for the interactions.
** The statistical evaluation showed significantly different values for the diet with the highest lipid level (28 %) compared to that of the second highest level (25 %).

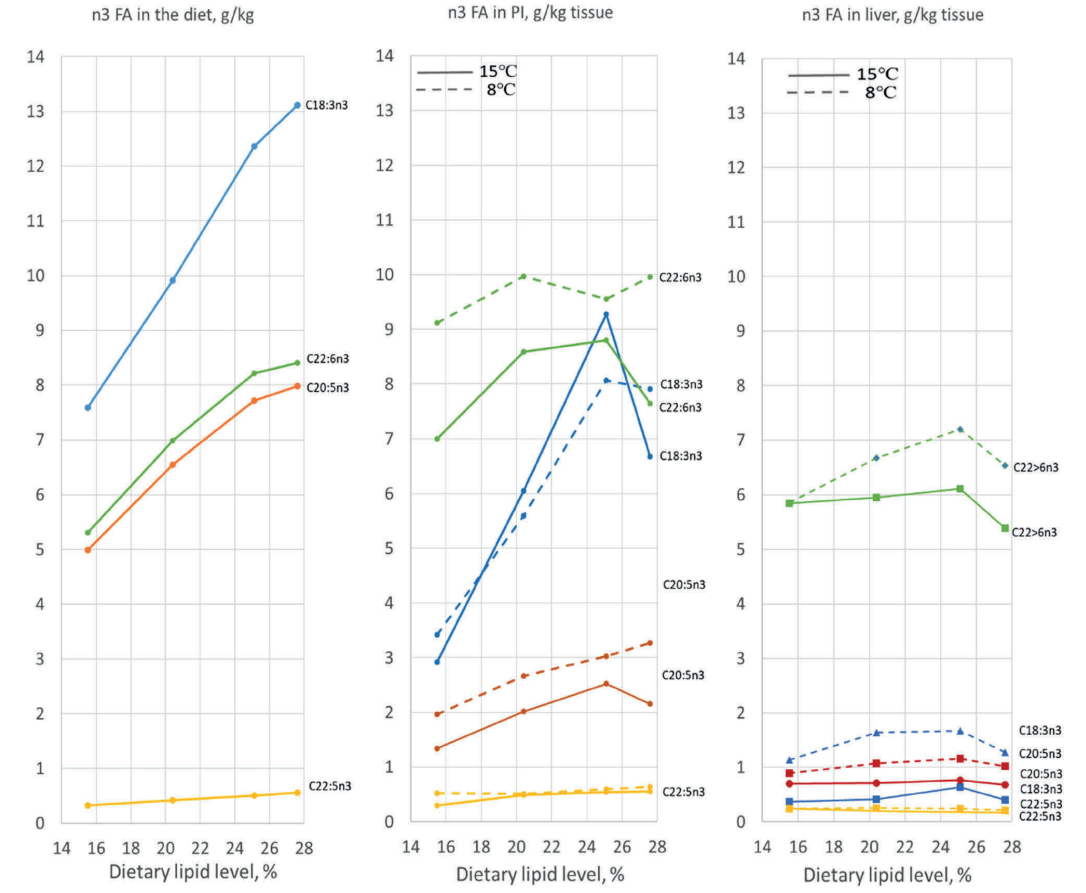

Figure 5. Level of *n*3 fatty acids in the diet (left), and in tissue from pyloric intestine (middle, PI) and liver (right), expressed as g per kg. The results of two-way ANOVA are shown in Tables 5 and 6.

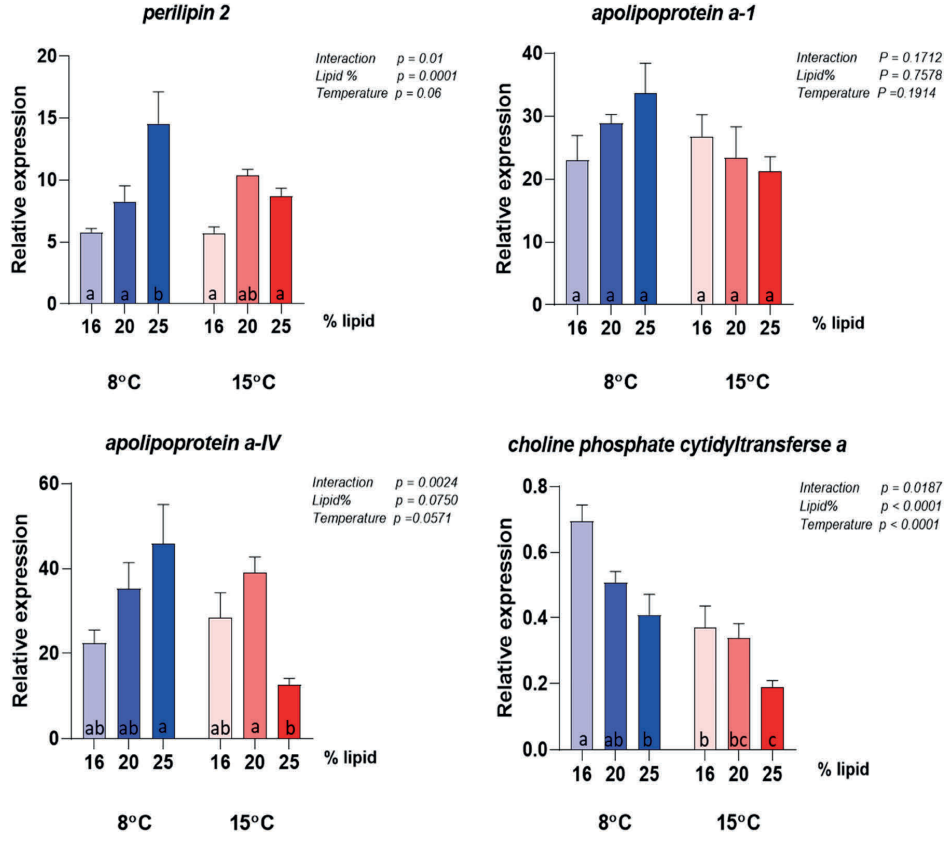

Figure 6. Expression of biomarker genes for choline requirement. Data are mean normalised expression levels + SEM. Different letters denote statistically significant differences among diet groups.

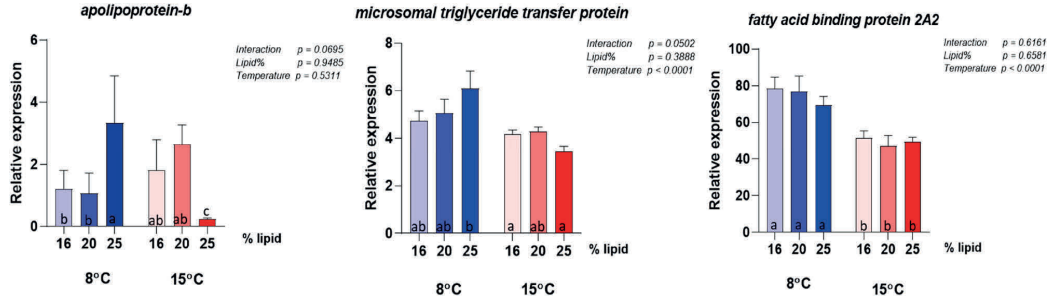

Figure 7. Expression of genes involved in lipid and fatty acid transport. Data are mean normalised expression levels + SEM. Different letters denote statistically significant differences among diet groups.

with low melting point, such as plant oils, are highly digestible and are only slightly influenced by the environmental temperature. On the other hand, for lipids with high melting point, which means high level of saturated and/or long-chain polyunsaturated fatty acids, overall digestibility is lower and is affected to a larger extent by temperature[36,50,51].

The observed increase in digestibility of protein and energy with increasing lipid level, and to a more pronounced degree for ash digestibility, was supposedly related to an increase in gut passage time induced by the increase in lipid level, allowing more time for digestion and absorption. This is a well-known relationship in animals[52–54].

Effects on lipid content in the pyloric caeca and liver

The results observed for fatty acid composition in the diet, pyloric caeca and liver, illustrate that the pyloric caeca markedly modulate the fatty acid profile of the diet and the liver. This

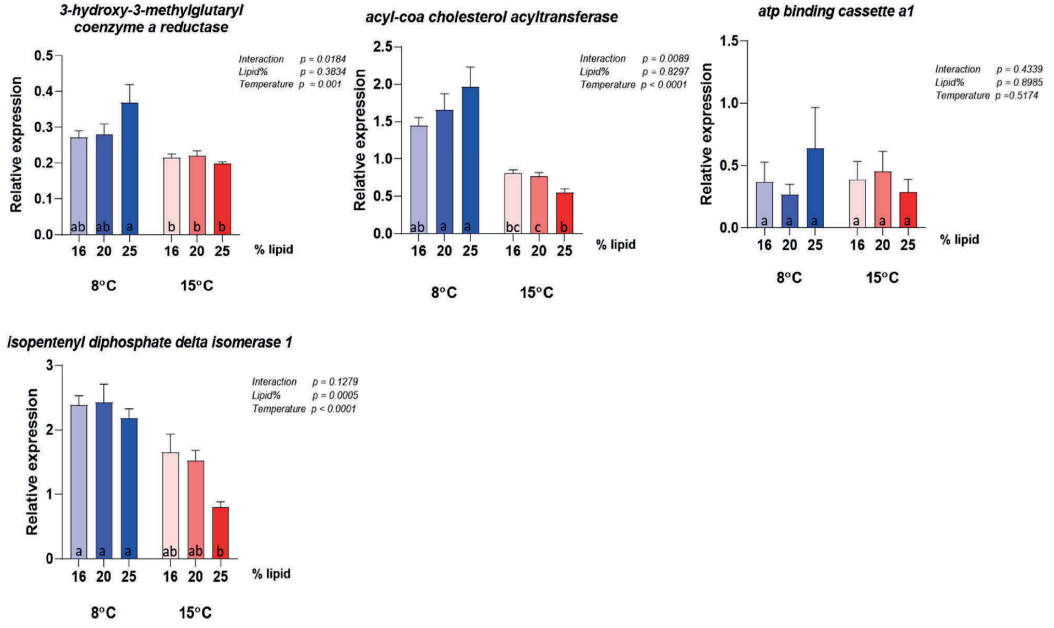

Figure 8. Expression of genes involved in cholesterol metabolism. Data are mean normalised expression levels + SEM. Different letters denote statistically significant differences among diet groups.

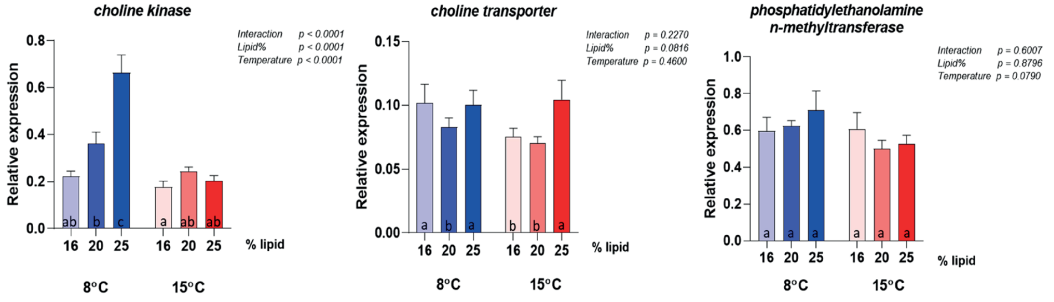

Figure 9. Expression of genes involved in phosphatidylcholine synthesis. Data are mean normalised expression levels + SEM. Different letters denote statistically significant differences among diet groups.

regards in particular the *n*3 fatty acids, for which there seemed to be a substantial conversion from 18:3*n*3 and 20:5*n*3 to 22:5*n*3. This observation is in line with the results of Bou *et al.*[5], which showed a clear difference between the fatty acid composition of the diet and that of tissue from the MI. In the present work, the conversion of the *n*3 fatty acids seemed to be affected the most by lipid level of the diet. This observation was possibly a consequence of the increasing severity of the steatosis which developed in the enterocytes. The abrupt change in the general development of this relationship between the diet containing 25 and 28 % of fat, most clearly illustrated for the results expressed as g/kg, supports this consideration. The marked difference in fatty acid composition between the liver and the pyloric caeca, and the greater, and seemingly opposite effect of temperature, more marked and inverse for the liver, underlines the different roles of these two organs. These results also call for better understanding of the pathways and metabolism of lipid between organs in fish. Knowledge on the transport routes from the intestine to the peripheral tissues and internal organs is weak. Although a lymphatic system has been described in fish, with zebrafish as the main model[55], an intestinal lymphatic system has not been identified in Atlantic salmon. As there are species differences among vertebrates regarding lymph vessels in the intestine, e.g. a lymphatic system is absent in chicken[56], efforts should be made to identify lipid transport routes for Atlantic salmon. Such information would help understand the trafficking and metabolism of lipid in this important domesticated animal.

Effects on gene expression in the pyloric caeca

Among the assessed genes, *plin2*, *apoA-I*, *apoA-IV* and *pcyt1α* showed the greatest effects of lipid and temperature and are

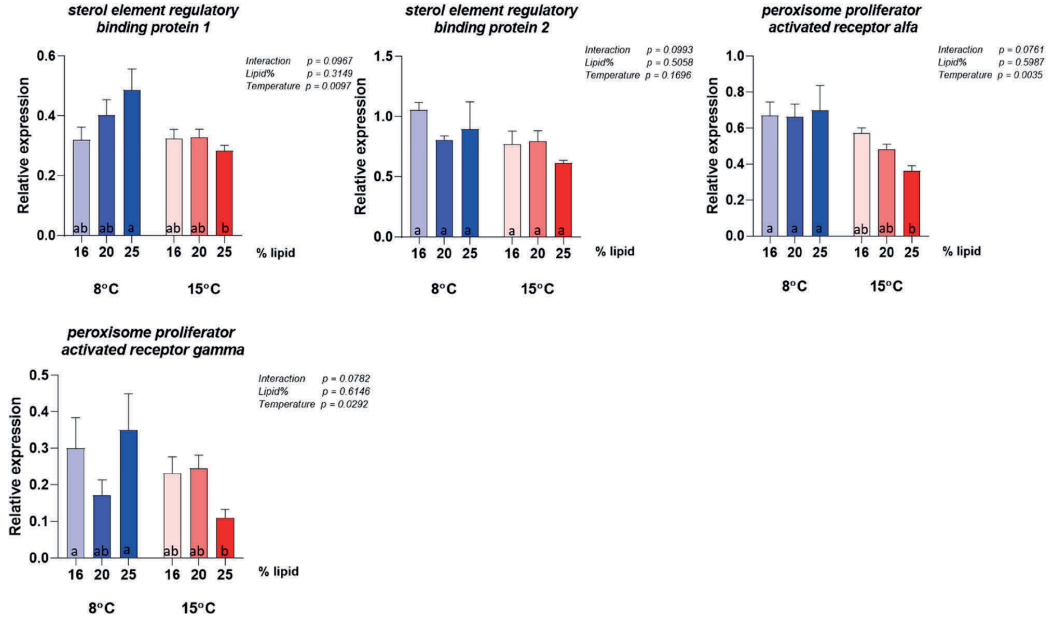

Figure 10. Expression of genes coding for nuclear receptors involved in regulation of lipid and sterol metabolism. Data are mean normalised expression levels + SEM. Different letters denote statistically significant differences among diet groups.

therefore considered to comprise the most sensitive biomarkers for choline requirement, in accordance with the observations in the study of Hansen *et al.*[10] In Hansen *et al.*'s dose-response study, in which dietary lipid level was the same (29 %) for all groups, the expression of *plin2* followed an inverse relationship with choline and degree of enterocyte lipid accumulation. The inverse relationship between intestinal *plin2* expression and steatosis has been observed in several recent studies in Atlantic salmon[9,11,57–59], and highlights that *plin2* is a sensitive biomarker of intestinal lipid accumulation[60]. In the present study, in which choline inclusion was kept equally low for all diets while the lipid level increased, *plin2* expression followed a clear dose-response relationship with lipid percentage at 8 °C, again showing an inverse relationship with the degree of steatosis. This result not only confirmed *plin2*'s important role as indicator of choline requirement, but it also confirmed that the requirement is influenced by dietary lipid level. At 15 °C, *plin2* followed the same pattern only for the first two lipid levels, while at 25 % the expression dropped. The cause of the difference in response at the low and high temperature is difficult to suggest based on the other results of this study, and no scientific literature is available offering further information. Concerning the two genes encoding respectively for ApoA-I and ApoA-IV, major protein components of lipoproteins, they showed at 8 °C the same picture observed for *plin2*. This response matches the results obtained in Hansen *et al.*'s latest work, in which *apoa-I* and *apoa-IV* were both suppressed by a too low choline supply, while their expression was higher when choline level was adequate to provide a proper assembly of lipoproteins and sufficient lipid transport. At 15 °C, the picture showed by the expression levels of the two genes, especially of *apoa-I*, again indicated that the result was affected by the environmental temperature, as confirmed by the significance of the interaction term. Among the genes involved in phosphatidylcholine production, *chk*, which regulates the first step of the cytidine (CDP)-choline pathway[35,36] and phosphorylates choline, showed the same expression pattern already discussed for *plin2* at both 8 and 15 °C. On the contrary, *pcyt1a*, involved in the generation of the high energy donor CDP-choline by regulating the second step of the pathway[44,46], showed a different picture, being strongly downregulated by the increasing lipid level at both water temperatures. The *chk* expression pattern was in agreement with results presented in Hansen's dose-response study[61], which showed that to a higher choline inclusion corresponds a lower *chk* expression. On the other hand, the opposite response of *pcyt1a*'s contrasted with the Hansen *et al.*[10]'s findings which showed similar responses in *pcyt1a* compared to the response in *chk*. Following the steps of the cytidine (CDP)-choline pathway, we can suggest, as possible explanation, that to a higher choline phosphorylation, corresponds a lower production of the high-energy donor CDP-choline. However, the complexity of the pathway, regulated by several rate-limiting enzymes[62], makes it difficult to discuss the effects of the diet on *pcyt1a* expression and more specific studies are therefore needed.

At 8 °C, the expression of other genes involved in lipid metabolism, such as *hmgcr*, the rate-limiting enzyme for cholesterol synthesis, *acat* which catalyses cholesteryl esters synthesis from cholesterol[63], the two transcription factors *srebp1* and *srebp2*, and *mtp*, which has a pivotal role in lipoprotein

formation, confirmed the direct relationship between dietary lipid level and choline requirement. At 15 °C, all these genes followed the same pattern observed for *plin2*, validating the hypothesis of an interaction between water temperature and lipid inclusion. As the steatosis, as well as the lipid accumulation in the pyloric tissue indicated increased choline requirement with increasing temperature, it is unlikely that the gene expression results observed for the fish fed at high temperature, indicated mitigating effects.

No impact of the increasing fat level was observed for the liver, neither as indicated by the HSI or histology. This outcome is in accordance with the findings achieved in our previous studies[9,10] which showed that liver lipid indicators are not suitable biomarkers for estimation of severity of lipid accumulation and, hence, for choline requirement[64].

Effects of lipid level and environmental temperature on choline requirement

According to the discussion above, the design of our experiment appears to be suitable for evaluation of possible effects of variation in dietary lipid content and temperature on choline requirement. To get an indication of the quantitative effects on choline requirement, the dose-response study conducted by Hansen *et al.*[10]'s work to estimate choline requirement in Atlantic salmon[10] can be used, although the fish was somewhat larger and kept in seawater. In Hansen *et al.*'s[10] work, a diet with a choline level of about 1900 mg/kg gave fish with an OSIPI of 2·0, while a diet with 1200 mg/kg resulted in a OSIPI of 2·4. Based on this observation, the results obtained from the present study provide an estimation of the effects on choline requirement given by the raise in lipid level from 16 to 25 %. With a dietary choline level of 1400 mg/kg, the shift of lipid level increased OSIPI by 0·8, regardless of the temperature. The effect of temperature, i.e. of an increase in OSIPI of 0·5, correspond to an estimated increase in choline requirement of about 900 mg/kg. As the conditions of the present and Hansen *et al.*'s study differ somewhat, the estimation of the effects of dietary lipid content and temperature, are high, indicating that lipid level in the diet, as well as temperature should be taken into account when diets are formulated. To be able to conclude the optimal level of choline in salmon diets for elimination of steatosis, a dose-response trial is needed with diets high in lipid and at high temperatures. Such an experiment should also observe endpoints regarding disease resistance as well, as choline is the primary source of methyl groups in epigenetic processes, which are essential for differentiation of immune cells.

Conclusions

The obtained results confirmed the relevance of dietary lipid level and water temperature as important drivers for intestinal lipid accumulation, showing clear effects on steatosis symptoms on a molecular, histological, biochemical level. In addition, the effects given by the water temperature seemed particularly significant when the interaction with the different lipid levels occurs. These findings represent the first steps towards the estimation of choline requirement in Atlantic salmon at later developmental stages and raised under different environmental conditions. However, further analyses and a dose-response feeding trial will be needed to investigate how this interaction occurs and influences the mechanisms and the pathways behind lipid metabolism and choline requirement.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/jns.2023.45.

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The author's contributions were as follows: D. S.: sampling, molecular and histological analyses, data evaluation and interpretation, statistical analyses and manuscript development; T. M. K.: data evaluation, interpretation and manuscript revision; G. M. B.: data evaluation, interpretation and manuscript review; A. K. G. H.: experimental design and manuscript review; Å. K.: project leadership, experimental design, fatty acid metabolism analyses, data evaluation and interpretation, and manuscript development.

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Paper II

Effects of dietary lipid source and fish size on induced steatosis in two Atlantic salmon (*Salmo salar* L) populations.

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Abstract

Choline is recognized as an essential nutrient for Atlantic salmon at all developmental stages, but dietary requirement is not well defined. Choline plays a critical role in lipid transport, and the clearest deficiency sign is intestinal steatosis. The present work, aiming to find whether lipid source and fish size may affect choline requirement, was one of a series of studies conducted to identify which production-related conditions may influence choline requirement. Six choline-deficient diets were formulated varying in ratios of rapeseed oil to fish oil and fed to Atlantic salmon of 1.5 and 4.5 kg. After eight weeks, somatic characteristics were observed, and severity of intestinal steatosis was assessed by histological, biochemical, and molecular analyses. Fatty acid composition in pyloric intestine, mesenteric tissue, and liver samples was also quantified. The increasing rapeseed oil level increased lipid digestibility markedly, enhancing lipid supply to the fish. Simultaneously, small fish consumed more feed, and consequently had a higher lipid intake. In conclusion, choline requirement was mainly affected by the dietary lipid load, which was influenced by the dietary lipid source, and therefore fatty acid profile, and by the fish size. The overall fatty acid composition of the observed tissues mirrored that of the diet.

Introduction

In recent decades, reduction in fish meal and fish oil in Atlantic salmon feeds has changed the dietary level of several essential nutrients. The consequences for the fish of such alterations are difficult to predict, since present knowledge concerning nutrient requirements in Atlantic salmon has severe gaps. It is therefore highly likely that today's feeds are suboptimal i.e., deficient or excessive, for some essential nutrients. Field observations of severe fat accumulation in the pyloric caeca of farmed Atlantic salmon, a condition known as intestinal steatosis, have recently been linked to deficient dietary supply of choline[1–3]. A mild degree of this condition seems to be a ubiquitous and underreported condition in cultivated Atlantic salmon, whereas cases of severe steatosis, categorized as lipid malabsorption syndrome (LMS), have also been reported[4].

The investigations conducted to explore potential explanations for LMS led to confirmation of choline ($C_5H_{14}NO_+$) as an essential nutrient for Atlantic salmon [1]. Choline has been acknowledged as essential for some fish species and other animals, particularly at early developmental stages[3,5,6,7]. Among its several functions, choline serves as a constituent of phosphatidylcholine, acetyl choline, and other cell components, in addition it is a key methyl group donor for production of S-adenosylmethionine (SAM) during methylation processes.

Phosphatidylcholine, a class of phospholipids composed of a choline head group and glycerophosphoric acid, is a major membrane constituent of all living cells[5]. Phosphatidylcholine also plays a critical role in lipid transport and absorption by being an essential component of lipoproteins. Lipoproteins facilitate lipid transport from the intestine to the systemic circulation, as well as in the formation and secretion of very-low-density lipoprotein (VLDL) from hepatocytes[8].

Symptoms of choline deficiency vary between fish species. The most apparent symptoms are related to impaired fat transport, comprising symptoms such as intestinal and hepatic steatosis, low growth rate, poor feed efficiency, and early death. Such symptoms have been observed in several cultivated species, including carp (*Cyprinus carpio*)[9], lake trout (*Salvelinus namaycush*)[10], rainbow trout (*Oncorhynchus mykiss*)[11], yellow perch (*Perca flavescens)*[12], channel catfish (*Ictalurus punctatus)*[13] cobia (*Rachycentron canadum*)[14], and, most recently, in Atlantic salmon (*Salmo salar L)*[3].

Typical signs of intestinal steatosis in Atlantic salmon are pale and swollen appearance of the pyloric caeca and hyper-vacuolization of the tissue caused by the excessive lipid accumulation within the enterocytes. In extreme cases lipid absorption is severely reduced, and ingested lipid may accumulate all along the intestinal tract, to be eventually discarded as whitish faeces, a condition known as steatorrhea[1].

According to present nutrient requirement guidelines, mostly based on observations of growth and hepatic steatosis, choline requirement of various fish species range between 400 and 1000 mg/kg feed. However, the work of Hansen et al. indicated that steatosis in the pyloric intestine is a more sensitive biomarker for estimating choline requirement. Hansen's dose-response feeding trial indicated a choline requirement of 3400 mg/kg in Atlantic salmon kept in seawater and weighing 200-400g[3]. The estimation was based on the observation of several biomarkers of lipid storage and transport, such as the organo-somatic indices of the pyloric and mid intestine, the histological evaluation of lipid vacuolation, as well as the expression levels of four genes with key roles in lipid metabolism, i.e., *apoA-IV, apoA-I, pcyt1α* and *plin2*.

The role of choline in lipid transport suggests that its dietary requirement may depend on several production-related conditions, such as life stage, size, growth rate of the fish, dietary lipid level and source, and environmental temperature. The present study was one of a series of screening studies underway to understand which biotic and abiotic conditions might affect choline requirement. Such knowledge would identify significant shifts in requirement levels to the extent that it is necessary to take them into account when formulating optimal diets with sufficient choline under the most demanding farming conditions. The aim of the present trial was therefore to find whether dietary fatty acid profile of the feed and fish size affect choline requirement importantly. A secondary aim was to strengthen understanding of fatty acid metabolism and transport in and between the intestine, mesenteric tissue, and liver.

Materials and Methods

Feeds

Six experimental feeds were formulated varying in ratios of rapeseed to fish oil, from 0:31% to 24:8%. The nutritional content was, otherwise, similar. Feed ingredients and nutrient composition are shown in Table 1a and 1b. The average dietary choline was 1523 mg/kg, a level which, according to choline requirements set by Hansen et al.[3], is severely deficient. This was a deliberate and necessary condition to evaluate the effects of specific factors on choline requirement. Yttrium oxide was added as an inert marker to estimate apparent nutrient digestibility. The experimental feeds were produced by extrusion (feed pellet size 9mm) at BioMar Feed Technology Centre (Brande, Denmark) using a BC 45 twin-screw extruder (Clextral, France).

95

96 ***Table 1a**. Feed ingredients and nutritional composition of the experimental feeds*

	Rapeseed oil level, %					
	0	5	9	14	19	24
Ingredients, %						
Fish Meal NA LT	13.0	13.0	13.0	13.0	13.0	13.0
Krill 56	2.0	2.0	2.0	2.0	2.0	2.0
Soya SPC	23.8	23.8	23.8	23.8	23.8	23.8
Pea Protein 65	7.5	7.5	7.5	7.5	7.5	7.5
Guar Meal	8.0	8.0	8.0	8.0	8.0	8.0
Wheat Milling quality	13.5	13.5	13.5	13.5	13.5	13.5
Fish Oil	31.2	26.5	21.8	17.1	12.4	7.7
Rapeseed Oil	0.0	4.7	9.4	14.1	18.8	23.5
Choline Chloride 70 %, dry	0.03	0.03	0.03	0.03	0.03	0.03
Vitamin and mineral mix*	2.1	2.1	2.1	2.1	2.1	2.1
Lucantin Pink CWD 10%, BASF	0.1	0.1	0.1	0.1	0.1	0.1
Yttrium oxide	0.1	0.1	0.1	0.1	0.1	0.1
Analyzed content						
Crude protein, %	38.6	38.3	38.6	38.3	39.7	39.6
Sum Fatty acids**, %	26.3	26.5	26.3	26.3	27.7	28.1
Choline, mg/kg	1490	1520	1680	1430	1500	1520

97 *The feeds were supplemented with standard vitamin and mineral premixes following NRC *guidelines*
98 *(2011) and BioMar standards to meet the requirements.*
99 ***See Table 1b for details*

100

Table 1b. *Fatty acid composition of the experimental feeds, % of diet.*

	Rapeseed oil level					
Fatty acid	0	5	9	14	19	24
C12:0	0.02	0.02	0.02	0.02	0.01	0.01
C14:0	1.88	1.58	1.32	1.02	0.87	0.60
C16:0	5.24	4.62	4.08	3.47	3.24	2.72
C18:0	1.28	1.18	1.08	0.99	0.97	0.89
C20:0	0.25	0.25	0.23	0.21	0.21	0.21
C22:0	0.18	0.20	0.18	0.20	0.21	0.22
C24:0	0.04	0.04	0.04	0.04	0.05	0.05
C16:1	1.91	1.59	1.32	1.06	0.88	0.62
C18:1	3.03	4.99	6.68	8.21	10.15	11.87
C20:1	0.42	0.42	0.42	0.41	0.43	0.45
C22:1n9	0.07	0.08	0.09	0.10	0.12	0.16
C22:1n11	0.27	0.24	0.22	0.20	0.19	0.18
C24:1	0.12	0.10	0.08	0.08	0.07	0.06
C18:2n6	1.04	1.90	2.56	3.30	4.02	4.65
C18:3n3	0.22	0.53	0.78	1.03	1.32	1.60
C18:3n6	0.06	0.06	0.05	0.04	0.03	0.03
C20:3n3	0.02	0.01	0.01	0.01	0.01	0.01
C20:3n6	0.05	0.05	0.03	0.03	0.02	0.01
C20:4n6	0.29	0.24	0.20	0.16	0.13	0.09
C20:5n3	3.79	3.16	2.55	2.03	1.70	1.17
C22:5n3	0.44	0.37	0.28	0.24	0.19	0.13
C22:6n3	1.63	1.38	1.11	0.92	0.80	0.59
Other*	4.03	3.49	2.98	2.55	2.11	1.80
Tot sum	26.3	26.5	26.3	26.3	27.7	28.1

**Including unidentified fatty acids, and cis and odd chain fatty acids*

Fish and rearing conditions.

The feeding trial was conducted at the LetSea research facility at Dønna, Norway, a facility approved by The Norwegian Animal Research Authority (NARA), operating following Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). Twelve steel-cages with a volume 125 m^3 (5x5x5m) volume each fitted with a standard net and equipped with a lift-up system for feed waste collection were used during the trial.

Atlantic salmon (*Salmo salar* L.) of two sizes were used, with average initial weights of 1.7 and 4.5 kg (CV=10%), respectively. As it is not possible to produce fish with the same background which differed in size, it was decided to compare fish produced from autumn (S0/Small) and spring smolt (S1/Large). The small fish, delivered by AquaGen AS, were smoltified and put to sea autumn 2020, while the large

fish, delivered from Salmobreed AS, were smoltified and put to sea spring 2020. Accordingly, the observed differences between the small and the large fish reflects the combined effects of fish size, fish breed and smoltification strategy.

The fish were pit tagged and randomly distributed to one of the six pens allocated for each size, 100 fish per pen. The feeds were randomly assigned to the pens. The fish were fed weighed amounts of feed twice a day, in excess. Uneaten feed was collected from a net under the pens for feed waste estimation. The feeding period lasted 55 days, from 2nd of Sep – 25th of Oct 2021, during which the fish were exposed to natural daylight and temperature conditions. Water salinity varied between 29 and 33 and the temperature ranged from 12 to 9°C and averaged at 10.5°C. Dissolved oxygen in the cages was measured daily, averaging at 80% throughout the experiment.

Sampling

At the end of the feeding period, twelve fish were randomly taken from each pen using a land net before being anesthetized by exposure to tricaine methane-sulfonate (MS-222) for a minimum of 5 minutes. After recording final body weight (FBW, g) and fork length (FL, cm) of the fish, the blood was sampled from the caudal vein and stored on ice. Blood was then centrifugated and the obtained plasma was collected in 2mL aliquots, frozen in liquid nitrogen and kept at 80℃. Following blood sampling, the fish were killed by a sharp blow to the head, in accordance with the Norwegian Animal Welfare act and opened ventrally. The whole gastrointestinal package was removed from the abdominal cavity and the gutted carcass was weighed. Fish without feed content along the gastrointestinal tract were discarded. The intestine was sectioned into three parts[15]: pyloric intestine (PI), which extends from the pyloric sphincter to the most distal pyloric caecum; mid intestine (MI), which is the area between the latter pyloric caecum and the beginning of the visible complex mucosal folds of the distal intestine; distal intestine (DI), comprised between the MI and the anus. A sample of the mesenteric fat from the PI area was collected. The three gut sections were opened longitudinally, and the digesta collected, snap frozen in liquid N_2 and stored at -80℃ until further enzyme activity analyses. The tissues sections were weighed, and samples collected. Tissue samples for histological analyses were first fixed in 10% neutral buffered formalin (4% formaldehyde) for 24h, and subsequently transferred to 70% EtOH for storage until processing. Samples for RNA extraction were rinsed in sterile saline water, submerged in RNAlater®, incubated at 4°C for 24h, and subsequently stored at −20°C until analysis. The remainder of each tissue section was frozen in liquid N_2 and stored at -80℃ for further enzyme activity analysis. The remaining fish in each pen were weighed individually. Estimates of growth performance, feed intake and mortality represent all fish in each pen.

Welfare assessment

At the end of the feeding trial ten of the remaining fish were sampled randomly from each pen for welfare scoring (render score). At first, the number of Salmon lice (*Lepeophtheirus salmonis*) on each fish was counted. The parasites were classified based on their observed life stage as non-motile, Chalimus, motile and adult female. Lice released from the fish during anesthetization were counted and added to the total count for every fish. Considering the size difference, the number of lice was calculated per cm^2 of fish surface. The surface area of the fish was calculated based on their weight following the formula of O'Shea et al.[16]. Thereafter, fish were evaluated for external indicators such as scale loss, skin lesions, eye damage and cataract, according to the scoring system developed by Kolarevic et al[17]. Each fish was evaluated for every welfare indicator by assigning a score between 0 and 2, where 0 represents a good condition, while 2 stands for a bad condition. The dorsal and caudal fin evaluation was conducted using the scoring system developed by Hoyle et al.[18] where the level of visible damages ranges from 0, no damages, to 5, complete erosion of the fin. Mortality was also used as a welfare indicator and the number of dead fish was collected along the feeding trial.

Histology

The tissue samples collected from the pyloric caeca and the DI of the twelve fish sampled from each tank, were processed at the Norwegian University of Life Sciences (NMBU) using standard histological techniques: dehydration in graded ethanol, clarification in xylene, embedding in paraffin, and sectioning of 5 µm thick sections. The sections were further dewaxed, re-hydrated, stained with haematoxylin and eosin, and scanned using a Philips Ultra-Fast Scanner controlled using the Image Management System version 3.3 within the Philips IntelliSite Pathology Solution version 3.2 (Philips, Norway). Scans were then randomized and evaluated through a PC screen. The selected histological variables were submucosal infiltration of the DI and enterocytes steatosis in the PI. Concerning submucosal infiltration, the appearance of the DI was assessed based on those features which are characteristic of soybean meal-induced enteritis, respectively: changes in mucosal fold height and width, cellularity of the submucosa and lamina propria, and supranuclear vacuolization in the enterocyte[19]. A scoring system with a scale of 0-4 was used where 0 represented a normal condition, and 1 to 4 represented mild, moderate, marked and severe changes, respectively. The severity of steatosis was scored according to the proportion of tissue affected by the presence of lipid-like vacuoles, swollen and irregular cells and condensed nuclei, as Normal (≤10 %), Mild (10–25 %), Moderate (25–50 %), Marked (≥50 %) and Severe (≥75 %) as shown by Siciliani et al.[20]

RNA extraction, cDNA synthesis and gene expression analyses

Gene expression analysis was performed on 144 tissue samples collected from the pyloric caeca. RNA was extracted from pyloric caeca samples weighing 20-30mg using an Precillus homogenizer, TRIzol® reagent (Invitrogen, ThermoFisher Scientific), and chloroform according to the manufacturer's protocol. RNA was extracted with PureLink RNA mini kit (Invitrogen, ThermoFisher Scientific), and treated on column with PureLink DNase (Invitrogen, ThermoFisher Scientific). From each sample 2µl was analysed in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) to assess the RNA purity and concentration. The integrity of the RNA was verified on selected samples with a 2200 TapeStation (Agilent Technologies). Total RNA was then stored at -80 degrees for further analyses. Before cDNA synthesis RNA samples were pooled three by three according to their origin tank. Afterwards, 1 µg of each RNA pool and Superscript IV VILO (Invitrogen, ThermoFisher Scientific) in 20 µL reactions were used to conduct the cDNA synthesis. The same process was performed to achieve negative controls, omitting RNA and enzymes. cDNA was then diluted at 1:10 and stored at -20 degrees. The primers used in the qPCR reactions were obtained from literature and previous works conducted in the research group[1,3]. Additional information concerning gene names, primer source, efficiency, and size, is shown in Supplementary Table S1. The efficiency (E) of the PCR reaction was assessed for each gene assay using serial dilutions of a pool of randomly selected cDNA samples. Additionally, when required, primer optimization was carried out by testing selected primer pairs at a range of temperatures in a single reaction. A LightCycler LC96 (Roche Diagnostic) was used to perform DNA amplification and gene expression analyses. Each reaction mix contained 2µl PCR-graded water, 5µl of LightCycler 480 SYBR Green I Master mix (Roche Diagnostics), and 0.5 µl of both forward and reverse primer. Every sample was analyzed in duplicate alongside a no-template control. The three-step qPCR program featured a first enzyme activation at 95 degrees for 5 min, a following 40-45 cycles of 95 degrees (10s), 55, 58, 60, or 63 degrees (10s, depending on the single gene), and 72 degrees (15s). Quantification cycle (Cq) values were calculated using the second derivative method. The specificity of the qPCR reactions was confirmed by evaluating the melting curve of qPCR products and the band pattern on the agarose gel after electrophoresis. RNA polymerase II (*rnapoll*), hypoxanthine phosphoribosyl transferase 1 (*hprt1)* and glyceraldehyde- 3-phosphate dehydrogenase (*gapdh*) were evaluated for use as reference genes according to their stability across and within the treatments[21]. Target gene expression was normalized to the geometric mean of *rnapoll*, *hprt1* and *gapdh*. Mean normalized expression of the target genes was calculated from raw Cq values by relative quantification. The target genes selected to be assessed in this study correspond to the same set of genes involved in lipid transport and metabolism that Hansen et al.[3] and Siciliani et al.[20] identified as

more receptive to steatosis symptoms and therefore to choline deficiency: *plin2, apoA-I, apoA-IV and pcyt1α.*

Chemical analyses

Feed and faeces samples were analyzed for dry matter (DM), ash, crude protein (CP), crude fat (CF), fatty acids and starch at LabTek, Norwegian University of Life Sciences, Ås, Norway. Dry matter was established by drying samples to a constant weight at 105 °C. Ash content was assessed by combustion at 550 °C [22]. Total nitrogen, identified as crude protein was analysed by the semi-micro-Kjeldahl method (Kjeltec-Auto System, Tecator, Höganäs, Sweden). Fatty acid composition was analysed with the FAME method described by O`fallon, J.V 30[23]. Gross energy was recorded using the Parr 1271 Bomb calorimeter (Parr, Moline, IL, USA). Analyses of yttrium content in feed and faeces were conducted by pre-digestion with concentrated ultrapure HNO3 at 250°C using a Milestone microwave UltraClave III (Milestone Srl, Sorisole, Italy). Samples were then diluted (to 10% HNO3 concentration), and yttrium was determined by inductively coupled plasma optical emission spectrometry (ICP-OES analysis) with a PerkinElmer Optima 5300 DV (PerkinElmer Inc., Shelton, CT, USA).

Calculations

Fish growth was estimated as thermal growth coefficient (TGC) = $1000 * [BW1^{1/3} – BW0^{1/3})/ddg]$ in which BW0 and BW1 are the initial and final body weight and ddg is daydegrees (no of feeding days (D) x average temperature in ◦C) and specific growth rate (SGR)= (ln BW1/ln BW0)/D) x 100. The condition factor (CF) was estimated as: CF=100 x (FBW)/FL) (FL: (fork length $(cm)^3$). The organ somatic indices (OSI) were calculated as: (organ weight g/body weight g) x 100). Apparent digestibility (AD) for each nutrient was determined by using Yttrium oxide (Y_2O_3) as an inactive marker and estimated as follows: ADn = 100 – (100 x (Mfeed/Mfaeces) x (Nfeed/Nfaeces)), where M represents the percentage of Yttrium oxide in feed and feces and N represents the percentage of a specific nutrient in feed and feces. To estimate the fatty acid (FA) content in the diets and organs, tridecanoic acid (13:0) was used as internal standard and added to the samples, 0.25 mg per 500 mg sample. The following formulas were applied for the estimations: FA (mg) = (peak area FA/peak area 13:0) x RF (response factor) x 13:0(mg); and: FA (mg/g feed or tissue) = FA (mg)/weighted sample (g). The RF is proportional with the number of active carbons in the fatty acid chain and varies among different fatty acids. Content of digestible fatty acids in the diet was calculated as for other nutrients based on fatty acid in the faeces and in the diet employing level of Yttrium in the faeces and diet.

Statistical analysis

Histological observations

The scores for both submucosal infiltration (DI enteritis) and enterocyte steatosis were categorical variables, and the impact of fish size and rapeseed oil level on the distribution of the histological scores among the diet groups were explored by ordinal logistic regression run in the R statistical package (version 4.2.1; 2022) using the polr (proportional odds logistic regression) package within the RStudio interphase (version 2023.06.1+524). Differences were examined based on odds ratios of large fish and rapeseed oil level having different histology scores compared to the small fish and the 0%-rapeseed oil diet.

Welfare scores

Statistical analyses of scale loss, skin lesions, eye damage and cataract as well as the sea lice number were performed using the unpaired t-test and fish size was considered as main variable. All data are means ± SEM. The level of significance was set to P<0.05.

Other results

For the main body of results a Bayesian approach was selected for the statistical analysis. This approach provides uncertainty awareness and robust reasoning for resource demanding studies aiming to describe dose-response relationships even when the number of observations is relatively small and without replications[24]. Although other approaches are available to define linear regressions functions from studies without replications[25], the Bayesian represents the most reliable approach to the influence of the outliers, enabling proper handle uncertainty on the key parameters and uncertainty in the models[26].

For simplicity, assume n observations for every studied dependent variable, denoted as $y_i, i \in \{1, \dots, n\}$ and the vector of corresponding independent variables $\boldsymbol{x}_i$. Further, for every studied dependent variable, 5 different models are suggested corresponding to 5 hypotheses on the dependence between the dependent and independent variables. More specifically $Y_i \sim N(\mu_k(\boldsymbol{x}_i), \sigma^2), k \in \{1, \dots, 5\}$ corresponding to models $m_1, \dots, m_5$ with:

1. $m_1: \mu_1(\boldsymbol{x}_i) = \beta_0$ – no effects of $\boldsymbol{x}_i$ on y_i (intercept only model).
2. $m_2: \mu_2(\boldsymbol{x}_i) = \beta_0 + \beta_{oil} RapeOil_i$ – intercept and a linear dependence on $RapeOil_i$ variable.
3. $m_3: \mu_3(\boldsymbol{x}_i) = \beta_0 + \beta_{size} Size_i$ – intercept and a linear dependence on $Size_i$ variable.
4. $m_4: \mu_4(\boldsymbol{x}_i) = \beta_0 + \beta_{size} Size_i + \beta_{oil} RapeOil_i$ – intercept and a linear dependence on $RapeOil_i$ and $Size_i$ variables.
5. $m_5: \mu_5(\boldsymbol{x}_i) = \beta_0 + \beta_{size} Size_i + \beta_{oil} RapeOil_i + \beta_{size,oil} Size_i \times RapeOil_i$ – intercept and a linear dependence on $RapeOil_i$ and $Size_i$ variables as well as the interaction effect between them.

Since models $m_1, \dots, m_5$ were addressed corresponding to 5 hypotheses on the relations between the dependent and independent variables to choose from, model uncertainty should also be carefully addressed. For the model priors $p(m_1), \dots, p(m_5)$, a uniform prior was used in the model space corresponding to all model priors being $p(m_1) = \cdots = p(m_5) = \frac{1}{5}$. This gives us a fully non-informative prior preference across the considered models. Further, default priors from *R-INLA*[27] library are assumed for the parameters. These priors for the fixed effects are standard independent normal with the mean of 0 and the variance of 1000, i.e., $N(0{,}1000)$ and are quite flat and are very weakly informative. For the intercept, $N(0, \infty)$ priors were used, which are completely flat and uninformative. Such a choice of priors is meant to reduce subjectivity of the models. A Bayesian framework does not require fit tests explicitly as we have hypotheses corresponding to m1 with no effects at all and if the evidence coming from the data is not strong enough, we shall prefer this simpler m1 to more complicated models.

For inference, the integrated nested Laplace approximations[28] implemented in *R-INLA* library were used to obtain the quantiles of all the marginal posterior distributions of interest. Also, for the Gaussian regression with Gaussian priors, exact marginal likelihoods $p(\boldsymbol{y} \mid \boldsymbol{x}, m), m \in \{m_{1,\dots,}m_5\}$ can be computed by *R-INLA* and allow to compute the marginal posterior model probabilities $p(m \mid \boldsymbol{x}, \boldsymbol{y}) = \frac{p(\boldsymbol{y}|\boldsymbol{x}, m)p(m)}{\sum_{m' \in \{m_{1,\dots,}m_5\}} p(\boldsymbol{y}|\boldsymbol{x}, m')p(m')}, m \in \{m_{1,\dots,}m_5\}$ through renormalization and thus accurately account for model uncertainty in the post-selection inference. Since uniform model priors are used selecting the model with respect to posterior marginal probabilities coincides with the selection according to Bayes factors between models m [29] and m': $BF(m, m') = \frac{p(\boldsymbol{y}|\boldsymbol{x}, m)}{p(\boldsymbol{y}|\boldsymbol{x}, m')}$. In this paper for each dependent variable Bayes factor between the most probable and second most probable models are reported as BF12.

Supplementary Table S2 explains terms and abbreviations used in the output from Bayesian statistics which are relevant for the present work.

The term Bayesian factor, BF12, is key to the interpretation of the results. In the notion of Kass and Raftery (1995)[29], the values of Bayes factors indicate evidence for relationship as follows: Values between **1** and **3.2**: *Negligible*; between **3.2** and **10**: *Substantial*; Between **10** and **100**: *Strong*; >**100**: *Decisive*.

Most of the standardized residuals of the finally selected models showed satisfactory characteristics regarding the requirement for homogeneity in normality and variance according to the p-values of Kolmogorov-Smirnov test (KS)[30] with the null hypotheses that standardized residuals come from a

309 homoscedastic standard normal distribution, i.e. for KS >0.05 we cannot reject the null, while for
310 results showing KS<0.05, the null is rejected, and interpretation of individual effects should be done
311 with caution, although we still have valid conclusions from the marginal posterior model probabilities
312 and Bayes factors on the explored set of models for each response.

313 Comparisons across models for different dependent variables described in the Results chapter were
314 made based on visual examination of the graphs, considering results for which the 95% credible
315 intervals are not overlapping as significantly different. These conclusions are only made for the models
316 where the assumptions on the residuals are satisfied.

317 **Results**

318 *Growth performance*

319 Growth performances were overall good. Small fish grew on average 1.4 kg reaching a final body
320 weight of 3.1, the large grew 1.9 kg and reached 6.4 kg, giving an averaged SGR of 1.04 and TGC of 4.3
321 for the small fish, and 0.63 and 3.4 for the large, respectively. See Supplementary Table S3 for details
322 and explanation of parameters. The statistical analyses of the results for TGC showed decisive
323 evidence for m3 (BF12= 919; Model probability (Prob) = 0.999, meaning that fish size but not dietary
324 fatty acid composition affected the result decisively. Regarding FCR, which averaged 1.1 and 1.2, and
325 CF, which averaged 1.50 and 1.52, respectively, the analyses showed decisive evidence for m1 (BF12:
326 5157 and 2408; Prob: 1.000 and 0.999, respectively), meaning that neither fish size nor dietary lipid
327 source affected these variables importantly. Feed consumption over the observation period,
328 expressed as percent of initial body weight, was 90% for the small, 42% for the large, meaning a much
329 higher feed and lipid load for the small than the large fish.

330 *Nutrient digestibility*

331 Results regarding nutrient digestibility, best model, probabilities, and BF12, are shown in Table 2a and
332 b, whereas further statistical characteristics are presented in Supplementary Table S4. The statistics
333 indicated high model probability for all observed nutrients, i.e., p>0.844, and correspondingly high
334 BF12, i.e., > 9.6 (8 nutrients showed substantial evidence levels, 14 nutrients showed strong evidence
335 levels, and 1 decisive level). The exceptions were 16:1 and 18:2 which showed lower model
336 probabilities. Regarding digestibility of crude protein, the evaluation showed substantial evidence for
337 model m3 (BF12: 6; Prob: 0.863), i.e., effect of fish size but not of rapeseed oil level in the feed. For
338 digestibility of fatty acids, the evaluation indicated effects only of rapeseed oil level, and not of fish
339 size, i.e. m2, for most, but not all, as follows: For the saturated fatty acids, as well as 20:1, 22:1, 18:2

and 18:3, evidence for m2 was strong, whereas the digestibility of the polyunsaturated fatty acids were not affected by rapeseed oil level (best model: m1).

Lipid digestibility in quantitative terms: the digestibility of sum of fatty acids was greatly affected by rapeseed oil level, increasing 13 %-units as the level increased from 0 and the 24% in the feed. The greatest elevating effects were seen for saturated fatty acids, with a difference between the feed with the lowest and highest rapeseed oil level of more than 20%. The longer and more unsaturated fatty acids showed higher digestibility, but still with clear, elevating effect of rapeseed inclusion level for 20:1 and 22:1, 18:2, and 18:3. The acids 16:1 and 18:1 showed the same trend, whereas the very long an unsaturated fatty acid appeared unaffected by rapeseed oil level in the feed. See Supplementary Table S4 for further statistical details.

Table 2a. *Digestibility (%) of crude protein (CP), Sum of fatty acids (FA), saturated and mono-unsaturated given as averages of small and large fish.*

	CP	Sum FA	14:0	16:0	18:0	20:0	16:1n7	18:1n9	20:1	22:1n9
Rapeseed oil level										
0	86.8	81.0	75.0	60.5	41.5	52.5	96.5	93.0	89.5	80.0
5	86.2	81.0	73.0	58.5	37.5	50.5	95.0	93.5	88.0	81.5
9	88.1	87.5	81.0	68.5	47.0	56.0	98.0	97.0	94.0	89.0
14	86.1	89.0	82.0	70.5	48.0	56.5	98.0	97.0	93.5	91.0
19	87.8	92.0	87.5	79.0	57.0	64.0	98.5	98.0	95.5	94.0
24	86.6	94.0	91.0	85.0	63.5	70.0	98.5	98.0	95.5	95.5
Statistics										
Best model	m3	m2	m2	m2	m2	m2	m1	m1	m2	m2
Probability	0.863	0.961	0.944	0.922	0.886	0.844	0.901	0.473	0.530	0.929
BF12	6	25	18	12	10	10	12	1	1	14
Model evidence	Subst	Strong	Strong	Strong	Strong	Strong	Strong	Negl	Negl	Strong

Table 2b. *Digestibility of n-6 and n-3 fatty acids.*

	18:2n6	20:4n6	18:3n3	20.5n3	22:5n3	22:6n3
Rapeseed oil level						
0	89.0	97.0	90.0	98.0	97.0	96.0
5	92.5	96.5	94.5	97.5	95.0	95.0
9	96.0	98.5	97.0	99.0	97.5	97.0
14	96.0	98.0	97.5	99.0	97.5	97.0
19	97.0	98.0	98.0	99.0	97.5	97.0
24	97.0	97.5	98.5	99.0	96.5	96.5
Statistics						
Best model	m2	m1	m2	m1	m1	m1
Probability	0.893	0.902	0.893	0.944	0.804	0.928
BF12	12	9	14	19	4	13
Model evidence	Strong	Subst	Strong	Strong	Subst	Strong

Plasma biochemistry

The observed plasma biomarkers showed decisive evidence for model m3 for alanine amino transferase (ALT), i.e., showing effect of fish size, but not of rapeseed oil level. The ALT averaged 25 and 17 U/l for the small and the large fish, respectively. The other observed plasma biomarkers showed no clear effects neither of fish size nor rapeseed oil level, i.e., m1 was the best model. The observed averages were for free fatty acids (FFA) 0.32 mmol/l, glucose (Glu) 6.8 mmol/l, cholesterol (Chol) 7.5 mmol/l, and for triglycerides (TG) 2.7 mmol/l. See Table S5 for statistical details.

Organ indices and lipid content

Results regarding organosomatic indices (OSI) of the PI, DI, Mes, and LI, as well as lipid concentration in PI, Mes, and LI are shown in Table 3 (See Supplementary Tables S3 and S5 for further details and statistics). The identified best models showed high probability with correspondingly high BF12, i.e., above 44, characterized as decisive evidence, for all these results. The exception was lipid level in Mes and liver which showed lower best model probability.

For lipid concentration in the PI tissue, model m4 was the best and showed high probability, i.e., showing clear, increasing effect of rapeseed oil level, higher values for the small fish, as well as an interaction between the two fish sizes, i.e. the difference between the small and the large fish increased with increasing rapeseed oil level in the diet. For the OSIPI m3 was the best model, showing higher values for the small fish than the large, but no clear effect of rapeseed oil level in the diet. Regarding the distal intestine (DISI) and OSIMes, and Mes Lipid, no clear relationship neither to diet rapeseed oil level, nor to fish size were observed (best model: m1). and liver (LISI), neither the organosomatic index nor lipid content were clearly affected by rapeseed oil level or fish size. Model m1 was the best for both. Although the model evidence was low for Mes Lipid and LI Lipid, it is worth mentioning that m4 was the best model for both, i.e., indicating a trend of effect of rapeseed oil level, fish size, as well as interaction between the two. Liver glycogen which averaged 1.9 % of the liver tissue, seemed unaffected by rapeseed oil level as well as fish size (data not shown).

Table 3. *Results for sampled fish regarding effects of fish size (0=small, 1=large) and feed rapeseed oil level on sum of identified digestible fatty acid (∑Dig. FA), somatic index (% of body weight) of pyloric intestine (OSIPI) cleaned of mesenteric tissue (Mes), distal intestine (OSIDI), Mes (OSIMes), and liver (OSILI), as well as lipid content (Sum of fatty acids, g/kg tissue) of PI, Mes, and LI (PI Lipid, Mes Lipid, LI Lipid)*

		∑dig. FA	OSIPI	PI Lipid	OSIDI	OSIMes	Mes Lipid	OSILI	LI Lipid
		g/kg feed	%	g/kg	%	%	g/kg	%	g/kg
Fish size	*Rapeseed oil level*								
0	0	189	2.6	66	0.5	6.2	610	1.21	34
0	5	197	2.9	90	0.4	6.6	589	1.27	30
0	9	207	2.6	77	0.5	6.3	830	1.26	35
0	14	214	2.8	107	0.5	6.5	688	1.33	35
0	19	238	2.7	121	0.5	6.5	726	1.39	46
0	24	251	2.9	129	0.5	6.6	648	1.29	55
1	0	181	2.0	69	0.6	6.4	633	1.23	27
1	5	187	1.9	62	0.5	6.3	694	1.27	29
1	9	211	2.3	84	0.4	7.0	736	1.23	30
1	14	217	2.2	66	0.5	6.8	697	1.29	32
1	19	242	2.4	87	0.5	7.3	796	1.26	32
1	24	250	2.3	97	0.5	6.7	728	1.32	36
Statistics									
Best model		m2	m3	m4	m1	m1	m4	m1	m4
Probability		0.919	0.978	0.77	0.921	0.999	0.459	0.999	0.436
BF12		11	45	7	12	898	2	2161	2
Model evidence		Strong	Strong	Strong	Strong	Decisive	Negl	Decisive	Negl

Histology

The histology scores showed significant effects of fish size (p=0.008) on steatosis symptoms in the pyloric caecal enterocytes (Figure 1). The severity of the symptoms increased significantly with increasing level of rapeseed oil in the feeds (p<0.035). Assigning a score of 0 for normal appearance, 1 for mild, 2 for moderate, 3 for marked and 4 for severe, and fitting a first-degree linear regression line to the results, gives the following equations for the small fish: y = 0.0532x + 3.1111, for the large fish: y = 0.0699x + 2.3452. The equations give average score for small and large fish fed 0 rapeseed oil equal to 3.1 and 2.3, respectively, i.e. a difference of 0.8, and for fish fed 24% rapeseed oil of 4.3 and 4.0, i.e. a difference of 0.3. The functions indicate a difference in score between small fish fed 0 and 24% rapeseed oil of 1.2, and for the large fish a score of 1.7.

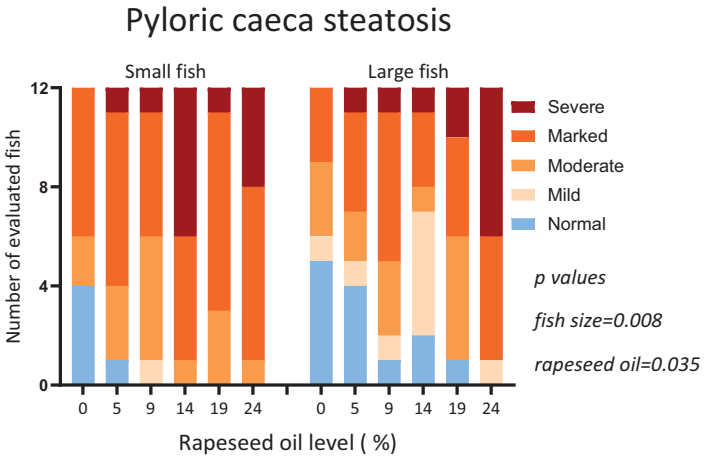

***Figure 1**. Distribution of the histology scores for enterocyte steatosis of the pyloric caeca tissue among the treatment groups. X-axis presents rapeseed oil level for the two fish sizes: small (S1 = autumn smolts) and large (S0 = spring smolts) fish. Table insert presents results for an ordinal logistic regression of impact of fish size and rapeseed oil level on the distribution of the histological scores among the diet groups for pyloric caeca enterocyte steatosis.*

The histological evaluation of inflammatory markers in PC and DI showed that most of fish displayed normal and healthy morphology. Some individuals in all diet groups showed mild to moderate submucosal cellularity in the DI (Figure 2). A significant effect of fish size was discerned (p=0.005; Figure 2) for occurrence of distal intestinal enteritis with more of the large fish observed with changes. No significant effects of dietary rapeseed oil level were observed on inflammatory changes (p=0.37).

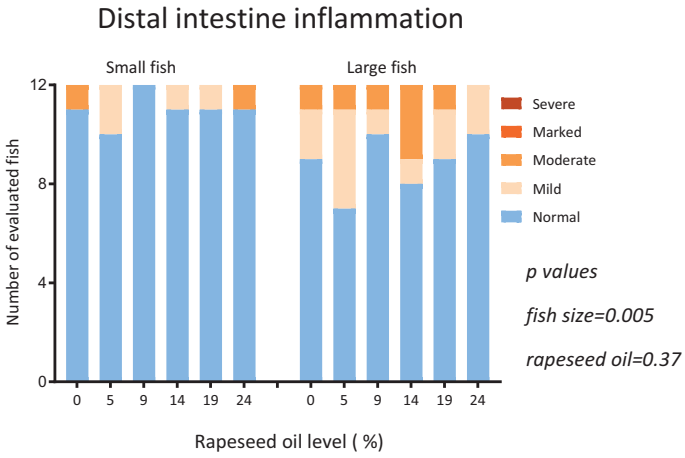

Figure 2*. Distribution of histology scores for inflammatory cell infiltration in the submucosa and lamina propria of distal intestine among the treatment groups. X-axis presents rapeseed oil level for the two fish sizes: small (S1 = autumn smolts) and large (S0 = spring smolts) fish. Table insert shows results for an ordinal logistic regression of impact of fish size and rapeseed oil level on the distribution of the histological scores among the diet groups for distal intestine submucosal infiltration.*

Gene expression

The gene expression results (Supplementary Table S6) showed high probabilities and high values of BF12, respectively: strong for *apoA-I* and *apoA-IV,* and decisive and substantial for *pcyt1α* and *plin2*. In detail: for *apoA-I, pcyt1α and plin2* the best model was m1, meaning no clear effect of either fish background or rapeseed oil level. The best model for *apoA-IV* was m3, showing clear effect of the fish size, being more expressed in the large than in the small fish, but no clear effects of rapeseed oil level (Figure 3).

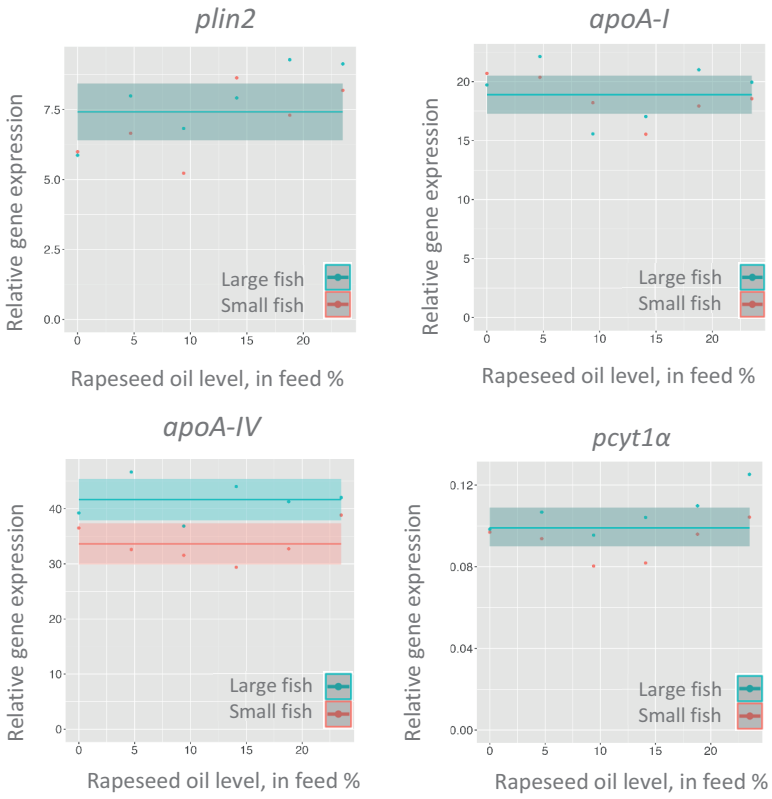

Figure 3. *Relative expression of biomarker genes for choline requirement. The curves show the estimated regression lines with indication of 95% credible intervals for posterior mean.*

F*atty acid profile and content in feed, pyloric intestine, mesenteric adipose tissue, and liver*

Table 3 and Figure 4 shows results regarding sum of fatty acids given as g digestible fatty acid/kg diet and g/kg tissue. Figure 5a and 5b show results regarding concentration of fatty acid in samples of the PI, Mes, and LI as well as absorbable fatty acid in the feed, given as % of sum of fatty acids for fatty acids present at levels >1%. The fatty acid results expressed as g/kg diet or tissue are shown in Supplementary Figure S1a and b. All results of the fatty acid analyses, including statistics, are shown in Supplementary Table S7a and S7b. As described under the chapter Statistics in Materials and Methods, the comparisons presented below of the models for the different dependent variables were based on visual examination of the graphs, considering results for which the 95% credible intervals

are not overlapping as significantly different, and conclusions are only made for the models where the assumptions on the residuals are satisfied.

Sum of digestible fatty acids in the diet and sum of fatty acids in PI, Mes, and LI, expressed in g/kg, increased with increasing rapeseed oil level in the diet. The Mes tissue was clearly a lipid storage organ, with more than 60% fat (589 – 830 g/kg). Lipid level in the PI was low (62 - 129 g/kg), but higher than of the liver (27 – 55 g/kg). Clear but minor effects of fish size were observed.

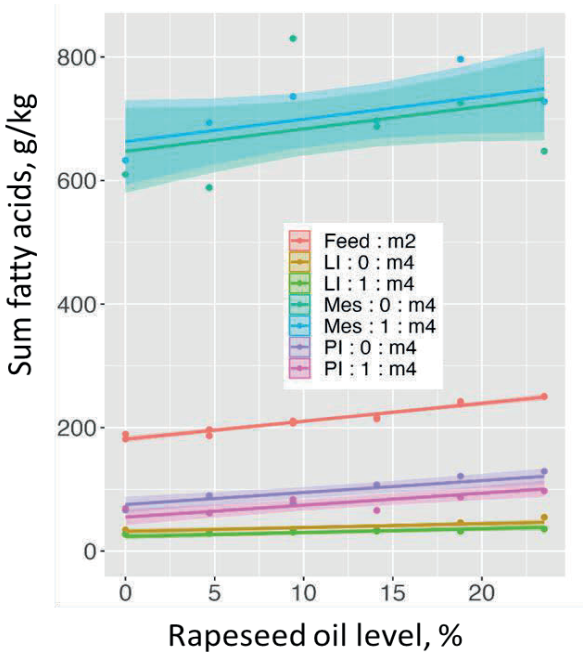

Figure 4. *Effects of the increasing dietary rapeseed oil level on the sum of fatty acids in pyloric caeca (PI), liver (LI), mesenteric fatty tissue (Mes) and on the sum of absorbable fatty acids in the feed (Feed) (Unit: g/kg feed or tissue), for small (0) and large (1) fish. The legend in the figure presents the best model for the data. The curves show estimated regression on dietary rapeseed oil level with indication of 95% credible intervals for the posterior means, allowing comparison of the results: lines and parts of lines for which the 95% range do not overlap differ significantly.*

At low rapeseed oil level, the lipid fraction of the PI tissue, through which the digestible fatty acids enter the body, contained lower concentrations (% of sum of fatty acids) of 14:0, 16:0*, 16:1*, 20:4n6, 20:5n3*, and 22:6n3 compared to the absorbable fat in the feed. At higher levels of rapeseed oil the difference was less or absent. For the fatty acids marked with an asterix, evidence for effect of fish size was observed. For 18:0, 18:3n3*, and 22:5n3 the picture was opposite, with higher levels of such fatty acids in the PI than in the feed. Again, the difference was greatest at low rapeseed oil level except for 22:5n3 for which the difference did not differ with rapeseed oil inclusion. For 18:1*, 18:2n6*, 20:1, the level in PI was lower than in the feed at low rapeseed oil level, higher at high levels.

In Mes, the tissue supposedly first in line to receive fatty acids after crossing the intestine, 14:0, 16:0, 16:1, 20:4n6, 20:5n3, and 22:6n3 showed concentrations lower than in the diet but higher than in the PI. For 18:0 the distance to the diet observations was greater, i.e., the concentration was much higher than for PI, diminishing with increasing rapeseed oil level, whereas 18:3n3 was lower in the Mes than in the diet, increasing with increasing rapeseed oil level. For 18:1 and 18:2n6 the level in the Mes was lower than in PI, and lower than in the diet. 22:5n3 was lower than in the PI, but higher than in the diet, i.e., intermediate between the PI and the diet. 20:1 showed a mixed picture with lower levels than in PI for all rapeseed oil levels, and lower levels than in the diet at the low rapeseed oil inclusion level, higher at the high levels.

In LI, an organ playing a key role in metabolism of fatty acids after absorption and transport to the peripheral circulation, the evidence for model m2 was high for all fatty acids, i.e. the level correlated with the level in absorbed fatty acids. The exceptions were C12:0 and C20:0, for which m1 showed high evidence, i.e. neither rapeseed oil level nor fish size affected the level importantly. For the fatty acids 14:0, 16:0, 16:1, 18:1, 18:2, and 18:3, the level in the liver was lower than in the absorbed lipid, for 18:0, 20:4, 22:5 and 22:6 it was higher, whereas for 20:1, 20:5 the level was quite similar.

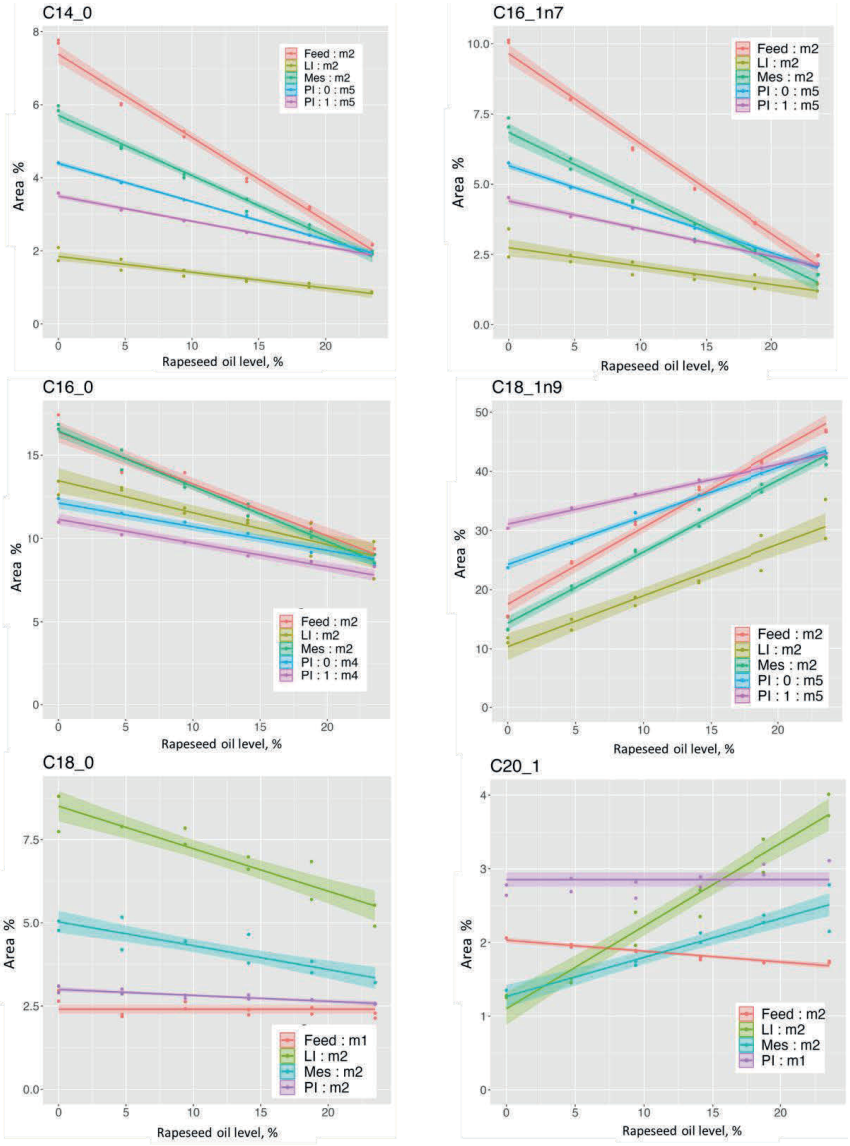

Figure 5a. *Effects of the increasing dietary rapeseed oil level on the relative level of saturated and mono-unsaturated fatty acids (% of sum of fatty acids, Area %) indicated on the left above the graphs, in absorbed fat, pyloric caeca (PI), mesenteric fatty tissue (Mes), and liver (Unit: % of sum fatty acids), representative for small (0) and large (1) fish. The legend in the figure indicates whether fish size clearly affected the results and the best model selected for the data. For fatty acids not clearly affected by fish size, average cures are presented. For fatty acids showing significant effects of fish size, separate curves are shown. The curves show the estimated regression lines with indication of 95% credible intervals for posterior means. The curves show estimated regression on dietary rapeseed oil level with indication of 95% credible intervals for the posterior means allowing comparison of the results: lines and parts of lines for which the 95% range do not overlap differ significantly.*

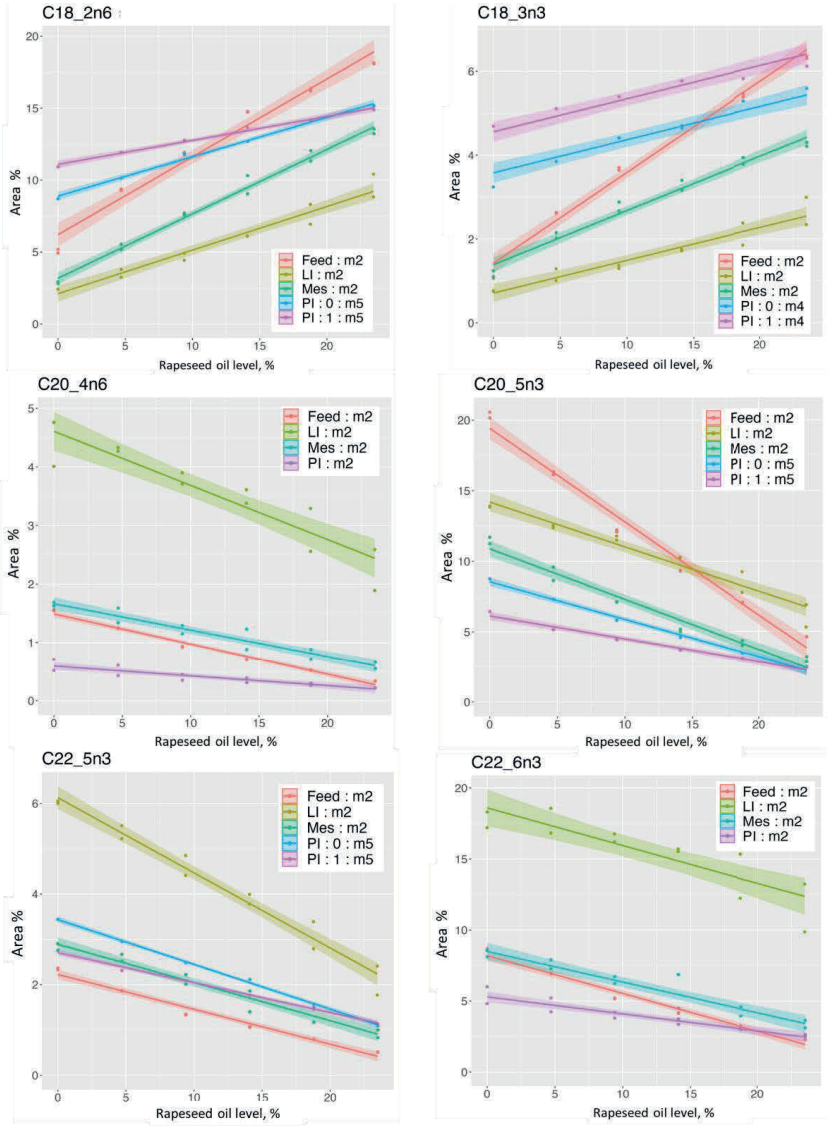

Figure 5b. *Effects of the increasing dietary rapeseed oil level on the relative level (% of sum of fatty acids, Area %) of poly-unsaturated fatty acids indicated on the left above the graphs, in absorbed fat, pyloric caeca (PI), mesenteric fatty tissue (Mes), and liver (Unit: % of sum fatty acids), representative for small (0) and large (1) fish. The legend in the figure indicates whether fish size clearly affected the results and the best model selected for the data. For fatty acids not clearly affected by fish size, average cures are presented. For fatty acids showing significant effects of fish size, separate curves are shown. The curves show the estimated regression lines with indication of 95% credible intervals for posterior means. The curves show estimated regression on dietary rapeseed oil level with indication of 95% credible intervals for the posterior means allowing comparison of the results: lines and parts of lines for which the 95% range do not overlap differ significantly.*

Welfare

Sea lice infestation was a significant challenge for the fish during the experiment, as it is for most Norwegian salmon farms. The number of lice, recorded as motile lice as well as adult female lice, was significantly higher for the smaller fish than the large (Figure 6). Scale loss showed the same picture. The scores for the other observed welfare indicators, i.e., skin lesions, eye damage, cataract and fin erosion were not affected either of fish size or rapeseed oil level. The complete dataset of the welfare assessment is given in Supplementary Table S8. Among the 1200 fish used in the trial, 50 died during the feeding period, randomly distributed among the treatments.

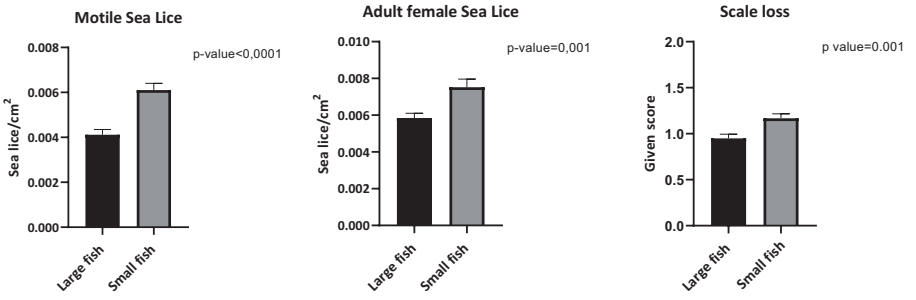

Figure 6. *Results of welfare scores observed at termination of the feeding experiment.*

Discussion

Effects of dietary lipid source on choline requirement

While planning the current study, the idea was to investigate the effects of dietary lipid source on choline requirement, by employing diets with constant lipid level and varying ratio of fish oil to rapeseed oil, i.e. the main marine and plant oil used in salmon diets. However, the variation in lipid source affected the lipid and fatty acid digestibility to a much greater degree than expected, and made the diets differ in total content of digestible lipid. The lipid digestibility of the diet without rapeseed oil was quite low, 81%. Similarly low digestibility coefficients have been observed in other studies conducted on Atlantic salmon. Karalazos et al [31], comparing diets varying in proportion of lipid sources, observed that the diet with the highest fish oil content was also the one showing the lowest lipid digestibility, 82%. On the other hand, increasing rapeseed oil level increased lipid digestibility. Another

study, conducted by Ng et al [32], showed that varying the proportion of rapeseed oil and crude palm oil in salmon feeds caused a variation in lipid digestibility of 3% for total fat, and 11% for C16:0. In the present study, the increasing rapeseed oil level enhanced lipid digestibility and therefore dietary lipid supply to the fish. This observation should be seen in relation to the results obtained from our recent, related study, which showed that dietary lipid level is the main factor influencing choline requirement in Atlantic salmon [20].

According to our previous studies, the histological scoring of enterocyte steatosis in the pyloric caeca is among the most sensitive biomarkers of choline deficiency. As this biomarker was the most sensitive in the present study, we decided to use it in order to obtain information on the magnitude of impact of the treatments on choline requirement [33]. The increasing rapeseed oil level from 0 to 25% increased histology score of 1.2 and 1.7 units, respectively for the small and the large fish. By employing the dose-response relationship between dietary choline level and histological steatosis score observed in Hansen et al.'s study [3] and illustrated in Figure 7, we obtained a semi-quantitative indication of the effects of the treatments on choline requirement: for the large fish of about 650 mg/kg, for the small fish about 450 mg/kg.

The results concerning the lipid content in the pyloric intestine mirrored the histological assessment, showing increasing lipid level with increasing rapeseed to fish oil ratio. At the same time, the lipid content was higher in the smaller fish compared to the large ones. Also the mesenteric and hepatic lipid levels seemed to correspond to the histological scores and pyloric intestine lipid content, but with weaker statistical evidence. On the contrary, the relative weight of the pyloric intestine (OSIPI), which is a sensitive biomarker for choline deficiency in Hansen et al.'s study [3], did not show clear effect of the rapeseed to fish oil ratio. The explanation for this apparent discrepancy may be fewer observation points and substantially larger variance in the present than in Hansen et al.'s study. The larger fish size and the exposure to the natural environmental variations may also have contributed to the overall variation in the present work.

As mentioned above, the effects of increasing ratio of rapeseed to fish oil may partly be related to the enhanced lipid digestibility, which increased lipid supply to the fish from 28.3 to about 33.0%. In our previous study[20], a 5% increase in the level of digestible lipid in the feed increased histological scores of about 0.7 units at 8°C, and 1.5 at 15°C, corresponding to shifts in choline requirement of 200 and 600 mg/kg, respectively. Based on these observations, our conclusion is therefore that the present study, in which the environmental temperature averaged 10.5°C, indicates that the ratio of fish oil to

rapeseed oil had minor effects on choline requirement and that the main cause of observed effect of lipid source on choline requirement was the supply of digestible lipid.

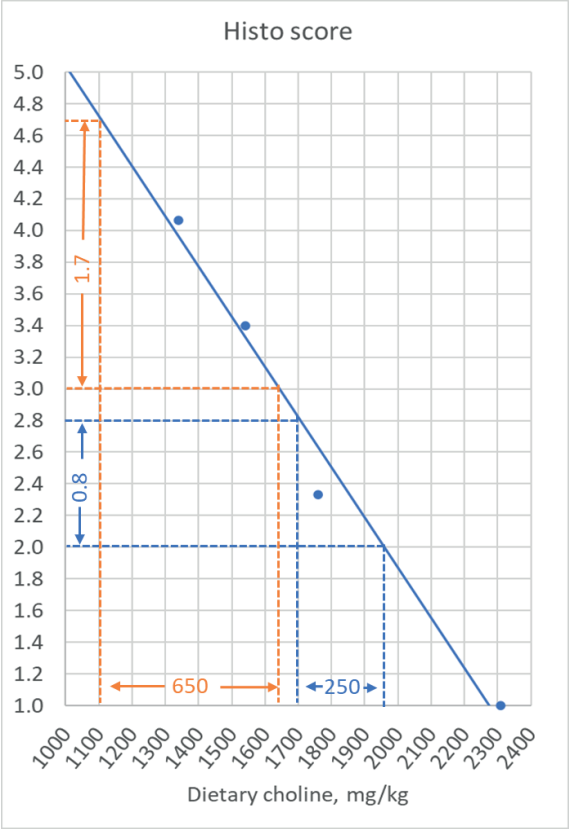

Figure 7. *The curve in the figure illustrates the dose-response relationship between dietary choline level and intestinal steatosis, as previously published by Hansen et al.*[3]*. The dotted lines illustrate the strategy used for estimation of effect on choline requirement in the present study. The increase in rapeseed oil from 0 to 24% increased the score by 1.7 units (orange line) in the large fish. The figure indicates that this shift corresponds to a shift in choline requirement of about 650 mg/kg. The shift in the small fish was 1.2, corresponding to a shift of 450 mg/kg (not illustrated). The difference between the two fish sizes, when fed the lowest rapeseed oil level, was 0.8 units (blue line), i.e. lower in the small than the large fish, corresponding to a difference in choline requirement of about 250 mg/kg. At the highest rapeseed oil level, the difference was 0.3, corresponding to a difference in choline requirement of about 100 mg/kg (not illustrated). As there were great differences in the experimental conditions between Hansen et al.'s experiment and the present, these estimates should be taken as indications of magnitude of effects rather than accurate estimates.*

Effects of fish size on choline requirement

The effect of fish size on choline requirement was calculated by using the same dose-response relationship between dietary choline level and histological steatosis score previously published [3]. Accordingly, fish size induced a variation of 0.5 units, corresponding to a 200 mg/kg higher dietary choline requirement in small compared to large fish. However, as daily feed intake and weight gain, as percent of body weight, were higher in small fish, also lipid consumption was relatively higher in small compared to large fish. At present, no experimental studies addressing effects of feed intake on steatosis symptoms and choline requirement in fish have been found. However, a life cycle field survey observing prevalence and severity of intestinal steatosis in farms along the Norwegian coast, clearly indicated that in periods with lower feed intake, i.e. when temperature is low, symptoms of steatosis are mostly absent, supposedly due to lower lipid supply and reduced demand for lipid transport capacity [34]. The present results therefore suggest that the effect of fish size, which was rather small, most likely was due to difference in lipid supply, and that effect of the differences in developmental stage, smoltification strategy, and genetic background was minor. Further studies are needed to confirm these suggestions.

Effects on gene expression

The gene expression biomarkers observed to be most sensitive to variation in choline supply in Hansen et al.[3]'s work comprise *plin2*, *apoA-I*, *apoA-IV* and *pcyt1α.* In particular, a clear relationship between *plin2* expression, a general marker for the lipid load of non-adipogenic cells, and severity of intestinal steatosis, has been documented in several recent studies conducted in salmon [1,20,35–38]. In the present study, higher expression of *apoA-IV* was observed in the large than the small fish, but otherwise no clear effects of rapeseed oil level or fish size were detected. In our most recent work by Siciliani et al.[20], in which dietary lipid level increased from 16 to 26% in choline-deficient diets (1600 g/kg), expression levels of *plin2*, *apoA-I*, *apoA-IV* and *pcyt1α* showed clear dose-response relationships with dietary fat inclusion and intestinal steatosis. However, the study also showed that interactions between the dietary lipid level and the two assessed water temperatures, 8 and 15°C, influenced the expression pattern of these genes. The present study was conducted at decreasing temperatures in the range between the two temperatures studied in the previous work. Hence, a discussion of the absence of effect of rapeseed oil level and fish size must wait until the knowledge is strengthened.

Fatty acid metabolism

The fatty acid composition of the pyloric intestine (PI), mesenteric fat (Mes), and liver (LI), provide information which strengthens the knowledge of lipid metabolism in these tissues in relationship to

fatty acid composition of the diet. The transport routes and chemical form of lipid from the intestinal lumen to the peripheral tissues in fish are not well described yet. The work of Denstadli et al. [39], employing isotopes of C10:0 and C18:1 in a force-feeding trial with Atlantic salmon, indicates that both lipid transport route and chemical form may differ depending on the chain length of the fatty acids supplied and time after feeding. A route via the portal vein and liver is suggested for both medium and long chain fatty acids. However, a more direct route to the peripheral tissues is also indicated. The proportion of free and bound fatty acids changed with time after feeding. According to the results of the studies of effects of choline supply, it is also likely that dietary choline and lipid level, as well as feed intake, may affect lipid transport route and chemical form[1,20,35,36]. In the following discussion of fatty acid profile of the PI, Mes, and liver, it should be kept in mind that the results were obtained from fish under severe choline deficient conditions which may have affected the route of transport, storage, as well as lipid metabolism in the observed tissues. The fact that the fatty acids may be oxidized, elongated, integrated into phospholipids or triglycerides, and supposedly also selectively released from the tissues, makes discussion of the results difficult. The fatty acid profile, i.e. % of total, of the Mes, overall, was closer to that of the feed, than the profile of the PI. For instance, the 14:0 level, expressed as % of total lipid, was lower in the PI fat than in the absorbed lipid, while 18:0 was higher. The 14:0 might therefore have been elongated to 18:0. Similarly, the observation that level of 20:5 was lower in PI than in the feed, whereas 22:6 was higher, indicates that 20:5 might have been converted to 22:6 in the PI. Further discussion of these aspect requires more thorough studies of fatty acid metabolism in PI and Mes.

These observations suggest that the fatty acids accumulating in the enterocytes of the PI are, to some degree, selected for storage or for passage through. Another possibility is that fatty acids stored in the enterocytes are metabolized and thereby altering the profile of the PI tissue. Recent publications, including relevant results over fatty acid metabolism in gut cell culture studies[40,4142,43,44] support this suggestion. The fact that the fatty acid profile of the Mes was more similar to that of the lipid absorbed from the feed, than to that of the PI, even if the PI precedes the Mes in the lipid transport route, may be explained by the quantities of lipid passing through the enterocytes compared to the quantity which is stored. The quantity stored in Mes was many times higher than the quantity stored in the intestine, and therefore the amounts stored in PI would not necessarily affect the lipid profile of the Mes.

The liver is commonly regarded as the key organ in lipid metabolism in an animal [45–47]. Accordingly, although correlations with level of fatty acids in the diet were observed, the liver differed the most from the levels in the absorbed lipid, supposedly reflecting substantial selectivity and high rate of metabolism of the fatty acids supplied to the organ. For instance, the relative level of C20:4n-6 and

C22:6n3 was higher in the liver than in in the absorbed fat, indicating metabolic conversion from their precursors C18:2n6 and C18:3n3.

Conclusions

The Increasing dietary rapeseed oil level, and the higher feed intake of small fish induced moderate increases in severity of intestinal steatosis. The increase in rapeseed to fish oil ratio enhanced lipid digestibility and consequently lipid supply. Small fish showed relatively higher feed consumption, and consequently higher lipid intake. As increasing dietary lipid level has been shown to increase severity of steatosis, the conclusion based on the present results is that neither lipid source nor fish size affect choline requirement importantly.

Although the overall fatty acid composition of all the observed tissues reflected that of the absorbable fat in the feed, the fatty acid composition of the mesenteric tissue resembled it more closely compared to the pyloric intestine, whereas the composition in the liver showed greater differences.

The present findings strengthen the basis for designing further experiments addressing choline requirement. A dose-response experiment with the aim to define choline requirement should be conducted using a fast-growing salmon breed, raised at a temperature on the high end of the normal range, and fed to diets containing the highest lipid level which would be used in commercial feeds.

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Author contributions

The author's contributions were as follows: D.S.: sampling, molecular analyses, data evaluation and interpretation and manuscript development and revision, A.H.: statistical analyses, manuscript development and manuscript revision, B.R.: participation to fatty acid metabolism analyses and manuscript review, E.M.C.: histological analyses, data evaluation, interpretation, and manuscript revision, V.G.T.: sampling, molecular analyses, manuscript development and revision, E.C.V.: molecular analyses, manuscript revision, A.K.G.H: experimental design and manuscript revision, H. H.: planning and conducting the feeding trial, T.M.K.: supervision, data evaluation, interpretation, and manuscript revision, Å.K: project leadership, experimental design, fatty acid metabolism analyses, data evaluation and interpretation, and manuscript development.

Conflict of interest

None

Data availability statement

Raw data supporting the results presented in the paper are available upon request.

Figures' legend

Figure 1. Distribution of the histology scores for enterocyte steatosis of the pyloric caeca tissue among the treatment groups. X-axis presents rapeseed oil level for the two fish sizes: small (S1 = autumn smolts) and large (S0 = spring smolts) fish. Table insert presents results for an ordinal logistic regression of impact of fish size and rapeseed oil level on the distribution of the histological scores among the diet groups for pyloric caeca enterocyte steatosis.

Figure 2. Distribution of histology scores for inflammatory cell infiltration in the submucosa and lamina propria of distal intestine among the treatment groups. X-axis presents rapeseed oil level for the two fish sizes: small (S1 = autumn smolts) and large (S0 = spring smolts) fish. Table insert shows results for an ordinal logistic regression of impact of fish size and rapeseed oil level on the distribution of the histological scores among the diet groups for distal intestine submucosal infiltration.

Figure 3. Relative expression of biomarker genes for choline requirement. The curves show the estimated regression lines with indication of 95% credible intervals for posterior mean.

Figure 4. Effects of the increasing dietary rapeseed oil level on the sum of fatty acids in pyloric caeca (PI), liver (LI), mesenteric fatty tissue (Mes) and on the sum of absorbable fatty acids in the feed (Feed) (Unit: g/kg feed or tissue), for small (0) and large (1) fish. The legend in the figure presents the best model for the data. The curves show estimated regression on dietary rapeseed oil level with indication of 95% credible intervals for the posterior means, allowing comparison of the results: lines and parts of lines for which the 95% range do not overlap differ significantly.

Figure 5a. Effects of the increasing dietary rapeseed oil level on the relative level of saturated and mono-unsaturated fatty acids (% of sum of fatty acids, Area %) indicated on the left above the graphs, in absorbed fat, pyloric caeca (PI), mesenteric fatty tissue (Mes), and liver (Unit: % of sum fatty acids), representative for small (0) and large (1) fish. The legend in the figure indicates whether fish size clearly affected the results and the best model selected for the data. For fatty acids not clearly affected by fish size, average cures are presented. For fatty acids showing significant effects of fish size, separate curves are shown. The curves show the estimated regression lines with indication of 95% credible intervals for posterior means. The curves show estimated regression on dietary rapeseed oil

level with indication of 95% credible intervals for the posterior means allowing comparison of the results: lines and parts of lines for which the 95% range do not overlap differ significantly.

Figure 5b. Effects of the increasing dietary rapeseed oil level on the relative level (% of sum of fatty acids, Area %) of poly-unsaturated fatty acids indicated on the left above the graphs, in absorbed fat, pyloric caeca (PI), mesenteric fatty tissue (Mes), and liver (Unit: % of sum fatty acids), representative for small (0) and large (1) fish. The legend in the figure indicates whether fish size clearly affected the results and the best model selected for the data. For fatty acids not clearly affected by fish size, average cures are presented. For fatty acids showing significant effects of fish size, separate curves are shown. The curves show the estimated regression lines with indication of 95% credible intervals for posterior means. The curves show estimated regression on dietary rapeseed oil level with indication of 95% credible intervals for the posterior means allowing comparison of the results: lines and parts of lines for which the 95% range do not overlap differ significantly.

Figure 6. Results of welfare scores observed at termination of the feeding experiment.

Figure 7. The curve in the figure illustrates the dose-response relationship between dietary choline level and intestinal steatosis, as previously published by Hansen et al.3. The dotted lines illustrate the strategy used for estimation of effect on choline requirement in the present study. The increase in rapeseed oil from 0 to 24% increased the score by 1.7 units (orange line) in the large fish. The figure indicates that this shift corresponds to a shift in choline requirement of about 650 mg/kg. The shift in the small fish was 1.2, corresponding to a shift of 450 mg/kg (not illustrated). The difference between the two fish sizes, when fed the lowest rapeseed oil level, was 0.8 units (blue line), i.e. lower in the small than the large fish, corresponding to a difference in choline requirement of about 250 mg/kg. At the highest rapeseed oil level, the difference was 0.3, corresponding to a difference in choline requirement of about 100 mg/kg (not illustrated). As there were great differences in the experimental conditions between Hansen et al.'s experiment and the present, these estimates should be taken as indications of magnitude of effects rather than accurate estimates.

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

Gene name	Gene symbol	Forward primer	Reverse primer	Amplicon size	Annealing temperature	Efficiency	Acc. number
Target genes							
Perilipin2	*plin2*	CCCAGGTCTACTCCAGCTTC	CAGCGACTCCTTCATCTTGC	104	60	2.0	BT072598
Apolipoprotein A-I	*apoA-I*	CTGGTCCTCGCACTAACCAT	TGGACCTCTGTGCAGTCAAC	144	60	2.0	NM_001123663
Apolipoprotein A-IV	*apoA-IV*	CAGGACCAGTCTCAGCAACA	GTTGACTTCCTGTGCCACCT	131	60	1.9	BT048822
Choline-phosphate cytidylyltransferase	*pcyt1a*	CGGGTCTATGCAGATGGAAT	GCTCGTCCTCGTTCATCACT	166	60	2.1	BT045986
Reference genes							
RNA polymerase II	*rnapolI*	CCAATACATGACCAAATATGAAAGG	ATGATGATGGGGATCTTCCTGC	157	60	2.0	BG936649
hypoxanthine phosphoribosyl transferase 1	*hprt1*	CCGCCTCAAGAGCTACTGTAAT	GTCTGGAACCTCAAACCCTATG	255	60	1.9	BT043501
glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	AAGTGAAGCAGGAGGGTGGAA	CAGCCTCACCCCATTTGATG	96	60	2.1	BT050045

Table S2. *Explanation of terms and abbreviations used for the Bayesian statistics.*

	Explanations
Best model	Model with the highest posterior probability
Prob	Posterior probability of the best model
BF12	Bayes factor between the best model and the model with the second highest posterior probability
Model efficiency	Values of BF12 indicating evidence for the selected models
Negl	Value indicating negligible evidence for the selected model (BF12 between 1 and 3.2)
Subst	Value indicating substantial evidence for the selected model (BF12 between 3.2 and 10)
Strong	Value indicating strong evidence for the selected model (BF12 between 10 and 100)
Decis	Value indicating decisive evidence for the selected model (BF12 >100)
B0	Posterior mode of the intercept
B0L	Lower bound of 95% credible interval for the intercept
B0U	Upper bound of 95% credible interval for the intercept
BS	Posterior mode of the effect for Size
BSL	Lower bound of 95% credible interval for the effect for Size
BSU	Upper bound of 95% credible interval for the effect for Size
BR	Posterior mode of the effect for Rape Oil
BRL	Lower bound of 95% credible interval for the effect for Rape Oil
BRU	Upper bound of 95% credible interval for the effect for Rape Oil
BRS0	Posterior mode of the effect for Rape Oil for small fish
BRS0L	Lower bound of 95% credible interval for the effect for Rape Oil for small fish
BRS0U	Upper bound of 95% credible interval for the effect for Rape Oil for small fish
BRS1	Posterior mode of the effect for Rape Oil for large fish
BRS1L	Lower bound of 95% credible interval for the effect for Rape Oil for large fish
BRS1U	Upper bound of 95% credible interval for the effect for Rape Oil for large fish
KS	P-value for the Kolmogorov – Smirnov test for the residuals

Table S3. *Results of statistical evaluation of effects of fish size and rapeseed oil level in the feed on final weight (BW), conditions factor (CF), growth (TGC), yield, organosomatic indices of the pyloric organ package (OSIPI1) and pyloric intestine cleaned of fatty tissue (OSIPI2), mid intestine (MISI), and distal intestine (DISI)*.*

	Best model	Prob	BF12	Model efficiency	B0	B0L	B0U	BS	BSL	BSU	KS
BW	m3	1.000	11465	Decis	8.75	8.72	8.78	-0.72	-0.76	-0.67	0
TGC	m3	0.999	919	Decis	1.37	1.33	1.41	0.187	0.13	0.244	0
FCR	m1	1.000	5157	Decis	1.12	1.08	1.17	0	0	0	0.05
CF	m1	0.999	2408	Decis	0.92	0.91	0.93	0	0	0	0
OSIPI1	m1	0.995	208	Decis	2.02	2.00	2.04	0	0	0	0.05
OSIPI2	m3	0.978	45	Strong	1.16	1.13	1.18	0.158	0.12	0.195	0.3
MISI	m1	0.999	898	Decis	0.19	0.18	0.19	0	0	0	0
DISI	m1	0.921	12	Strong	0.4	0.38	0.41	0	0	0	0
LISI	m1	0.999	2161	Decis	0.82	0.81	0.83	0	0	0	0

** Explanations to the abbreviations are given in Table S2.*

Table S4. *Results of statistical evaluation of digestibility of crude protein, sum of fatty acids (FA) and fatty acid**

	Best model	Prob	BF12	Model efficiency	B0	B0L	B0U	BS	BSL	BSU	KS
Crude protein	m3	0.863	6	Subst	88.1	87.3	89.0	-2.41	-3.63	-1.19	0.811
Sum FA	m2	0.961	25	Strong	80.2	78.4	82.0	0.62	0.49	0.75	0.210
12:0	m1	0.857	9	Subst	91.3	89.7	93.0	0	0	0	0.110
14:0	m2	0.944	18	Strong	72.8	69.9	75.7	0.74	0.54	0.95	0.988
15:0	m2	0.935	15	Strong	66.2	62.9	69.5	0.87	0.64	1.10	0.996
16:0	m2	0.922	12	Strong	57.0	53.2	60.8	1.14	0.87	1.40	0.997
17:0	m2	0.916	11	Strong	54.3	49.9	58.8	1.30	0.99	1.62	0.989
18:0	m2	0.886	10	Strong	36.9	31.8	42.0	1.03	0.67	1.39	0.928
20:0	m2	0.844	10	Strong	49.3	45.0	53.5	0.77	0.47	1.07	0.812
22:0	m1	0.421	1	Negl	-1.0	-8.9	6.9	0	0	0	0.300
24:0	m2	0.862	7	Subst	38.8	33.0	44.7	1.21	0.80	1.62	0.967
16:1n7	m1	0.901	12	Strong	97.4	96.5	98.4	0	0	0	0.000
18:1n9c	m1	0.473	1	Negl	96.1	94.7	97.5	0	0	0	0.001
20:1	m2	0.530	1	Negl	88.9	86.7	91.1	0.32	0.17	0.48	0.038
22:1n9	m2	0.929	14	Strong	80.2	77.7	82.7	0.71	0.53	0.88	0.131
22:1n11	m1	0.583	2	Negl	91.6	89.6	93.5	0	0	0	0.001
24:1	m2	0.933	15	Strong	68.4	65.2	71.7	0.83	0.60	1.05	0.249
18:2n6c	m2	0.893	12	Strong	90.9	89.3	92.5	0.32	0.20	0.43	0.811
18:3n3	m2	0.893	14	Strong	92.2	90.5	93.8	0.32	0.21	0.44	0.759
18:3n6	m1	1.000	20799	Decis	100	100	100	0	0	0	0.000
20:2	m1	0.863	6	Subst	97.5	96.8	98.1	0	0	0	0.000
20:3n3	m1	0.756	5	Subst	42.9	37.4	48.5	0	0	0	0.454
20:3n6	m1	0.858	6	Subst	95.8	94.5	97.1	0	0	0	0.000
20:4n6	m1	0.902	9	Subst	97.7	97.1	98.4	0	0	0	0.000
20:5n3	m1	0.944	19	Strong	98.5	98.0	99.1	0	0	0	0.000
22:5n3	m1	0.804	4	Subst	96.8	96.1	97.6	0	0	0	0.000
22:6n3	m1	0.928	13	Strong	96.4	95.8	97.0	0	0	0	0.000

** Explanations to the abbreviations are given in Table S2.*

Table S5. *Results of statistical evaluation of effects of fish size and rapeseed oil level in the feed on plasma biomarkers**

	Model	Prob	BF12	Model efficiency	B0	B0L	B0U	BS	BSL	BSU	KS
TG	m1	0.957	22	Strong	2.7	2.6	2.8	0	0	0	0
FFS	m1	0.686	2	Negl	0.32	0.29	0.34	0	0	0	0
Chol	m1	0.992	128	Decis	7.5	7.3	7.7	0	0	0	0
Glu	m1	0.920	12	Strong	6.8	6.6	7.0	0	0	0	0
ALT	m3	0.995	228	Decis	17.3	15.3	19.4	7.4	4.5	10.3	0

** Explanations to the abbreviations are given in Table S1. ALT=Alanine transferase, FFS: free fatty acids, Glu=glucose, Chol=Cholesterol, TG=triglycerides.*

Table S6. *Results of statistical evaluation of effects of fish size and rapeseed oil level in the feed on expression of genes selected for responsibility to level of supply of choline.*

	Best model	Prob	BF12	Model Efficiency	B0	B0L	B0U	BS	BSL	BSU	KS
apoA-I	m1	0.954	22	Strong	18.9	17.7	20.1	0	0	0	0.383
apoA-IV	m3	0.965	32	Strong	41.7	38.9	44.4	-8.1	-11.9	-4.2	0.832
pcyt1a	m1	0.997	294	Decis	0.099	0.092	0.106	0	0	0	0.628
adph	m1	0.844	8	Subst	7.42	6.675	8.162	0	0	0	0.436

** Explanations to the abbreviations are given in Table S2.*

Table S7a. *Results of evaluation of effects of fish size and rapeseed oil level in the feed on fatty acid content in absorbed fat (Feed), mesenteric fatty tissue (Mes), pyloric tissue (PI) and liver (LI) expressed as area % of sum of fatty acids**

	Best model	Prob	BF12	B0	B0L	B0U	BS	BSL	BSU	BR	BRL	BRU	BRS0	BRS0L	BRS0U	BRS1	BRS1L	BRS1U	KS
C12:0 Feed	m2	1.000	13826	0.118	0.114	0.122	0	0	0	-0.004	-0.004	-0.003	0	0	0	0	0	0	0.764
C14:0 Feed	m2	0.996	221	7.39	7.14	7.63	0	0	0	-0.228	-0.245	-0.211	0	0	0	0	0	0	0.687
C16:0 Feed	m2	0.988	85	16.4	15.8	17.0	0	0	0	-0.311	-0.355	-0.267	0	0	0	0	0	0	0.985
C16:1n7 Feed	m2	0.994	169	9.65	9.32	9.98	0	0	0	-0.321	-0.345	-0.298	0	0	0	0	0	0	0.182
C18:0 Feed	m1	0.992	234	2.40	2.27	2.54	0	0	0	0	0	0	0	0	0	0	0	0	0.048
C18:1n9c Feed	m2	0.973	36	17.6	16.1	19.0	0	0	0	1.296	1.191	1.4	0	0	0	0	0	0	0.053
C18:2n6c Feed	m2	0.985	68	6.20	5.37	7.03	0	0	0	0.541	0.483	0.599	0	0	0	0	0	0	0.063
C20:0 Feed	m1	0.736	3	0.619	0.586	0.652	0	0	0	0	0	0	0	0	0	0	0	0	0.125
C20:1 Feed	m2	0.998	720	2.03	1.99	2.07	0	0	0	-0.015	-0.017	-0.012	0	0	0	0	0	0	0.501
C18:3n3 Feed	m2	0.996	236	1.42	1.19	1.66	0	0	0	0.217	0.2	0.233	0	0	0	0	0	0	0.036
C20:3n3 Feed	m1	0.582	1	0.023	0.015	0.031	0	0	0	0	0	0	0	0	0	0	0	0	0.226
C20:4n6 Feed	m2	0.999	965	1.49	1.43	1.55	0	0	0	-0.052	-0.056	-0.048	0	0	0	0	0	0	0.550
C20:5n3 Feed	m2	0.986	70	19.4	18.6	20.2	0	0	0	-0.663	-0.719	-0.608	0	0	0	0	0	0	0.402
C22:5n3 Feed	m2	0.998	526	2.22	2.12	2.33	0	0	0	-0.077	-0.084	-0.07	0	0	0	0	0	0	0.989
C22:6n3 Feed	m2	0.994	156	8.19	7.84	8.55	0	0	0	-0.266	-0.291	-0.241	0	0	0	0	0	0	0.761
C12:0 Mes	m1	0.997	360	0.038	0.033	0.044	0	0	0	0	0	0	0	0	0	0	0	0	0.028
C14:0 Mes	m2	0.996	279	5.71	5.54	5.87	0	0	0	-0.165	-0.176	-0.153	0	0	0	0	0	0	0.999
C16:0 Mes	m2	0.503	1	16.4	16.1	16.8	0	0	0	-0.331	-0.358	-0.304	0	0	0	0	0	0	0.758
C16:1n7 Mes	m2	0.988	85	6.85	6.52	7.18	0	0	0	-0.228	-0.251	-0.205	0	0	0	0	0	0	0.990
C18:0 Mes	m2	0.771	7	5.03	4.70	5.36	0	0	0	-0.072	-0.095	-0.048	0	0	0	0	0	0	0.793
C18:1n9c Mes	m2	0.938	15	14.4	13.2	15.5	0	0	0	1.202	1.121	1.282	0	0	0	0	0	0	0.989
C18:2n6c Mes	m2	0.961	25	3.18	2.76	3.60	0	0	0	0.448	0.418	0.477	0	0	0	0	0	0	0.767
C20:0 Mes	m1	0.999	808	0.466	0.437	0.495	0	0	0	0	0	0	0	0	0	0	0	0	0.045
C20:1 Mes	m2	0.983	65	1.27	1.11	1.42	0	0	0	0.053	0.042	0.064	0	0	0	0	0	0	0.148
C18:3n3 Mes	m2	0.996	280	1.39	1.21	1.57	0	0	0	0.129	0.117	0.142	0	0	0	0	0	0	0.875
C20:3n3 Mes	m2	1.000	2393	0.196	0.177	0.215	0	0	0	0.013	0.012	0.015	0	0	0	0	0	0	0.303

C20:4n6 Mes	m2	0.962	26	1.67	1.55	1.78	0	0	0	-0.045	-0.053	-0.037	0	0	0	0	0	0	0.326
C20:5n3 Mes	m2	0.989	94	10.9	10.3	11.5	0	0	0	-0.359	-0.4	-0.317	0	0	0	0	0	0	0.257
C22:5n3 Mes	m2	0.972	35	2.89	2.73	3.05	0	0	0	-0.084	-0.095	-0.073	0	0	0	0	0	0	0.385
C22:6n3 Mes	m2	0.955	22	8.49	7.88	9.11	0	0	0	-0.216	-0.259	-0.173	0	0	0	0	0	0	0.406
C12:0 PI	m1	0.996	311	0.037	0.03	0.043	0	0	0	0	0	0	0	0	0	0	0	0	0.078
C14:0 PI	m5	0.986	80	4.39	4.32	4.46	-0.894	-0.994	-0.794	0	0	0	-0.047	-35.9	35.8	-0.01	-35.8	35.8	0.708
C16:0 PI	m4	0.849	6	12.1	11.8	12.5	-0.98	-1.315	-0.645	-0.142	-0.163	-0.121	0	0	0	0	0	0	0.995
C16:1n7 PI	m5	0.972	60	5.66	5.54	5.78	-1.262	-1.435	-1.089	0	0	0	-0.071	-35.9	35.7	-0.01	-35.8	35.8	0.082
C18:0 PI	m2	0.827	6	3.00	2.93	3.07	0	0	0	-0.018	-0.023	-0.013	0	0	0	0	0	0	0.914
C18:1n9c PI	m5	0.975	48	24.2	23.4	25.1	6.833	5.624	8.039	0	0	0	0.378	-35.4	36.2	0.06	-35.7	35.9	0.882
C18:2n6c PI	m5	0.881	13	8.89	8.60	9.17	2.206	1.802	2.609	0	0	0	0.127	-35.7	35.9	0.02	-35.8	35.8	1.000
C20:0 PI	m1	0.923	12	0.337	0.325	0.35	0	0	0	0	0	0	0	0	0	0	0	0	0.056
C20:1 PI	m1	0.903	11	2.85	2.76	2.95	0	0	0	0	0	0	0	0	0	0	0	0	0.545
C18:3n3 PI	m4	0.726	3	3.58	3.33	3.82	0.978	0.738	1.22	0.079	0.064	0.094	0	0	0	0	0	0	0.349
C20:3n3 PI	m1	0.589	2	0.454	0.407	0.502	0	0	0	0	0	0	0	0	0	0	0	0	0.383
C20:4n6 PI	m2	0.700	5	0.594	0.525	0.662	0	0	0	-0.017	-0.022	-0.012	0	0	0	0	0	0	0.856
C20:5n3 PI	m5	0.835	7	8.56	8.24	8.87	-2.421	-2.865	-1.98	0	0	0	-0.124	-35.9	35.7	-0.02	-35.8	35.8	0.246
C22:5n3 PI	m5	0.951	35	3.44	3.37	3.50	-0.73	-0.828	-0.63	0	0	0	-0.044	-35.9	35.8	-0.01	-35.8	35.8	0.850
C22:6n3 PI	m2	0.520	1	5.29	4.92	5.67	0	0	0	-0.12	-0.146	-0.093	0	0	0	0	0	0	0.913
C12:0 LI	m1	1	18612	0.0	0.0	0.0	0	0	0	0	0	0	0	0	0	0	0	0	0
C14:0 LI	m2	0.811	4.4	1.8	1.7	2.0	0	0	0	-0.043	-0.052	-0.035	0	0	0	0	0	0	0.933
C16:0 LI	m2	0.889	9.0	13.5	12.7	14.2	0	0	0	-0.192	-0.245	-0.138	0	0	0	0	0	0	0.219
C16:1n7 LI	m2	0.516	1.3	2.7	2.4	3.0	0	0	0	-0.066	-0.087	-0.044	0	0	0	0	0	0	0.378
C18:0 LI	m2	0.639	1.8	8.5	8.0	9.0	0	0	0	-0.128	-0.16	-0.095	0	0	0	0	0	0	0.686
C18:1n9c LI	m2	0.815	4.7	10.4	8.1	12.6	0	0	0	0.86	0.701	1.02	0	0	0	0	0	0	0.392
C18:2n6c LI	m2	0.964	27	2.1	1.5	2.7	0	0	0	0.303	0.262	0.343	0	0	0	0	0	0	0.352
C20:0 LI	m1	0.998	534	0.3	0.3	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0.377
C20:1 LI	m2	0.994	162	1.1	0.9	1.3	0	0	0	0.112	0.097	0.128	0	0	0	0	0	0	0.46
C18:3n3 LI	m2	0.99	107	0.7	0.5	0.9	0	0	0	0.078	0.063	0.093	0	0	0	0	0	0	0.283
C20:3n3 LI	m1	0.994	191	0.5	0.4	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0.682

C20:4n6 LI	m2	0.822	4.9	4.6	4.3	4.9	0	0	0	-0.092	-0.116	-0.069	0	0	0	0	0	0	0.046
C20:5n3 LI	m2	0.965	27.6	14.2	13.5	14.9	0	0	0	-0.317	-0.365	-0.269	0	0	0	0	0	0	0.337
C22:5n3 LI	m2	0.637	1.8	6.1	5.9	6.4	0	0	0	-0.166	-0.184	-0.148	0	0	0	0	0	0	0.681
C22:6n3 LI	m2	0.862	11.8	18.6	17.3	19.9	0	0	0	-0.265	-0.358	-0.173	0	0	0	0	0	0	0.236

** Explanations to the abbreviations are given in Table S2.*

Table S7b. *Results of evaluation of effects of fish size and rapeseed oil level in the feed on fatty acid level in absorbed fat (Feed), mesenteric fatty tissue (Mes), pyloric tissue (PI) and liver (LI) expressed as g/kg feed or tissue.*

	Best	Prob	BF12	Model Efficiency	B0	B0L	B0U	BS	BSL	BSU	BR	BRL	BRU	KS
12:0 Feed	m2	0.999	2915	Decis	0.22	0.21	0.23	0	0	0	-0.005	-0.006	-0.005	0.251
14:0 Feed	m2	0.990	104	Decis	13.8	13.2	14.3	0	0	0	-0.35	-0.39	-0.32	0.994
16:0 Feed	m2	0.809	5	Subst	30.3	28.5	32.0	0	0	0	-0.31	-0.43	-0.19	0.967
18:0 Feed	m1	0.987	89	Strong	5.11	4.78	5.45	0	0	0	0	0	0	0.248
20:0 Feed	m1	0.997	436	Decis	1.3	1.25	1.37	0	0	0	0	0	0	0.319
16:1n7 Feed	m2	0.989	92	Strong	17.9	17.5	18.3	0	0	0	-0.51	-0.54	-0.48	0.984
18:1n9c Feed	m2	0.965	27	Strong	28.6	27.5	29.8	0	0	0	3.73	3.65	3.81	0.354
20:1 Feed	m1	0.606	2	Negl	3.9	3.81	4.07	0	0	0	0	0	0	0.145
18:2n6c Feed	m2	0.990	97	Strong	10.0	9.43	10.55	0	0	0	1.52	1.48	1.56	0.029
18:3n3 Feed	m2	0.997	331	Decis	2.08	1.94	2.22	0	0	0	0.58	0.57	0.59	0.687
20:3n3 Feed	m1	0.780	4	Subst	0.05	0.03	0.06	0	0	0	0	0	0	0.395
20:4n6 Feed	m2	0.999	680	Decis	2.8	2.71	2.83	0	0	0	-0.08	-0.09	-0.08	0.126
20:5n3 Feed	m2	0.982	56	Strong	36.1	35.2	37.0	0	0	0	-1.06	-1.13	-1.00	0.093
22:5n3 Feed	m2	0.997	336	Decis	4.1	4.00	4.26	0	0	0	-0.12	-0.13	-0.12	0.396
22:6n3 Feed	m2	0.991	106	Decis	15.2	14.7	15.6	0	0	0	-0.41	-0.45	-0.38	0.076
12:0 Mes	m1	0.978	63	Strong	0.28	0.24	0.32	0	0	0	0	0	0	0.893
14:0 Mes	m2	0.699	3	Negl	28.9	25.8	31.9	0	0	0	-0.55	-0.77	-0.34	0.093
16:0 Mes	m1	0.659	4	Subst	76.5	70.5	82.4	0	0	0	0	0	0	0.106
18:0 Mes	m1	0.963	29	Strong	21.4	20.3	22.5	0	0	0	0	0	0	0.079
20:0 Mes	m1	0.964	33	Strong	2.6	2.43	2.77	0	0	0	0	0	0	0.561
16:1n7 Mes	m2	0.695	2	Negl	36.8	32.9	40.6	0	0	0	-0.83	-1.10	-0.56	0.037
18:1n9c Mes	m4	0.807	5	Subst	220	187	250	-28.3	-54.4	2.2	5.83	3.83	7.81	0.997
20:1 Mes	m1	0.783	8	Subst	22.0	20.6	23.4	0	0	0	0	0	0	0.756
18:2n6c Mes	m4	0.517	1	Negl	79.5	68.0	90.8	-10.6	-21.4	0.6	1.97	1.27	2.67	0.851
18:3n3 Mes	m4	0.881	18	Strong	34.6	30.2	39.0	-8.89	-13.15	-4.58	0.72	0.46	0.99	0.851

20:3n3 Mes	m4	0.722	3	Negl	3.1	2.77	3.41	-0.843	-1.157	-0.53	0.07	0.05	0.09	0.998
20:4n6 Mes	m2	0.582	2	Negl	4.3	3.84	4.80	0	0	0	-0.11	-0.15	-0.08	0.849
20:5n3 Mes	m4	0.684	3	Negl	49.6	44.0	55.2	7.92	2.39	13.39	-1.44	-1.79	-1.10	0.530
22:5n3 Mes	m2	0.820	5	Subst	22.4	20.1	24.8	0	0	0	-0.55	-0.71	-0.38	0.275
22:6n3 Mes	m2	0.830	5	Subst	38.6	35.3	42.0	0	0	0	-0.77	-1.00	-0.54	0.259
12:0 PI	m1	0.999	1357	Decis	0.04	0.03	0.04	0	0	0	0	0	0	0.140
14:0 PI	m1	0.546	2	Negl	3.64	3.07	4.22	0	0	0	0	0	0	0.420
16:0 PI	m1	0.699	2	Negl	12.3	11.2	13.4	0	0	0	0	0	0	0.315
18:0 PI	m1	0.846	5	Subst	4.12	3.87	4.37	0	0	0	0	0	0	0.284
20:0 PI	m1	0.979	61	Strong	0.48	0.40	0.56	0	0	0	0	0	0	0.016
16:1n7 PI	m2	0.652	3	Negl	5.80	5.02	6.58	0	0	0	-0.16	-0.21	-0.10	0.951
18:1n9c PI	m4	0.733	5	Subst	5.05	-0.55	10.69	8.92	3.39	14.39	1.78	1.44	2.13	0.849
20:1 PI	m2	0.469	2	Negl	0.91	0.38	1.43	0	0	0	0.09	0.06	0.13	0.788
18:2n6c PI	m4	0.627	2	Negl	0.38	-1.49	2.25	2.88	1.04	4.71	0.62	0.51	0.74	0.910
18:3n3 PI	m2	0.687	2	Negl	0.91	0.22	1.60	0	0	0	0.19	0.14	0.24	0.808
20:3n3 PI	m2	0.932	23	Strong	0.13	0.06	0.20	0	0	0	0.02	0.02	0.03	0.880
20:4n6 PI	m2	0.976	59	Strong	1.4	1.31	1.47	0	0	0	-0.03	-0.03	-0.02	0.793
20:5n3 PI	m2	0.840	8	Subst	9.2	8.05	10.38	0	0	0	-0.24	-0.33	-0.16	0.916
22:5n3 PI	m2	0.924	16	Strong	2.4	2.21	2.65	0	0	0	-0.05	-0.07	-0.04	0.218
22:6n3 PI	m2	0.790	5	Subst	7.1	6.54	7.62	0	0	0	-0.12	-0.16	-0.08	0.107
12:0 LI	m1	1.000	18612	Decis	0.00	0.00	0.00	0	0	0	0	0	0	0.000
14:0 LI	m1	0.911	11	Strong	0.51	0.44	0.59	0	0	0	0	0	0	0.223
16:0 LI	m3	0.900	9	Subst	3.99	3.82	4.15	0.59	0.35	0.82	0	0	0	0.493
18:0 LI	m1	0.822	5	Subst	2.67	2.53	2.82	0	0	0	0	0	0	0.750
20:0 LI	m1	0.998	688	Decis	0.10	0.09	0.11	0	0	0	0	0	0	0.001
16:1n7 LI	m1	0.799	4	Subst	0.76	0.62	0.89	0	0	0	0	0	0	0.097
18:1n9c LI	m2	0.543	2	Negl	2.64	-0.34	5.62	0	0	0	0.50	0.29	0.71	0.111
20:1 LI	m2	0.741	3	Negl	0.27	-0.04	0.58	0	0	0	0.06	0.04	0.08	0.093
18:2n6c LI	m2	0.772	6	Subst	0.44	-0.40	1.28	0	0	0	0.16	0.11	0.22	0.092
18:3n3 LI	m1	0.634	2	Negl	0.68	0.43	0.93	0	0	0	0	0	0	0.001

20:3n3 LI	m1	0.767	3	negl	0.21	0.13	0.28	0	0	0	0	0	0	0.025
20:4n6 LI	m2	0.584	2	Negl	1.5	1.47	1.61	0	0	0	-0.02	-0.02	-0.01	0.988
20:5n3 LI	m4	0.383	1	Negl	4.5	4.20	4.82	0.58	0.27	0.89	-0.07	-0.09	-0.05	0.716
22:5n3 LI	m2	0.955	26	Strong	2.1	1.94	2.23	0	0	0	-0.05	-0.06	-0.03	0.222
22:6n3 LI	m3	0.875	13	Strong	5.5	5.29	5.72	0.77	0.47	1.08	0	0	0	0.990
SUM Feed	m2	0.919	12	Strong	181.3	176	187	0	0	0	2.90	2.52	3.27	0.795
SUM Mes	m4	0.459	2	Negl	663.2	592	731	-15.3	-60.4	33.1	3.63	-0.98	8.23	0.716
SUM PI	m4	0.770	7	Subst	55.0	42.1	68.3	20.4	7.4	32.7	1.94	1.13	2.75	0.702
SUM LI	m4	0.436	2	Negl	23.8	18.7	28.9	8.16	3.16	13.10	0.62	0.31	0.93	0.890

** Explanations to the abbreviations are given in Table S2.*

Table S8. *Sea lice counting express as sea lice/^{cm2}, welfare indicators (WI) and relative scoring based on Kolarevic and Hoyle scoring systems.*

Fish size	**Sea lice number/cm² of fish area**				**WI scored by Kolarevic et al.**				**WI scored by Hoyle et al.**	
	Non-motile	Chalimus	Motile	Adult female	Scale loss	Skin lesions	Eye damage	Cataract	Dorsal fin damage	Caudal fin damage
Small fish	0	0	0,006104	0,007522	1.16	0.37	0.083	0.25	1.25	1.4
Large fish	0	0	0,004115	0,005844	0.95	0.55	0.208	0.25	1.25	1.28

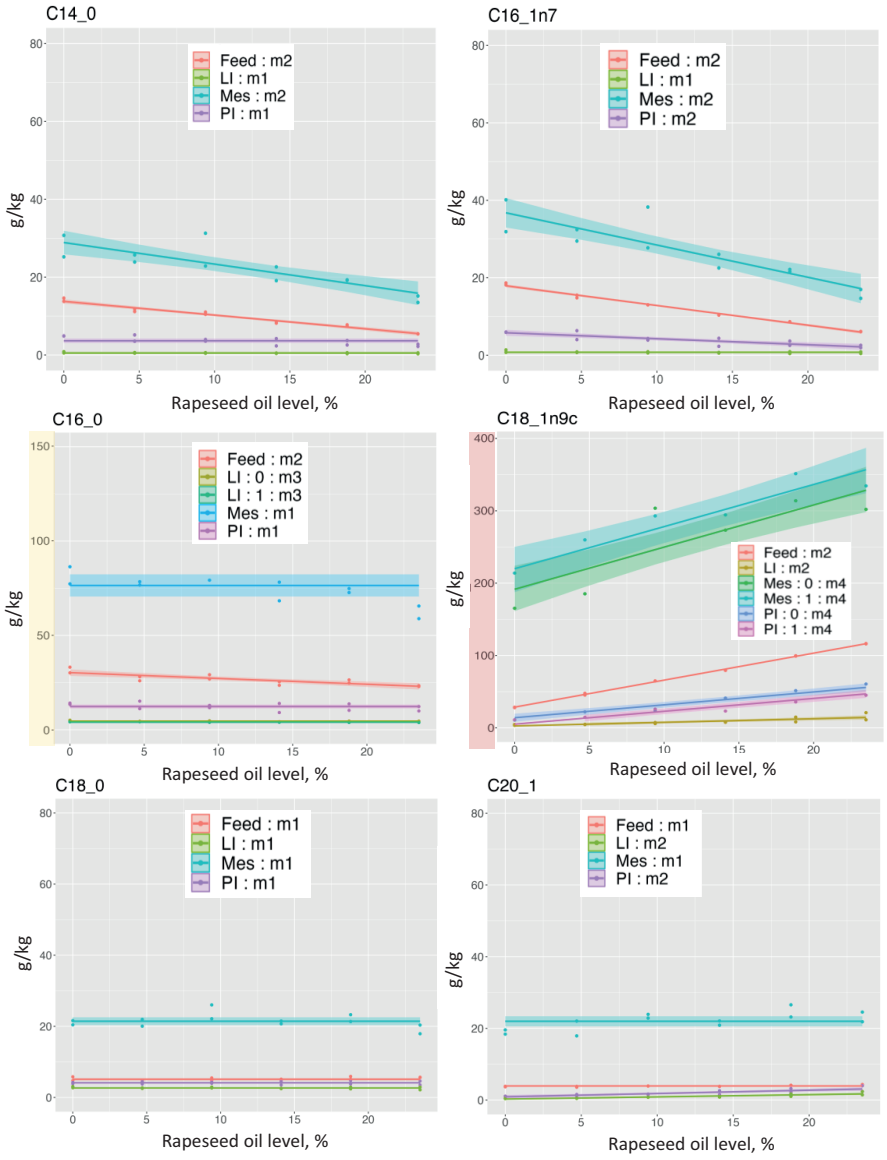

Figure S1a. *Effects of the increasing dietary rapeseed oil level on content of saturated and mono-unsaturated fatty acids indicated on the left above the graphs, in absorbed fat, pyloric caeca (PI), mesenteric fatty tissue (Mes), and liver (LI) (Unit: g/kg feed or tissue), respectively for small (0) and large fish (1). The legend in the figure indicates whether fish size clearly affected the results and the best model selected for the data. For fatty acids not clearly affected by fish size, average cures are presented. For fatty acids showing significant effects of fish size, separate curves are shown. The curves show estimated regression on diet rapeseed oil level with indication of 95% credible intervals for the posterior means.*

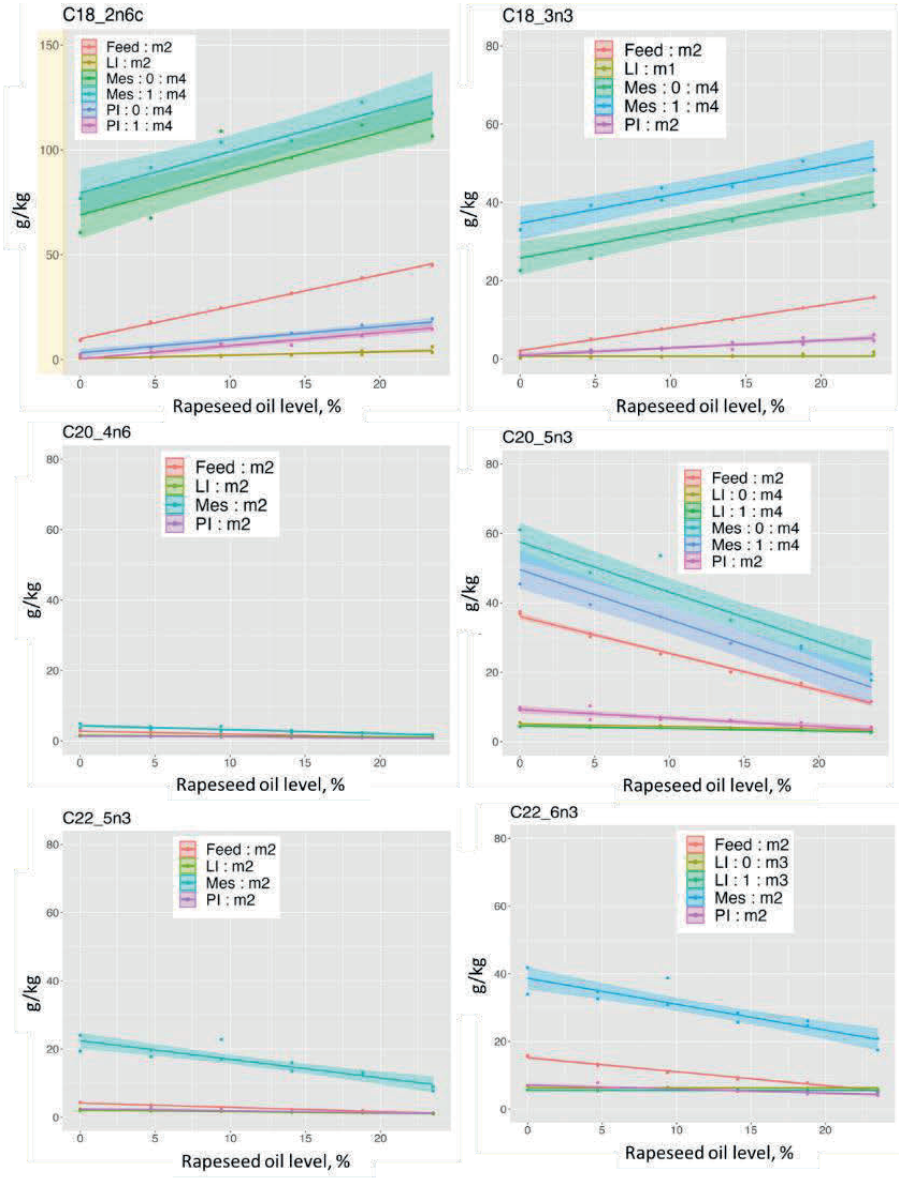

Figure S1b. *Effects of the increasing dietary rapeseed oi level on content of n3 and n6 fatty acids indicated on the left above the graphs, in absorbed fat, pyloric caeca (PI), mesenteric fatty tissue (Mes), and liver (LI) (Unit: g/kg feed or tissue), respectively for small (0) and large fish (1). The legend in the figure indicates whether fish size clearly affected the results the best model selected for the data. For fatty acids not clearly affected by fish size, average cures are presented. For fatty acids showing significant effects of fish size, separate curves are shown. The curves show estimated regression on diet rapeseed oil level with indication of 95% credible* intervals for the posterior means.

Paper III

Epigenetic changes in pyloric caeca of Atlantic salmon fed diets containing increasing levels of lipids and choline.

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Abstract

An earlier study of ours investigating the effect of dietary lipid levels on the choline requirement of Atlantic salmon, showed increasing severity of intestinal steatosis with increasing lipid levels. As choline is involved in epigenetic regulation by being the key methyl donor, pyloric caeca samples from the study were analyzed for epigenetic effects of dietary lipid and choline levels. The diets varied in lipid levels between 16 and 28%, with choline level between 1.9 and 2.3 g/kg. The diets were fed for eight weeks to Atlantic salmon of 25 g of initial weight. Using reduced representation bisulfite sequencing (RRBS), this study revealed that increasing dietary lipid level induced methylation differences in genes involved in membrane transport and signaling pathways, and in microRNAs important for the regulation of lipid homeostasis. Increasing choline level also affected genes involved in fatty acid biosynthesis and transport, lipolysis, and lipogenesis, as well as important immune genes. Our observations confirmed that choline is involved in epigenetic regulation in Atlantic salmon, as has been reported for higher vertebrates. This study showed the need for the inclusion of biomarkers of epigenetic processes in studies that must be conducted to define optimal choline levels in diets for Atlantic salmon.

Key words: Choline, lipids, Atlantic salmon, DNA methylation, steatosis

Introduction

Steady increase in the replacement of fishmeal and fish oil with plant ingredients and increased lipid levels in diets for farmed Atlantic salmon have caused challenges related to the health and welfare of the fish, and fish husbandry (Bjørgen et al. 2020; Hardy 2010; Krogdahl et al. 2010). Excessive lipid accumulation (steatosis) in the pyloric caeca and mid intestine is an example of such health challenges and is frequently observed in today's farmed salmon (Krogdahl et al. 2022). A mild intestinal steatosis is a ubiquitous condition. In extreme situations, dietary fat accumulate throughout the gastrointestinal tract and lipidic faeces are discarded and float in the surrounding waters (Penn 2011). With the huge changes in salmon diets in the past decades, fish have experienced changes in the contents of nutrients and other ingredient components, that is, non-nutrients and antinutrients, with subsequent challenges, such as disturbed nutrient digestion and absorption (Bjørgen et al. 2020; Hardy 2010; Krogdahl et al. 2010). Increased prevalence and severity of steatosis could therefore be a consequence of complications in lipid digestion and absorption caused by an insufficient supply of essential nutrients or increased supply of antinutrients. Recently, through a series of studies, choline was established as an essential nutrient for Atlantic salmon (Hansen, Anne K. G. et al. 2020; Hansen, Anne Kristine G. et al. 2020; Krogdahl et al. 2020; Penn 2011; Siciliani et al. 2023), and that plant based diets must be supplied with choline to prevent steatosis in Atlantic salmon.

Choline is necessary for many metabolic and physiological processes by being a component of lipoproteins, membrane lipids, and a precursor for the neurotransmitter acetylcholine (Korsmo, Jiang & Caudill 2019; Ueland 2011). In the main ingredients used in salmon diets, choline is present mostly as phosphatidylcholine (PC), which is hydrolyzed to lysophosphatidylcholine, absorbed, and then re-esterified to PC (Krogdahl et al. 2020). Choline chloride is the most common choline supplement, a form which is absorbed by enterocytes and further incorporated into PC via the Kennedy pathway, sequentially reacting with ATP and cytidine triphosphate, and finally fusing with diacylglycerol (Gibellini & Smith 2010; Krogdahl et al. 2020; van der Veen et al. 2017). Approximately 95% of choline in the body exists as PC, which is the main component of the phospholipid bilayer of cell membranes, and thereby influences signaling and transport across membranes (Ridgway 2016). PC is necessary for the transport of lipids from enterocytes (Krogdahl et al. 2020) and for the regulation of lipid, lipoprotein, and whole-body energy homeostasis (van der Veen et al. 2017).

Choline is the key source of methyl groups for DNA and histone methylation. Choline is oxidized to betaine, from which a methyl group is donated to homocysteine producing methionine, which is subsequently converted to the universal methyl donor S-adenosylmethionine (SAM) (Ueland 2011; Zeisel 2017). During DNA methylation, DNA methyltransferases (DNMTs) transfer a methyl group from SAM to the fifth carbon of a cytosine nucleotide, followed by a guanine nucleotide (CpG), to form 5-methylcytosine along with S-adenosylhomocysteine (SAH) (Metzger & Schulte 2016; Moore, Le & Fan 2013).

Epigenetic modifications, including DNA methylation, modulate gene expression without altering the genome sequence (Bird 2007). DNA methylation is the most stable and most studied epigenetic mechanism. Alterations in DNA methylation in different genomic regions differentially influence gene activity, resulting in diverse physiological and phenotypic changes (Metzger & Schulte 2016; Moore, Le & Fan 2013). DNA methylation can be modulated by environmental factors including diet and nutritional status (Amenyah et al. 2020; Dhanasiri et al. 2020). Macro- and micronutrients have been reported to influence DNA methylation and affect health and disease conditions (Anderson, Sant & Dolinoy 2012; Dhar et al. 2021; Jones et al. 2021; Saito et al. 2021). Several in vivo and in vitro studies have demonstrated that changes in choline intake alter global and gene-specific DNA methylation (Bai et al. 2022; Jiang, Greenwald & Jack-Roberts 2016; Korsmo, Jiang & Caudill 2019; Zeisel 2017).

In previous studies, we have demonstrated that the choline requirement of Atlantic salmon varies with variations in production-related conditions, such as dietary lipid levels (Hansen, Anne K. G. et al. 2020; Siciliani et al. 2023). In a study by Siciliani et al. (Siciliani et al. 2023), fish in fresh water, averaging 25 g at the start, fed choline-deficient diets varying in lipid levels from 16%, 20%, 25%, and 28% for eight weeks, showed clear, increasing effects of lipid level on intestinal steatosis, as well as a decreasing effect on lipid digestibility. Growth performance was, however, not affected by variations in the dietary lipid levels. These results support the hypothesis that dietary lipid levels affect choline requirements and are necessary for optimal feed utilization. Considering the importance of choline as a key methyl donor in DNA methylation, the present study employed reduced representation bisulfite sequencing (RRBS) to describe genome-wide DNA methylation patterns in pyloric caeca samples from the dose-response study mentioned above by Siciliani et al. (Siciliani et al. 2023).

Results

As described in detail by Siciliani et al. (Siciliani et al. 2023), a challenge was encountered at the start of the feeding period of the experiment. Among the newly delivered diets, the one with the highest lipid levels showed lipid leakage. A new batch was made. However, soybean lecithin was added to prevent leakage, resulting in higher choline levels. Hence, the diets with 16% (L16), and 25% (L25) lipid contained 1.9 g of choline/kg, and the diet with 28% (L28) lipid contained 2.3 g/kg of choline. See Siciliani et al. (2023) for further details regarding diet composition and Supplementary Table 1 for the analyzed nutrient content. However, “every cloud has a silver lining”. The event provided an opportunity to get indications also of effects of choline levels in epigenetic regulation in pyloric caeca tissues. L16 and L25 diets, which were similar in choline level, differed by 9% in lipid content, while the L25 and L28 diets, differed in choline by 0.4 g/kg, i.e., about 22%, but only 3% in lipid. Accordingly, the difference in results for the diets L16 and L25 indicates effects of lipid level, whereas that for L28 and L25 provides information elucidating the impact of a substantial difference in degree of choline deficiency confounded with a small difference in lipid level on the observed diet effects.

RRBS library characterization

Using the RRBS technique, we assessed epigenetic differences in the pyloric caeca of Atlantic salmon fed the L16, L25, and L28 diets. RRBS generated 21.6 - 40 million reads, with an average of 27.5 million raw reads per sample (Supplementary Figure 1). After quality and adaptor trimming, an average of 25.4 million reads was obtained per sample. Of them, an average of 10 million reads were uniquely mapped, with an approximately 39% mapping efficiency to the Atlantic salmon genome (Supplementary Figure 1). An average of 12.8 million reads were not mapped uniquely (approximately 50% of the trimmed reads), even though the average total mapping efficiency was 90%. Low unique mapping efficiency is a common factor experienced during RRBS studies, as reported for several fish species (Dhanasiri et al. 2020; Podgorniak et al. 2022; Saito et al. 2021).

Characterization of pyloric caeca methylome of Atlantic salmon

An average of 158 million cytosines were analyzed per sample, including an average of 27.7 million methylated cytosines in total (Table 1). Of those methylated cytosines, an average of 26.6 million were in CpG context. An average of 4.9 million was also observed for unmethylated cytosines in CpG context, totaling up to 31.5 million cytosines in both methylated and unmethylated cytosines in CpG context. The percentage of methylated cytosines calculated as a percentage from the total sum of methylated and unmethylated cytosines respectively in CpG, CHG, and CHH context (where H is A, C, or T) are given in the Table 1. Observed average percentage were 84.4, 0.9, and 0.8 methylated cytosines for CpG, CHG, and CHH contexts, respectively per diet group (Table 1). The percentage of methylated CpGs, CHGs and CHHs did not differ between the diet groups (Table 1).

Table 1. Global cytosine methylation levels detected in CpG, CHG and CHH contexts.

Diet group	Total number (million) of		% of methylated C's		
	Cytosines (C's) analyzed	Methylated C's (% of total C's)	CpG context	CHG context	CHH context
L16	151 ± 12	26.6 (17.6%)	84.6	0.9	0.8
L25	157 ± 7	27.1 (17.3%)	84.2	1.0	0.8
L28	166 ± 17	29.3 (17.6%)	84.5	1.0	0.8

Total number of cytosines analyzed ± SEM from n = 6 per diet group.

Differentially methylated CpGs (DMCs), their chromosomal distribution and annotation

Pairwise comparisons of DMCs among different diet groups are presented in Table 2. DMCs were presented for diets with higher lipid levels compared to diets with lower lipid levels in each comparison. The results presented below focus on the observed differences between diets L25 and L16, that is, the difference between the diets with similar choline levels and the greatest difference in lipid levels, and the difference between the diets with different choline levels and the smallest difference in lipid levels, that is, L28 and L25.

Table 2. General statistics on significantly differentially methylated cytosines in CpG context.

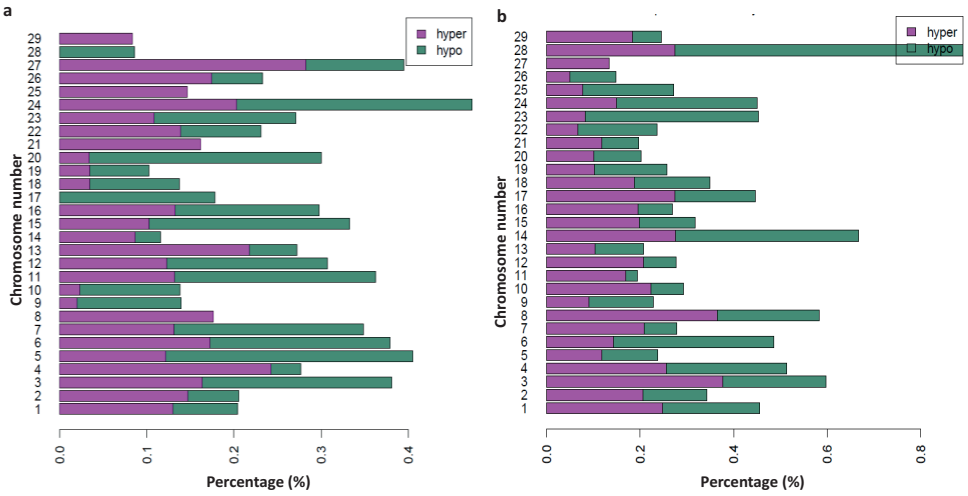
Comparisons	Differentially methylated CpGs (>10%, q < 0.1)

	Total	Hypermethylation*	Hypomethylation*
L25 vs L16	197	93 (47.2%)	104 (52.8%)
L28 vs L25	360	190 (52.8%)	170 (47.2%)

151 * The percentages of hypermethylation and hypomethylation are shown in parentheses.

152 The chromosomal distributions of DMCs that differed between diet groups are shown in Figure
153 1. Comparison of L25 vs L16 groups showed the highest DMCs distribution on chromosome
154 24, followed by 5 and 27 (Figure 1a), while comparison of L28 vs L25 showed the highest
155 distribution in chromosome 28 followed by 14 (Figure 1b).

156



157

158 **Figure 1**. Bar plots showing the percentage of hyper- and hypomethylated CpGs per
159 chromosome over all the covered CpGs in a given chromosome. (a) Comparison of L25 vs L16
160 diet groups and (b) L28 vs L25 diet groups. CpGs with $q < 0.1$ and methylation differences >
161 10% were considered as hyper- and hypomethylated.

162 Genomic feature analysis revealed that the number of DMCs in genic regions comprising the
163 promoter region (−1 kb to +100 bp from the transcription start site [TSS]), exons, introns, and
164 transcription termination sites (TTS, −100 bp to +1 kb) was higher than the number of DMCs
165 in intergenic regions for both pairwise comparisons (Figure 2).

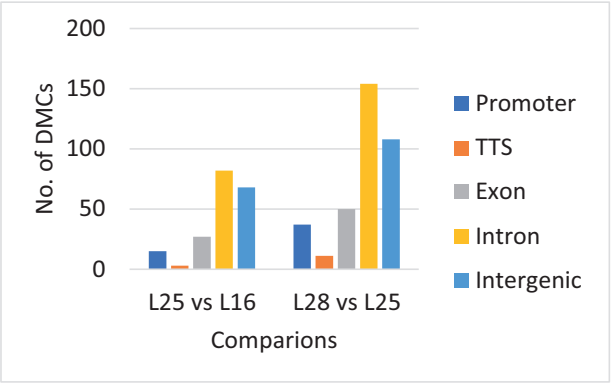


Figure 2. Genomic feature analysis of differentially methylated cytosines in each of the comparisons between diet groups.

Genes associated with DMCs

The total number of genes associated with DMCs for each comparison is shown in Table 3. Approximately 50% of the genes associated with DMCs could not be assigned a gene name/symbol since the Atlantic salmon genome is still not well annotated compared to the genomes of model fish species. The number of genes associated with DMCs followed the same order as the number of DMCs found in the pairwise comparisons.

Table 3. The total number of genes associated with DMCs in each pairwise comparison of diet groups.

Comparisons	Total no. of genes	Genes assigned with symbol/name*	Genes not assigned with symbol/name*
L25 vs L16	167	89 (53.3%)	78 (46.7%)
L28 vs L25	290	143 (49.3%)	147 (50.7%)

*Percentages of assigned and not assigned genes are presented in parentheses.

Gene ontology (GO) enrichment analysis of genes associated with DMCs in each comparison showed few enriched biological processes, molecular functions, and cellular components. The L25 vs L16 comparison showed enrichment of molecular functions related to transmembrane transporter activity, transcription regulator activity, oxidoreductase activity, and protein

184 binding activity (Supplementary Table 2). On the other hand, L28 vs L25 showed enriched
185 biological processes, including cellular response to stimulus (Supplementary Table 2).

186 Supplementary File 1 presents all genes associated with DMCs in each of the comparisons
187 between diet groups that were assigned to the gene symbol/name. Genes considered
188 functionally relevant to the topic of the present study are presented in Table 4.

189
190 Table 4. Selected genes associated with DMCs in the pyloric caeca genome of Atlantic
191 salmon fed diets with increasing lipid or choline content.

Gene symbol	Gene Name	25 vs 16*	28 vs 25*
Promoter			
tjap1	tight junction associated protein 1	4(4)	4(0)
slc22a15	solute carrier family 22-member 15	0	3(0)
LOC106597856	acyl-CoA desaturase-like (*scd*)	0	1(0)
LOC106564195	ARF GTPase-activating protein Git2 (*git2*)	1(1)	0
LOC106610877	kelch-like protein 28 (*klhl28*)	3(0)	0
sstr2a	somatostatin receptor 2a	0	2(2)
TTS			
LOC106580941	major facilitator superfamily domain-containing protein 1-like (*mfsd1*)	0	3(0)
Exon			
LOC123728301	potassium voltage-gated channel subfamily A member 1-like (*kcna1*)	2(0)	3(3)
Intron			
ssa-mir-212a-2	ssa-mir-212a-2	1(1)	0
irf4a	interferon regulatory factor 4a	0	3(0)
LOC106594380	arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 3 (*acap3*)	0	4(4)
agap3	ArfGAP with GTPase domain, ankyrin repeat and PH domain 3	0	2(2)
gabrb2	gamma-aminobutyric acid type A receptor subunit beta2	0	4(0)

192 *Total number of DMCs are presented with the number of hypermethylated cytosines in
193 parentheses.
194

195 Few but important, relevant genes were associated with DMCs in fish fed L25 compared to
196 those fed L16 (Table 4), that is, diets with 9% difference in lipid levels and similar choline
197 levels. A higher number of genes were associated with DMCs in fish fed L28 compared to

198 those fed L25, that is, diets with a difference in choline level of 0.4 g/kg (22%) and a 3%
199 difference in lipid level (Table 4).

200

201 ***L25 vs L16 – Effects of a 9% difference in lipid level***

202 Relatively few genes were associated with DMCs in fish fed L25 compared with those fed L16.
203 The promoter region of the tight junction associated peripheral protein *tjap1,* playing an
204 important role in the formation of tight junctions and may participate in vesicle trafficking
205 (Rodgers et al. 2013; Zihni et al. 2016), showed hypermethylation (Table 4).

206 The gene coding for ARF GTPase-activating protein Git2, *git2*, which is important for the
207 regulation of the toll-like receptor signaling pathway, uniquely showed hypermethylation of
208 the promoter region of L25 fish compared to the L16 fed fish.

209 The gene coding for kelch-like protein 28, *klhl28*, showed hypomethylation in the promoter
210 region of L25 fed fish compared to L16 fed fish. At present, there is little information available
211 on the specific function of *klhl28*, but members of the kelch-like protein family are known to
212 play important roles in ubiquitination processes mediated by E3 ligases, a processes involved
213 in almost all life activities of eukaryotes (Shi et al. 2019).

214 Small non-coding microRNAs which suppress the expression of targeting mRNAs were also
215 differentially methylated, i.e., were hypermethylated in the intron region of mir-212, which is
216 important for lipid homeostasis (Hanin et al. 2018), in fish fed L25 compared to those fed L16
217 (Table 4).

218 The gene encoding potassium voltage-gated channel subfamily A member 1 (*kcna1*), involved
219 in the regulation of voltage-dependent potassium ion permeability of excitable membranes
220 (Bachmann et al. 2020), showed hypomethylation of the exon region in fish fed L25 compared
221 to fish fed L16 (Table 4).

222

223 ***L28 vs L25 – Effects of a 22% difference in choline and 3% difference in lipid level***

224 A higher number and different genes were associated with DMCs in the L28 vs L25 comparison
225 than in the L25 vs L16 comparison (Table 4). Hypomethylation of the promoter region of *tjap1*
226 was observed, in contrast to the hypermethylation observed in the L25 vs L16 comparison.
227 Hypomethylation of the promoter region of the carnitine and betaine transporter *slc22a15* was

also observed in fish fed L28 compared to those fed L16 (Table 4). A member of the major facilitator superfamily (mfs) of transporters, *mfsd1* showed hypomethylation of cytosines in the transcription termination site in L28 fed fish compared to L25 fed fish. These transporters are important for the passage of a variety of small molecules across cell membranes, i.e., important for nutrient absorption (Massa López et al. 2019). The lysosomal transporter *mfsd1* has been shown to be vital for liver homeostasis and liver health in mice (Massa López et al. 2019). Their roles in the intestine remain to be evaluated.

Two genes belonging to the GTPase activating protein (GAP) family, *acap3* and *agap3* were hypermethylated in the intron region of fish fed L28 compared to the fish fed L25. GAPs bind to GTPase and accelerate the conversion of GTP to GDP, and this regulation of GTPase activity by GAPs causes a series of signaling changes (He et al. 2021). The GTPase activating protein *acap3* is specific to the small GTPase, arf6, and those arfGAPs can be activated by lipid kinase, phosphatidylinositol 4,5-bisphosphate, and phosphatidic acids (Honda et al. 1999). However, *acap3* has been mostly reported as a regulator of neuronal migration and growth (Miura et al. 2016; Miura & Kanaho 2017). Furthermore, *agap3* has also been identified as an NMDA-interacting signaling protein that is important for regulating NMDA receptors (Oku & Huganir 2013). NMDA receptors are glutamate-gated ion channels present in excitatory synapses in the central nervous system and are important for mediating sodium, calcium, and potassium ion flow in the cells (Fan, Jin & Wang 2014).

The promoter region of *scd*, a gene encoding acyl-CoA desaturase involved in fatty acid biosynthesis, showed hypomethylation in L28 fed fish compared to L25 fed fish (Table 4).

In contrast to the effect of lipid levels on hypermethylation of the intron region of mir-212, this microRNA was not significantly affected by DMCs in the L28 vs L25 comparison.

Among the immune genes associated with DMCs were transcription factor, *irf4a*, hypomethylated in the intron region, and somatostatin receptor, *sstr2a*, hypermethylated in the promoter region of fish fed L28 compared to those fed L25 (Table 4).

Several genes related to the nervous system and neurotransmitters showed differences in epigenetic regulation between the L28 and L25 diet fed fish, possibly linking to the role of choline as precursor for the neurotransmitter acetylcholine. In addition to the previously mentioned *acap3* and *agap3*, *gabrb2*, a multi-subunit chloride channel that mediates inhibitory synaptic transmission, showed differences in epigenetic regulation between L28 and L25 fed

fish. Hypermethylation of *kcna1* observed in the L28 vs L25 comparison, in contrast to hypomethylation of *kcna1* observed in the L25 vs L16 comparison (Table 4).

Discussion

Studies describing diet-related epigenetic changes in fish are few (Dhanasiri et al. 2020; Saito et al. 2021; Skjærven et al. 2018). The present study adds new information to the presently weak knowledge base. The comparison of the epigenetic differences in fish fed the L25 to L16 diet, differing by 9% in lipid content and with similar choline levels, suggests functional effects that deviate substantially from those observed in fish fed the L28 and the L25 diet, differing by 3% in lipid level and 22% in choline. Therefore, in the following discussion, the results for the comparison of the L25 and L16 diets are considered to represent the effects of lipid level, whereas differences in the results for L28 and L25 are considered mainly to represent the effects of variation in choline level.

Effects of dietary lipid level

The observed effects of increased dietary lipid levels are related to membrane transport and signaling pathways. The effect on hypermethylation of promoter of *tjap1*, a peripheral tight junction protein involved in control of paracellular permeability and movement of ions, macromolecules and immune cells (Rodgers et al. 2013; Zihni et al. 2016), may have contributed to the alterations in the lipid and fatty acid transport capacity in the pyloric caeca mucosa with different degrees of excessive lipid accumulation (see the results published by Siciliani et al (Siciliani et al. 2023). It is commonly used as a biomarker of intestinal integrity in broilers (Santos et al. 2021; Slawinska et al. 2019).

Small non-coding microRNA, mir-212 has been observed to be a key regulator of hepatic lipid homeostasis, suppressing multiple target genes leading to hepatic steatosis (Hanin et al. 2018). As such, the hypermethylation of the intron region of mir-212 observed with increasing dietary lipid levels may be related to the increase in steatosis symptoms with increasing lipid levels. Gene body methylation is positively correlated with gene expression rates, as it blocks the transcription of repetitive DNA elements and regulates alternative promoters and splicing (Jjingo et al. 2012; Maunakea et al. 2010). The *tjap1* has also been identified as a potential target of mir-212 (Burek et al. 2019). Therefore, epigenetic modulation of mir-212 could be

important for L25 fed fish as an attempt to regulate lipid homeostasis in pyloric caeca tissue with severe steatosis.

Effects of dietary choline level

The effects of increased dietary choline levels include the epigenetic regulation of several genes involved in fatty acid transport and metabolism, as summarized in Figure 3.

In contrast to the hypermethylation observed with the increased lipid levels and low choline levels, hypomethylation of the promoter region of *tjap1* was observed with increased choline levels. Generally, methylation in promoter regions is inversely correlated with gene expression rates (Jones 2012), but sometimes varies with promoter sequences and gene function (Weber et al. 2007). Thus, hyper- and hypomethylation likely cause differential regulation of expression of *tjap1* in respective diet fed fish. Our results indicate that the degree of methylation of the *tjap1* promoter may be used as an epigenetic biomarker in pyloric caeca to assess dietary choline levels. However, our observations need to be validated further by supporting gene expression and functional studies.

Hypomethylation of the promoter of the solute carrier, *slc22a15*, which is important for regulating systemic and tissue levels of betaine and carnitine (Yee et al. 2020), indicates that variation in choline levels has consequences for the regulation of betaine and carnitine transport. Betaine is an intermediate in the transfer of methyl groups from choline. The likely increase in the level of betaine, resulting from the elevation of choline levels, could have led to the epigenetic regulation of *slc22a15,* which is needed to regulate systemic and tissue levels of betaine. The effects on carnitine, which is involved in fatty acid transport, may also be underlying the observed effects of lipid levels on fatty acid levels in the intestinal mucosa (Longo, Frigeni & Pasquali 2016).

The hypomethylation of promoter of *scd* observed with increasing dietary choline level, a gene involved in regulation of the biosynthesis of monosaturated fatty acids (Paton & Ntambi 2009), could correspond to a reduction in content of MUFA in the pyloric caeca (See the results published by Siciliani et al (Siciliani et al. 2023). This observation may be related to a decrease in the severity of choline deficiency symptoms with increasing choline levels.

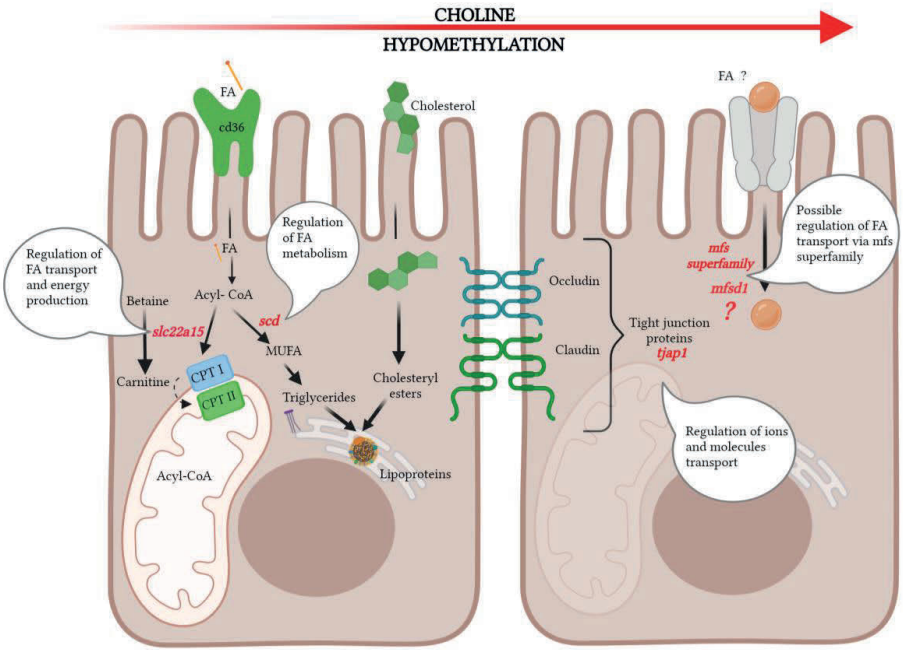


Figure 3. Increased dietary choline levels induced epigenetic regulation of several genes involved in fatty acid transport and metabolism. Peripheral tight junction protein *tjap1*, solute carrier, *slc22a15* and *scd* involved in fatty acid biosynthesis showed hypomethylation in promoter region and major facilitator superfamily transporter *mfsd1* showed hypomethylation in TTS with increased dietary choline levels. Figure created with Biorender.com.

Among the immune genes affected by the increase in choline levels, was transcription factor *irf4a*, which is vital for the regulation of immune cell differentiation, including T and B lymphocytes, macrophages, and dendritic cells (Nam & Lim 2016). The gene showed hypomethylation in the intron region with increasing dietary choline levels, indicating that dietary choline levels may play a role in salmon mucosal immune functions. Studies in mice have reported that *irf4* is a negative regulator of inflammation in diet-induced obesity and a key regulator of lipolysis and lipogenesis (Eguchi et al. 2011). The dominant somatostatin receptor, *sstr2a*, was hypermethylated in the promoter region of L28 with increasing choline levels. Somatostatin receptors mediate the action of somatostatin, which function as an endogenous inhibitory regulator of secretory and proliferative responses of target cells (Patel 1999) and *sstr2a* reported to regulate T cell IFNγ release from inflammatory cells in mice

(Elliott et al. 1999). These effects support the indications discussed above regarding the involvement of choline in mucosal immune function in Atlantic salmon. Such possible effects of choline should be investigated in follow up choline requirement studies.

Conclusion

Variation in dietary lipid level affected methylation of genes coding for membrane components and transporters and microRNAs important for lipid homeostasis. Variation in choline levels affected the methylation of genes involved in fatty acid biosynthesis and transport, lipolysis and lipogenesis, and genes related to immune functions, including genes involved in the differentiation of immune cells. Among the genes affected by choline level, the tight junction protein *tjap1* showed hypomethylation of the promoter with increased choline levels, indicating the possibility of its use as an epigenetic biomarker for dietary choline levels. Our observations support the vital role of choline in epigenetic regulation, as reported in higher vertebrates as well as in Atlantic salmon. These results call for research for revealing the role of choline in mucosal immune function in Atlantic salmon.

Materials and methods

Diet composition and feeding trial.

The samples processed in this study were part of a larger feeding trial conducted to estimate the influence of increasing lipid levels and water temperature on the choline requirement. All details regarding the feed production and feeding trial are presented in Siciliani et al. (Siciliani et al. 2023) and are briefly summarized below.

Three diets characterized by a high content of plant ingredients and an equally low choline level were formulated with increasing levels of lipids: 16% (L16), 25% (L25), and 28% (L28). To achieve iso-nitrogenous diets, ingredients with a low protein content were proportionally substituted with ingredients with a higher protein content in parallel with the increasing lipid level. The diets were supplemented with standard vitamins and mineral premixes and produced by extrusion. A summary of the diet compositions as analyzed is presented in Supplementary Table 1. The feeding trial was conducted at Nofima's Research Station in Sunndalsøra,

Norway. Atlantic salmon with an initial weight of 25 g were raised at a temperature of 8 °C using duplicate tanks per diet group.

After 8 weeks of feeding, three fish of mixed gender in the fed state were randomly sampled from each tank (n = 6 per diet group), anesthetized with tricaine methane sulfonate (MS-222), and killed by a sharp blow to the head, according to the Norwegian Animal Welfare Act guidelines. The abdominal cavity of the fish was opened longitudinally, and the internal organ package was removed. The mesenteric fat was removed from the pyloric intestine, and a small section from the pyloric caeca was sectioned, collected in a 1.5 ml Eppendorf tube, snap-frozen in liquid N_2 and stored at -80 °C.

Reduced representation bisulfite sequencing (RRBS)

DNA was extracted from 80-100 µg of pyloric caeca sample using an E.Z.N.A. insect DNA kit (Omega Bio-tek, Norcross, GA, USA), according to the manufacturer's protocol. DNA quantity was assessed using a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), whereas DNA quality was assessed using an Agilent 2200 TapeStation (Agilent technologies, Santa Clara, CA, USA). Library preparation was performed using the NuGen ovation RRBS methyl-seq system 1-16 (Tecan Genomics, Inc., Redwood City, CA, USA), according to the manufacturer's instructions. Genomic DNA was digested by incubating the samples with the MspI enzyme at 37 °C for 1 h. Following adapter ligation and the final repair, bisulfite conversion was performed according to the manufacturer's instructions. The resulting bisulfite-converted libraries were subjected to desulfonation and purification and then amplified with 12 PCR cycles. The quality and quantity of the resulting RRBS libraries were assessed using the TapeStation. Two sequencing runs were performed for two pools containing equal representation from the diet groups. Sequencing was performed on an Illumina NextSeq platform (San Diego, CA, USA) using a single-end 75 bp high-throughput sequencing kit with 4% Phix control DNA (Illumina) as an internal control, following the instructions for RRBS sequencing from the NuGen RRBS protocol.

Bioinformatics analysis of RRBS data

RRBS data analysis was performed as previously described (Dhanasiri et al. 2020). Briefly, quality and diversity adapter trimming of raw data was processed using Trim Galore (Babraham Bioinformatics) without the – rrbs option and using a specific script provided by

NuGen. This preserves the first base of the MspI fragment, which contains the CpG methylation measurement. Quality trimmed reads were aligned to the Atlantic salmon genome (http://www.ensembl.org, Ssal_v3.1, GCA_905237065.2) using Bismark aligner (Krueger & Andrews 2011) with directional options, and other default parameters. The percentage methylation of cytosines in CpG, CHG or CHH context were calculated by default method in Bismark aligner as follows: % methylation (context) = 100 * methylated Cs (context) / (methylated Cs (context) + unmethylated Cs (context)). Methylation information was extracted and used in the methylkit package (Akalin et al. 2012).

Differentially methylated cytosines in the CpG context in the genomes of Atlantic salmon between the diet groups were analyzed using the methylKit package following the recommendation from the developers. Sorted SAM files from the Bismark aligner used to create a methylRaw object for CpG methylation as per the instruction from methylKit. Descriptive statistics were performed on each sample to test methylation coverage and percent methylation. Then, all the samples were filtered based on read coverage, as recommended by the methylKit, to discard bases that have coverage bellow 10x, and bases that have above the 99.9^{th} percentile of coverage in each sample to avoid PCR bias. Replicate samples from the each pairwise comparison was merged to one object using unite () function with default settings in methylKit to produce bases/regions covered in all samples. DMCs were extracted by logistic regression using the statistical criteria of q-value $\leq$ 0. 1, and methylation differences $\geq$ 10%. The chromosomal distribution of DMCs was also analyzed for each comparison between the diet groups using the same package.

Genomic feature analysis and functional annotation of DMCs

Genomic feature analysis and annotation of DMCs were performed using the HOMER package (Heinz et al. 2010). Using default parameters, annotatePeaks.pl () function was used with Salmo_salar.Ssal_v3.1.dna.primary_assembly_fa for all the known 29 chromosomes and Salmo_salar.Ssal_v3.1.108.chr.gtf from ensembl. Overlapping annotations were prioritized by HOMER in the following order: transcription start sites (TSS, including promoter regions −1 kb to +100 bp from TSS) > transcription termination sites (TTS, from – 100 bp to +1 kb) > exon (coding) > 5′ UTR exon > 3′ UTR exon > CpG islands > repeats > introns > intergenic regions. HOMER identifies and assigns DMCs in intergenic regions to the gene with the nearest TSS.

Functional annotation of the genes associated with DMCs was performed using the g:Profiler online tool (Raudvere et al. 2019) and manually inspecting the Ensembl and NCBI (https://www.ncbi.nlm.nih.gov/) databases for the respective Ensemble gene Ids resulted from HOMER. Gene ontology enrichment analysis (GO) was performed using the same tool. All the known genes of the Atlantic salmon in the Ensembl database (Ensembl 107, Ensembl genome 54) were considered, and the threshold for determining GO terms was set as a Benjamini-Hochberg FDR (False Discovery Rate) value of 0.1. The enriched GO terms were then summarized by removing redundant GO terms using the REVIGO online tool (Supek et al. 2011).

Authors' contributions

AD: Laboratory work, bioinformatics analysis, data interpretation, and writing of the original draft of the manuscript. DS: Sample collection, laboratory work, bioinformatics analysis, and manuscript development. TMK: Project administration and manuscript development. ÅK: Project leadership, experimental design, and manuscript development. All the authors have read, revised, and approved the manuscript.

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Disclosure Statement

The authors reported no potential conflicts of interest.

Ethics Statement

All experiments involving Atlantic salmon were conducted in accordance with the Norwegian Animal Research Authority guidelines.

Data Availability Statement

The sequence data were deposited in the NCBI Sequence Read Archive (SRA) with reference number (PRJNA992799).

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Supplementary Table 1. Analyzed nutrients in the diets.

Diet	L16	L25	L28
Lipid, %	15.5	25.1	27.6
Choline, g/kg*	1.93	1.94	2.31
Dry matter, %	93.0	92.0	92.9
Crude protein, %	47.9	44.3	44.7
Nitrogen free extracts, %	22.4	15.4	13.4
Ash, %	8.6	6.8	7.5
Energy, MJ/kg	20.6	23.4	24.0
Yttrium, %	0.00044	0.00049	0.00103

Detailed dietary formulation published in Siciliani et al (Siciliani et al., 2023).

Supplementary Table 2. Enriched molecular function, biological process and cellular component of the genes associated with DMCs in each pairwise comparison of die groups.

Term Id	Name	Adjusted p value	Number of genes
L25 vs L16			
Molecular Function			
GO:0005249	voltage-gated potassium channel activity	0.014	5
GO:0005515	protein binding	0.091	34
GO:0008324	monoatomic cation transmembrane transporter activity	0.046	7
GO:0022857	transmembrane transporter activity	0.046	11
GO:0016491	oxidoreductase activity	0.091	7
GO:0022803	passive transmembrane transporter activity	0.046	7
GO:0030695	GTPase regulator activity	0.091	5
GO:0140110	transcription regulator activity	0.036	11
Biological process			
GO:0006813	potassium ion transport	0.082	5
L28 vs L25			
Biological process			
GO:0051716	cellular response to stimulus	0.04	37
GO:0048732	gland development	0.06	3
Cellular component			
GO:0016020	membrane	0.05	38

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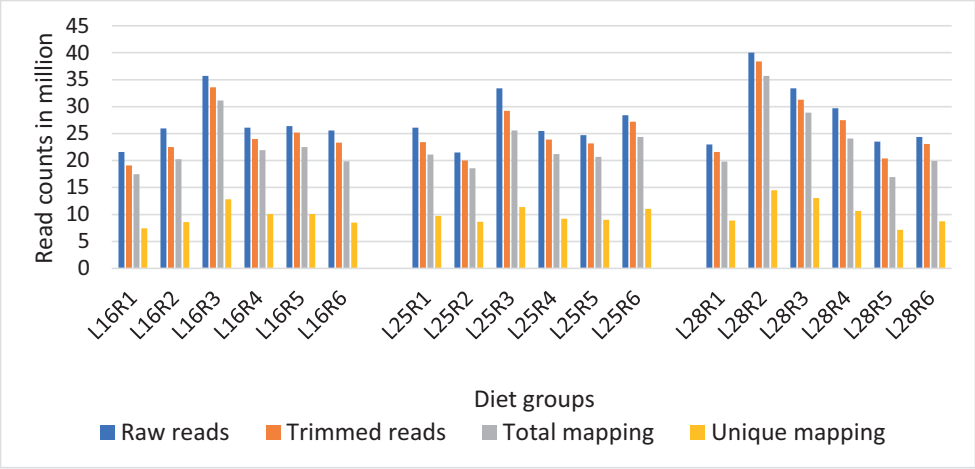
Supplementary Figures



Supplementary Figure 1. Number of raw, quality and adaptor trimmed, totally mapped, and uniquely mapped reads from the individual fish in each diet group.

Paper IV

A fish intestinal in vitro model for investigation of lipid metabolism and steatosis.

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Abstract

Choline is now recognized as essential for lipid transport in Atlantic salmon. Its deficiency leads to excessive lipid accumulation in the enterocytes, a condition known as steatosis. The knowledge over lipid metabolism and steatosis in fish remains limited, motivating the use of in vitro intestinal models to perform deeper explorations. This study aimed to create an in vitro steatosis model using RTdi-MI, a new cell line derived from the distal intestine of rainbow trout, exposed to varying oleic acid (OA) concentrations over different time points (24h, 72h, and 168h). Results indicated that the increasing OA concentration enhanced intracellular lipid droplet formation. Prolonged exposure to high OA concentration altered cell morphology, making them more rounded with nuclei moved towards the periphery of the cells by the accumulating droplets. Such morphology resembled that of Atlantic salmon's preadipocytes. Quantitative lipid analysis confirmed OA accumulation, which intensified with prolonged exposure and increased OA dose. Moreover, all cells, including controls, exhibited fatty acid metabolic activity. RTdi-MI cells expressed genes involved in lipid metabolism and synthesis similar to in vivo conditions. Collectively, our findings demonstrate the ability of RTdi-MI cells to accumulate OA in intracellular lipid droplets and mirror in vivo steatosis conditions, offering insights into fish intestinal lipid metabolism.

Introduction

The growing demand for high-quality plant-based ingredients in fish feeds has underscored the necessity for a better understanding of the impact of such compounds on fish intestinal health and nutrient metabolism [1,2]. In vivo feeding trials are considered the most dependable and robust method to investigate these effects. Nevertheless, in vitro models of the intestine are

gaining traction in fish nutrition research, aligning with the principles of the 3Rs by addressing ethical concerns, minimizing the use of experimental animals, and reducing the reliance on costly and time-consuming feeding trials [3].

The shift in proportion of fish meal and plant ingredients in diets for farmed carnivorous fish, such as the Atlantic salmon, has revealed severe knowledge gaps regarding lipid metabolism in fish. For instance, a recent study by Hansen et al. [4] demonstrated that choline is an essential nutrient for Atlantic salmon, and that choline deficiency is manifested as an excessive accumulation of dietary fat within the intestinal enterocytes, a condition known as intestinal steatosis [5]. While Hansen et al. [4,6] discovered that today's plant-based salmon diets must be fortified with choline to prevent diet-induced steatosis, and Siciliani et al. [7] suggested that the primary factor influencing this gut disorder is the dietary lipid load, the molecular and cellular processes underlying intestinal lipid uptake, metabolism, and steatosis remain poorly understood. Within this framework, cell-based models of the intestinal epithelium show great potential as study tools for examining how dietary components impact the physiological processes occurring in the fish intestine during lipid transport and metabolism.

Currently, the rainbow trout intestinal epithelial cell line, RTgutGC, is the most studied immortal cell line derived from the fish gut [8]. Two additional rainbow trout cell lines were very recently derived from the proximal (RTpi-MI) and the distal (RTdi-MI) intestine [9]. These three models have shown characteristics typical of the in vivo intestinal tissue. For instance, when grown on a permeable membrane, RTgutGC, RTpi-MI and RTdi-MI can form a selectively permeable polarized barrier which resembles the intestinal epithelial layer. Additionally, they can form tight junctions and desmosome cell-to cell adhesions [8,10–13]. According to Pasquariello et al. [9], RTpi-MI and RTdi-MI are heterogenous cell lines, showing markers of mature enterocytes, stem cells and fibroblasts. High seeding density seems to induce maturation, represented by the upregulation of alkaline phosphatase gene (*ialp*), while genes related to the stem-cell niche, such as *sox9, hopx* and *lgr5*, are suppressed [9,10]. Responsiveness of the RTgutGC cells to bile stimulation has illustrated the resemblance to the in vivo epithelial counterparts [14]. When cultivating the RTpi-MI and RTdi-MI cells on more organotypic platforms in co-culture with fibroblasts, the epithelial barrier developed morphological characteristics closely resembling the in vivo intestinal epithelium with cubic or cylindrical shaped enterocytes, occasional goblet cells and a basal membrane [15,16]

Studies on dietary fat transport and metabolism using human cell lines demonstrate the great value of such cell-based model systems [17]. For instance, Nauli and Whittimore [18] exposed Caco-2 cells, a cell line derived from human epithelial colorectal adenocarcinoma [19], grown on a permeable membrane system to a lipid mixture containing oleic acid at varying concentrations.

The results highlighted the efficient formation of lipoproteins at the basolateral side of the membrane, offering the development of an in vitro model for investigating dietary fat absorption and intestinal metabolism of drugs, vitamins, and other liposoluble substances. In parallel with Nauli and Whittimore's [18] experiment on intestinal cell lines, other studies have aimed to create in vitro systems capable of replicating the steatosis conditions observed in vivo. Given the pivotal role of the liver in lipid metabolism, most of these studies have employed human hepatocytes. For example, Gomez-Lechon [20] utilized HepG2, a human hepatocyte-derived cell line, to establish an experimental model of hepatocellular steatosis. Their study demonstrated that HepG2 cells exposed to high concentrations of oleic and palmitic acids exhibited behavior similar to that of liver tissue in humans afflicted by fatty liver syndrome.

To the best of our knowledge, only one study has been conducted to explore lipid metabolism in fish intestinal cell lines. This study, conducted by Selvam et al. [21] , investigated the intracellular trafficking of three classes of fatty acids—oleic acid, arachidonic acid, and palmitic acid—in the RTgutGC cell line. Selvam et al. [21] demonstrated that RTgutGC can undergo lipid absorption and transport processes similar to in vivo mammalian and fish models. This includes synthesizing triglycerides (TAG) and free fatty acids (FFA), producing lipid droplets, and expressing a set of genes involved in lipoprotein and lipid droplet formation. In light of the findings from Selvam et al. [21], the question arises as to whether it is possible to develop an in vitro model capable of replicating the steatosis condition observed in vivo, facilitating the investigation of the cellular, molecular, and biochemical mechanisms underlying lipid accumulation in the salmonid intestine. The present study addresses this question by subjecting the RTdi-MI cell line to high concentrations of oleic acid. Oleic acid represents the principal unsaturated fatty acid found in rapeseed oil; the main source of plant-based fat used in salmonid diets.

Materials and Methods

Routine RTdi-MI cell culture

To attain an amount of cells adequate to perform the planned experiments, RTdi-MI cells were cultured following the guidelines described by Minghetti et al., 2016 [22] to culture RTgutGC. The cells were cultured in a commercial cell culture medium Leibovitz' L-15 (Gibco/Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, USA) in 75 cm^2 flasks (Greiner Bio-One, USA) at 19°C under normal atmosphere. When 90-95% confluency was reached, the cells were split into two flasks. RTdi-MI at passages between 55 and 80 were applied in the current study. First, confluent flasks were washed twice with Versene (Thermo Fisher Scientific), thereafter cells were detached using 0.25% trypsin in phosphate buffer (PBS;

Thermo Fisher Scientific). Prior to cell seeding to conduct the exposure experiments, cells were counted using an automated cell counter (Countess II automated cell counter, Thermo Fisher Scientific). The seeding concentrations were different depending on the specific experiment.

Preparation of exposure solution

Oleic acid (OA) conjugated with bovine serum albumin (BSA) was purchased from Sigma-Aldrich (Merck, USA). Conjugation of OA to BSA ensures the fatty acid (FA) transport within the cell membrane. Lipogenic exposure solutions were freshly prepared by diluting OA+albumin with Leibovitz' L-15 medium. The final solutions, which OA concentration varied according to the experiment, were vortexed for 30s and immediately added to the washed cell monolayers.

Cell viability assay and cell morphology evaluation

First, a cell viability assay was performed to assess the effects of a wide range of OA concentrations on cells' metabolic activity. In parallel, the cell morphology after OA exposure was evaluated by light microscopy. RTdi-MI cells were seeded at a density of 75,000 cells/cm^2 in 24 multi-well cell culture plates (Greiner Bio-One). Two plates were seeded in parallel based on the percentage of FBS present in the exposure solution, either 5% or 1%, in order to assess the effects of two different FBS concentrations on cell viability and proliferation. The exposure experiment was performed for three time points, 24h, 72h, and 168h (1 week). Every plate contained triplicates of each exposure concentration, respectively 250µM, 125µM, 62.5µM, 31.25µM, 15.62µM and 7.8µM and two L-15 controls containing either 5 or 1% FBS. Wells without cells were also included to subtract the background fluorescence.

After seeding, cells were incubated at 19°C for 48h to allow attachment and development of a confluent monolayer on the multi-well plate surface. Following the incubation, the media was aspirated, and fresh exposure solutions were applied to the cells and incubated again at 19°C for the different time periods. After each selected time point was reached (i.e. 24h, 72h, 168h), the cells morphology was investigated by light microscopy and images were captured using a EVOS™ M5000 Imaging System (Invitrogen, Switerzarld). The exposure solution was aspirated from the wells and a solution of 5% (v/v) AlamarBlue (AB; Thermo Fisher Scientific) and 4µM of 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (CFDA-AM; Thermo Fisher Scientific) prepared fresh in PBS, was added to each well. The AB assesses cell viability by measuring the metabolic activity, while CFDA-AM estimates the membrane integrity [23]. The plates were wrapped in aluminum foil to avoid exposure to light and incubated at 19°C. After 30 minutes fluorescence was recorded simultaneously for both AB and CFDA-AM using a Cytation 5 multi-well plate reader (Biotek, USA), at excitation/emission wavelengths of 530/595 nm and 485/530 nm for AB and CFDA-AM, respectively. Afterwards, the AB/CFDA-AM solution was

aspirated and a 1.5% (v/v, in PBS) Neutral Red solution (NR; Sigma-Aldrich, St. Louis, MO, USA), was added to the wells to assess lysosome integrity. After a 2h incubation the Neutral Red solution was discarded, and the fixative solution (0.5% v/v formaldehyde and 1% w/v CaCl2) was added. Following few seconds, the fixative solution was discarded, the extraction solution (1% v/v acetic acid and 50% v/v ethanol) applied, and the plates were kept in dark on an oscillator shaker for 10 min. Afterwards, the fluorescence was recorded at excitation/emission wavelengths of 530/635 nm. Results obtained are reported as % viability based on fluorescent units (FU) of the control and calculated using following equation: % of control = (FU ex. cells - FU ex.no cells) X 100/ (Average [FU con. – FU con.no cells]). Where "ex" stands for "exposed cells" and "con" for "control cells".

BODIPY assay

The presence of intracellular neutral lipids and lysosomal integrity were evaluated using the BODIPY and the LysoTracker (Invitrogen) staining, respectively.

In light of the results obtained from the cell viability assay, we decided to perform further experiments by using only the highest and one of the lowest OA levels in the culture media. Since after 168h of exposure cell metabolism was reduced of 35%, we decided to perform the BODIPY assay for lipid droplet visualization only on cells exposed for 72h.

RTdi-MI cells were seeded at a density of 50.000 cells/cm^2 in 96 multi-well cell culture plates (Greiner Bio-One). After 48h of incubation, the cells were exposed in triplicates to each exposure concentration, respectively 250µM, 31.25µM, and a L-15 control containing 1% of FBS.

Following a 72h exposure, cell monolayers were washed twice in PBS and incubated for 1 hour at 19°C in the dark in a 75 nM (13.34 dilution factor) solution of LysoTracker in L-15. To combine the stainings, after 40 minutes of incubation a 4µM BODIPY solution was added to the wells. Furthermore, after 10 minutes, the Nuclear blue staining (Invitrogen) (40.6 µM), to highlight the nuclei, was also added. When the total exposure was over, the solutions were aspirated, monolayers were washed twice in PBS and then kept in L-15. The fluorescence of each well was quantified using the Cytation 5 multi-well plate reader at respective excitation/emission wavelengths of 544/590 nm for the Lysotracker and 490/503 nm for BODIPY. Cells area and intensity ratio of the fluorescence were measured by montage of images captured using a 20x objective and stitched using Gen5 software (AgilentBioTek, USA) to allow the entire well surface to be viewed for quantitative surface area analysis.

Fatty acid quantification

To perform the quantification of the fatty acids retained within the cells, RTdi-MI cells were seeded at a density of 75,000 cells/cm^2 in 12 multi-well cell culture plates (Greiner Bio-One). After 48 hours from seeding, the cells were exposed to the lipidic solutions. Nine replicates of each exposure concentration, respectively 250µM, 31.25µM, and a L-15 control containing 1% of FBS. According to the results obtained from the viability assay, further experiments were conducted using medium containing 1% FBS, since the higher concentration (5%) was found to influence excessively lipid supply to the cells and their proliferation.

The exposure experiment was performed for three time points, 24h, 72h, and 168h.

After each time point was reached, the monolayers were washed twice with PBS and then harvested in PBS combined with 0.1% of bovine serum albumin (BSA). After centrifugating the sample for 5 minutes at 1000g, the supernatant was discarded, and the cell pellets were frozen at -80°C for further analyses.

Quantification of the FA retained within the cells was performed by gas chromatography (GC) as previously described by Bou et al. [24]. Briefly, lipids were extracted from each sample following the Folch's method [25] and using chloroform as solvent. Cell pellets were diluted 1:20 (v/v) with chloroform/methanol (2:1; v/v) and 0.2 equivalents (of the total volume of the mixture) of water were added to the solution. To achieve cells' membrane disruption, samples were sonicated performing 3 pulses of 10 seconds each with a 5 second gap between every pulse. During the gaps samples were kept on ice to avoid excessive heating. Samples were then briefly vortexed and centrifuged for 10 minutes at speed 1000g to achieve phase separation. An upper water/methanol phase separated from the lower chloroform phase in a volume ratio 40:60 (v/v). The lower phase, containing the lipids, was then carefully collected by penetrating with a glass Pasteur pipette the non-extractable residues at the interphase to minimize the collection of particular material. Retrieved samples were stored in 2 ml glass vials and concentrated by evaporation under a gentle stream of N_2. The extracted lipids were resuspended in a lower volume of chloroform and spotted on thin-layer chromatography plates (20cm x 10 cm, Silica Gel) and developed in hexane-ethyl ether-acetic acid (80:20:10). The amount of triacylglycerides (TAG), free fatty acids (FFA) and phospholipids (PL) was developed under iodine vapor and visualized under UV light. The spots corresponding to TAG, FFA and PL were firstly separated by using a pencil to draw a straight line and then scraped off with a razor blade. The silica powder was transferred into a 20 ml glass tube using a funnel and dissolved with 3 ml dichloromethane. the extract was dried under N2 gas, and the residual lipid extract was trans-methylated overnight with 2',2'-dimethoxypropane, methanolic HCl and benzene at room temperature. The methyl esters were separated and analyzed in a GC (Hewlett Packard 6890; HP) with a split injector, using an SGE BPX70 capillary column (length 60 m, internal

diameter 0·25 mm and film thickness 0·25µm; SGE Analytical Science), flame ionization detector and HP Chem Station software. The carrier gas was He, and the injector and detector temperatures were both 280°C. The oven temperature was raised from 50 to 180°C at the rate of 10°C/min, and then raised to 240°C at a rate of 0·7°C/min. Individual FA methyl esters were identified by reference to well-characterized standards. The relative amount of each FA was expressed as a percentage of the total amount of FA in the analyzed sample, and the absolute amount of FA per gram of cell pellet was calculated using C23:0 methyl ester as the internal standard.

RNA extraction, cDNA synthesis and gene expression analyses

Gene expression analyses were performed to further assess the cellular responses to the OA exposures. RTdi-MI cells were seeded at a density of 75,000 cells/cm^2 in 6 multi-well cell culture plates (Greiner Bio-One). After 48h of incubation, the cells were exposed in triplicates to each exposure concentration, respectively 250µM, 31.25µM, and a L-15 control containing 1% of FBS. The exposure experiment was conducted for three time points, respectively 24h, 72h and 168h (1 week). After each time point was reached, the monolayers were washed twice in PBS and harvested in TRIzol® reagent (Invitrogen) to perform RNA extraction. Chloroform was then added according to the manufacturer's protocol. Obtained RNA was DNase treated (TURBO™, Ambion, Thermo Fisher Scientific) and purified with PureLink RNA mini kit (Invitrogen). From each sample 1µl was analysed in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA) to assess the RNA purity and concentration. The integrity of the RNA was verified with a 2100 Bioanalyzer using an RNA Nano Chip (Agilent Technologies, USA). Total RNA was then stored at -80°C until further analyses. Afterwards, 0.8 µg of each RNA pool, oligo $(dT)_{20}$ primers, and Superscript III in 20 µL reactions (Invitrogen) were used to conduct the cDNA synthesis. The same process was performed to achieve negative controls, omitting RNA and enzymes. cDNA was then diluted at 1:10 and stored at -20°C. The primers used in the qPCR reactions were designed by the use of Primer3web version 4.0.0.

The efficiency (E) of the PCR reaction was assessed for each gene assay using serial dilutions of a pool of randomly selected cDNA samples. Additionally, primer optimization was carried out by testing primer pairs at a range of temperatures in a single reaction. A LightCycler LC96 (Roche Diagnostics, Switzerland) was used to perform DNA amplification and gene expression analyses. Each reaction mix contained 2µl PCR-graded water, 2µl diluted cDNA template, 5µl of LightCycler 480 SYBR Green I Master mix (Roche Diagnostics), and 0.5 µl (10 µM) of both forward and reverse primer. Every sample was analyzed in duplicate alongside a no-template control. The three-step qPCR program featured a first enzyme activation at 95 degrees for 5 min, a following 40-45 cycles (according to the gene of interest) of 95 degrees (10s), 55, 58, 60,

or 63 degrees (10s, depending on the single gene), and 72 degrees (15s). Quantification cycle (Cq) values were calculated using the second derivative method. The specificity of the qPCR reactions was confirmed by evaluating the melting curve of qPCR products and the band pattern on the agarose gel after electrophoresis. Ribosomal protein 20 (rps*20*), hypoxanthine phosphoribosyl transferase 1 (*hprt1)* and actin beta (*actb*) were evaluated for use as reference genes according to their stability across and within the treatments[26]. The expression of target genes was normalized to the geometric mean of *rps20, hprt1* and *actb*. Mean normalized expression of the target genes was determined through relative quantification using raw Cq values. The genes under investigation in this study play pivotal roles in lipid droplet formation and fatty acid biosynthesis [4,7,6] . Supplementary Table S1 provides further details, including gene names, primer source, efficiency, and amplicon size. Additionally, the DNA of several cells' samples were pooled together and the expression of the selected genes in such pool was compared to the expression level in a pool of DNAs extracted from the distal intestinal tissue of rainbow trout.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism 10 (GraphPad Software, USA). In light of the dose-response nature of the study, the cell viability results were assessed using a non-linear regression analysis. The gene expression and FA quantification results were subject to two-way analyses of variance (ANOVA) with OA concentration and exposure time as class variables. Data were assessed for normality using the Shapiro–Wilk test and log-transformed to obtain normal distribution. The level of significance for all analyses was set at $P < 0.05$, and P-values between 0.05 and 0.1 were considered to indicate a trend in effects as indicated in the text.

Results

Cell viability assay and cell morphology evaluation

The cell viability assay was performed with six different OA concentrations - 250μM, 125μM, 62.5μM, 31.25μM, 15.62μM and 7.8μM - and a L-15 control containing either 5 or 1% FBS. The exposure experiment was performed along three time points, 24h, 72h, and 168h (1 week). The highest FBS percentage (5%) enhanced cells growth excessively, leading to a high number of dead cells at the end of the exposure period. On the other hand, 1% of FBS maintained the cells alive for the whole exposure period without significantly influencing their growth. Therefore, further experiments were conducted by using 1% of FBS in the exposure media. The cell viability results described herein are only relative to the cells grown with 1% of FBS.

Cells' viability (Figure 1), as indicated by the AB, CFDA-AM and neutral red assays, was not influenced by the increasing OA concentration at 24h (p=0.96, p=0.33, p=0.18) and 72h of exposure (p=0.30, p=0.78, p=0.95). However, after 168h of exposure, a decrease in cell viability was recorded. According to the neutral red (lysosome integrity) and AB assay (metabolic activity), cell viability was reduced by 25%, while the CFDA-AM (membrane integrity) showed a reduction of 35% (p=0.0002, p=0.0019, p=0.5166). 30% is considered as the international standard cut-off limit used as acceptable reduction in in vitro cytotoxicity tests [28]. In light of the high viability of the cells, it was not possible to identify the EC_{50}.

The cells exposed to OA showed accumulation of both stress granules (SG) and intracellular lipid droplets (LD) around the nuclei. Both structures increased in amount with the increasing OA concentration (Figure 2). The increasing exposure time increased the number of LDs and induced a change in cell morphology. After 168h of exposure to both OA concentrations the cells became more rounded in shape and most of the smaller lipid droplets fused, creating larger ones (Figure 3). Additionally, the nuclei, more centrally located in the control cells, were located peripheral and close to the cell membrane in cells with large intracellular LDs. Other than morphological changes, the highest OA concentration induced signs of cells detachment from the well after 168h.

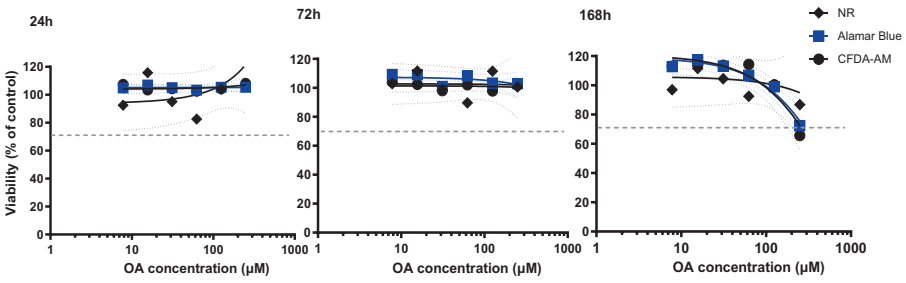


Figure 1. *Non-linear lines showing cell viability of RTdi-MI cells exposed to different concentrations of OA. Lysosomal integrity (NR) and cells' metabolic activity (Alamar Blue) and membrane integrity (CFDA-AM) are given as percentage compared to control cells set to 100%. Dotted lines indicate 30% reduced cell viability.*

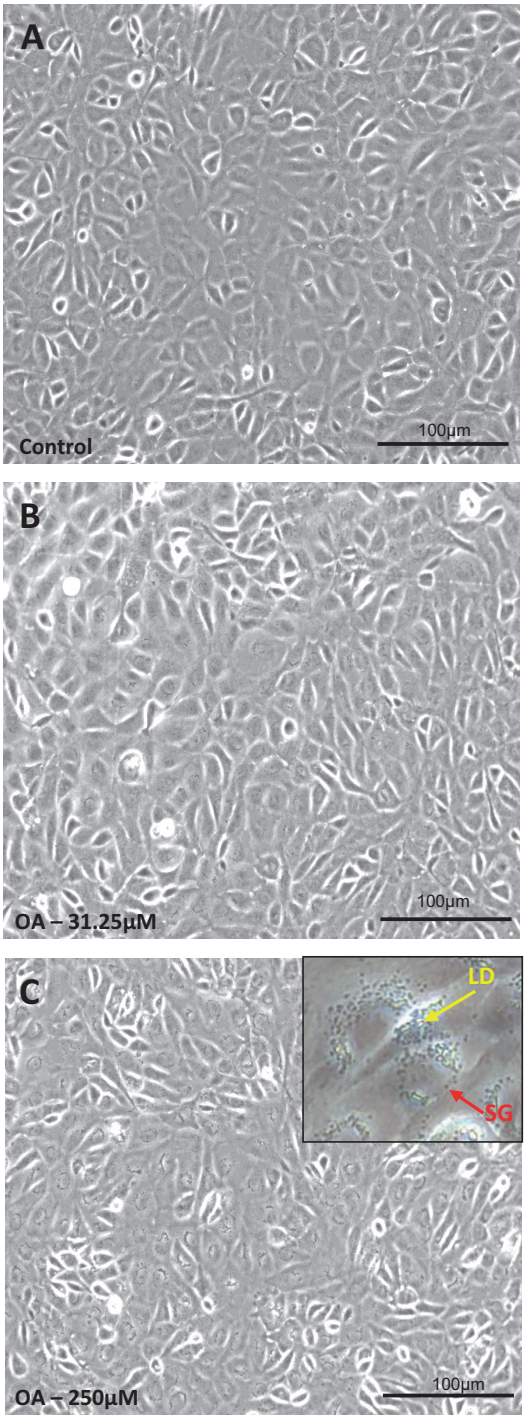

A
Control
100µm
B
OA – 31.25µM
100µm
C
LD
SG
OA – 250µM
100µm

Figure 2. Microphotographs of RTdi-MI cells after 24h of exposure. A) Untreated cells with normal morphology, B) cells exposed to 31.25µM oleic acid (OA) and C) cells exposed to 250µM OA. Effects of OA exposure were accumulation of both stress granules (SG) and intracellular lipid droplets (LD) around the nuclei, as indicated by the red and yellow arrow in the detail insert in C), respectively. Both structures increased in amount with the increasing OA concentration.

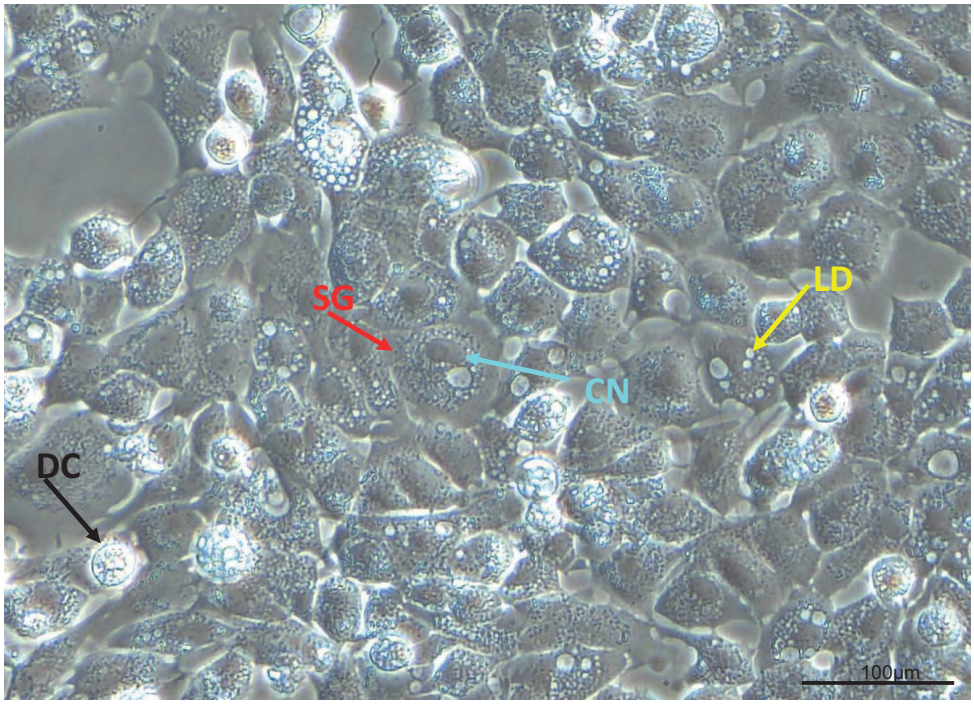


Figure 3. RTdi-MI cells as seen in the microscope when exposed to 250µM concentration of OA for 168h (1 week). Effects of the long OA exposure were accumulation of both stress granules (SG) and large intracellular lipid droplets (LD) around the nuclei, as indicated by the red and yellow arrow respectively. Both structures increased in amount with the increasing OA concentration. The blue arrow indicates a cell nucleus (CL) moved to the periphery of the cell by the accumulating LDs (FN). The black arrow points to a detached cell (DC).

BODIPY and Lysotracker assay

In light of the results obtained from the cell viability assays, we decided to perform additional experiments by exposing cells to the highest and one of the lowest OA concentrations i.e., 250µM and 31.25µM. Following 168 hours of exposure, cell membrane integrity exhibited a 35% reduction, accompanied by the initial manifestations of cell detachment. Consequently, we opted to conduct the BODIPY assay and the Lysotracker assay exclusively on cells exposed for 72 hours. The BODIPY and Lysotracker assays (Figure 4) showed a clear dose-response relationship between the OA level in the culture media and the total lipid accumulation, expressed as surface area of the cell ($p=0.0007$). The average surface area per cell was 3827 ± 129 µm², 14770 ± 1496 µm² and 25584 ± 1148 µm² respectively for the control cells and

for those exposed to 31.25μM and 250μM OA concentrations. On the other hand, the increasing OA level did not influence significantly (p=0.4) the intensity ratio per cell, which was respectively 1.41±0.17x10^7, 1.31±0.82x10^7 and 1.49±0.13x10^7± for the control cells and for those exposed to 31.25μM and 250μM OA concentrations.

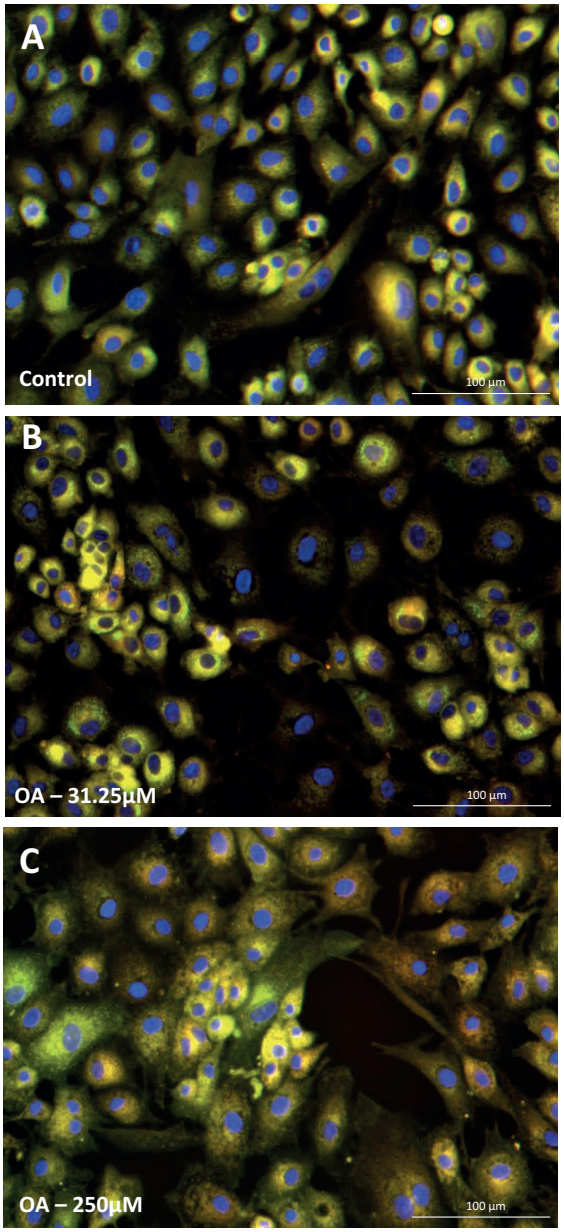


Figure 4. *Untreated cells with normal morphology and regular size (A) and cells exposed to a 31.25μM (B) and 250μM (C) concentration of OA. The nuclei are stained in blue (DAPI), lysosomes in orange (Lysotracker) and neutral lipids in green (BODIPY). The increasing OA concentration increased cells' area, whereas no effects was given on the intensity ratio of the fluorescence.*

Fatty acid quantification

To further explore the capacity of the cells to accumulate and metabolize OA, we extended our investigation beyond visual and semi-quantitative assessments of intracellular lipid levels to include the absolute quantification of intracellular FA concentrations. The complete dataset showing both absolute and relative FA distribution in the PL, TAG and FFA fractions is presented in Supplementary Tables S2-S4. The sum of the total FA, presented as µg FAs/g of cell pellet and comprising the PL, TAG and FFA fractions, increased about 2-fold for both OA concentrations 24h after exposure (Figure 5). For cells treated with the low OA exposure, total FA levels returned to control levels after 72 and 168h, whereas for the high OA exposure, FA levels remained 75% and 33% higher than control levels after 72 and 168h, respectively. The amount of FA in control cells remained low and stable at 24 and 72h but increased somewhat after 168h.

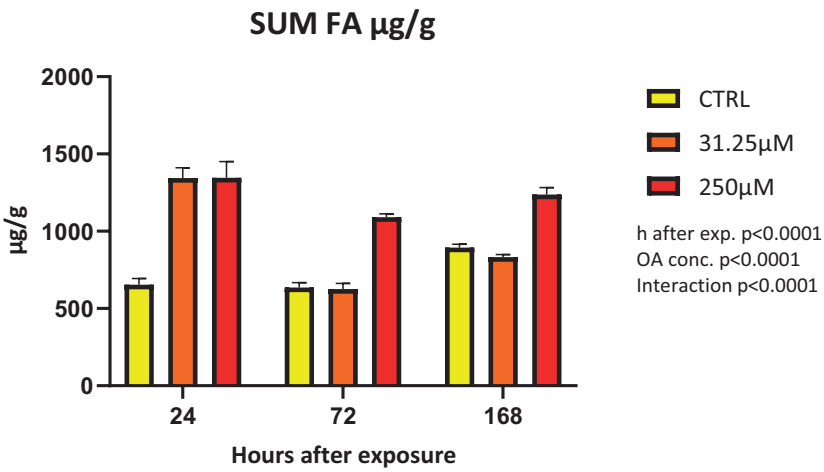


Figure 5. *Distribution of the total sum of FA presented as µg/g of cell pellet along the exposure period and in the control cells (CTRL) and OA treatments (31.25 and 250µM). Data are presented as normalised means + SD. The level of significance was set to p < 0.05.*

When considering the distribution of FAs into the three lipid fractions, we observed that control cells had relatively similar FA levels in the three fractions (TAG, PL, FFA) during the experiment, although the relative proportion of FAs in the TAG fraction increased slightly at the 168h time point as compared to earlier time points (Table 1). A similar pattern was observed for cells exposed to low OA levels (31.25µM). In contrast, cells exposed to high OA (250µM) showed the largest FA proportion in the FFA fraction after 24h, whereas the proportion of FAs in the PL fraction increased with time in culture.

Hours after exposure	CTRL			31.25µM			250µM		
	24	*72*	*168*	*24*	*72*	*168*	*24*	*72*	*168*
PL (%)	38	37	36	37	38	35	31	35	36
TAG (%)	27	28	31	28	27	31	27	31	29
FFA (%)	35	35	33	35	35	34	42	34	35

Table 1. *Relative distribution of FA within the different lipid fractions i.e., phospholipids (PL), triglycerides (TAG) and free fatty acids (FFA) and along the exposure period and in the control cells (CTRL) and OA treatments (31.25 and 250µM).*

Concerning the intracellular level of OA, the time and dose-response pattern reflected the pattern observed for the sum of FAs to a large extent. OA levels were increased about 300 and 400% after 24h compared to control levels for cells exposed to the low and high OA dose, respectively (Figure 6). For the low OA exposure, OA levels returned to control levels after 72 and 168h, whereas for the high OA exposure, OA levels remained 3.5-5-fold higher than control levels throughout the time course of the experiment.

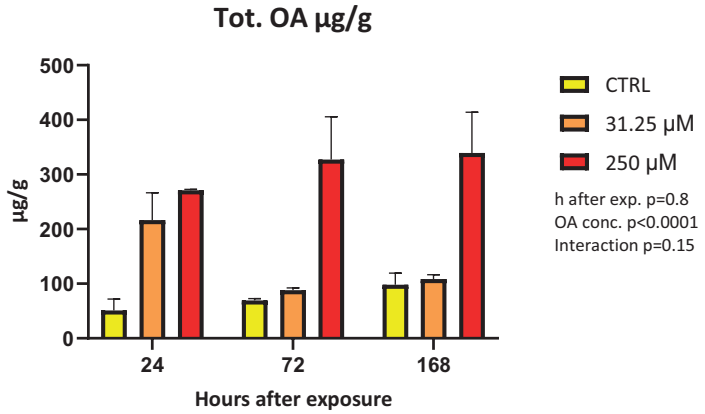


Figure 6. *Absolute distribution of OA presented as µg/g of cell pellet along the exposure period, in the control cells (CTRL) and OA treatments (31.25 and 250µM). Data are presented as normalised means + SD. The level of significance was set to p < 0.05.*

When considering the distribution of OA into the three lipid fractions (Table 2), we observed that control cells had >80% of the OA distributed to the PL fraction at all three time points, whereas low and even OA levels were found in the TAG and FFA fractions. The increased OA levels observed in cells exposed to the low OA dose at 24h was reflected in a different distribution of OA; the proportion of OA in the TAG fraction was higher and the PL fraction showed correspondingly lower relative levels. At later time points, OA distribution in the three lipid fractions reflected the distribution observed for control cells, corresponding to the similar OA levels observed in control and low OA treated cells at these two time points. Cells treated

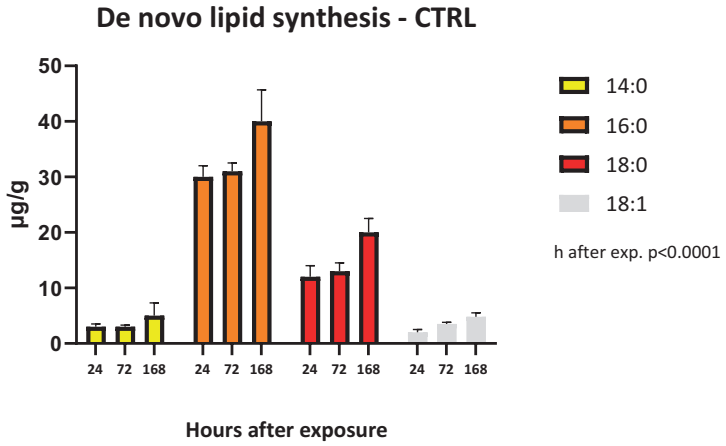
with the high OA dose displayed a different OA profile. Almost half of the OA was found in the TAG fraction after 24h, and OA levels in the TAG fraction remained high throughout the experiment.

	CTRL			31.25µM			250µM		
Hours after exposure	*24*	*72*	*168*	*24*	*72*	*168*	*24*	*72*	*168*
PL (%)	84	81	83	68	81	83	45	48	55
TAG (%)	8	11	9	25	12	9	49	48	39
FFA (%)	8	8	8	7	7	8	7	4	6

Table 2. *Relative distribution of OA within the different lipid fractions i.e., phospholipids (PL), triglycerides (TAG) and free fatty acids (FFA) and along the exposure period and in the control cells (CTRL) and OA treatments (31.25 and 250µM).*

Most of the other FAs displayed time, OA-dose, and lipid fraction response patterns similar to those observed for the total sum of FA (Supplementary Tables S2-S4). For instance, most FAs were found in increased amounts at the 24h time point for cells treated with OA, reflecting metabolism of OA into other FAs. Relatively, the most abundant FAs in the PL, TAG, and FFA fractions were PL: C16:0, C18:0, C18:1n-9, C20:4n-6, C22:4n-6; TAG: C16:0, C18:0, C18:1n-9, C18:3n-6, C20:3n-6; FFA: C16:0, C18:0, C18:3n-6, C20:3n-6.

Considering those FA which are products of de novo lipid synthesis, we observed an increasing concentration of C14:0, C16:0, C18:0 and C18:1-7 in the TAG fraction of the control cells with increasing time after exposure (Figure 7).



460

461 *Figure 7. Absolute distribution of those FAs which are products of the novo lipid synthesis presented as µg/g of cell pellet along the exposure period, in the control cells (CTRL). Data are presented as normalised means + SD. The level of significance was set to p < 0.05.*

462

463 ## Gene expression

464 The gene expression analyses were conducted on a panel of genes involved in lipid droplet
465 formation and fatty acid metabolism.

466 Firstly, we compared RTdi-MI expression levels of the selected genes to those of the
467 corresponding rainbow trout distal intestinal tissue (Table 3). All the genes were similarly
468 expressed in both cells and in vivo intestinal samples. The only exception was apolipoprotein A-
469 IV (*apoaIV*), which was highly expressed in the intestinal tissue, but not in the cell line.
470 Concerning the effects of OA concentration and exposure time, the expression of all target genes
471 increased with the increasing exposure time, and there was a significant interaction between
472 time and OA concentration for most of the genes (Figure 8). In addition, expression levels of
473 some genes showed dose-response relationships with the OA concentration in the medium.

474

Gene name	RTdi-MI (Cq)	RT tissue-DI (Cq)
Reference genes		
ribosomal protein 20	20	20.4
beta-actin	16.7	15.8
hypoxanthine phosphoribosyltransferase 1	24.5	23.9
Target genes		
perilipin2	23.6	22.4
elongase of very-long fatty acid 5	25.6	21.9
fatty acid desaturase 2	22.8	21
sterol regulatory binding protein 1	24.3	25.7
fatty acid synthase	16.5	16.8

peroxisome proliferator activated receptor gamma	24.1	25.1
apolipoprotein A-IV	35.3	18.9

Table 3. *Expression levels of the reference and target (assessed) genes in pooled samples of both RTdi-MI and distal intestine tissue of rainbow trout.*

The expression of the intracellular lipid droplet marker perilipin 2 (*plin2*) showed a dose-response relationship with OA medium concentration after 24h of exposure. Such relationship was less clear after 72 h and 168 h. Similarly to *plin2*, the expression of peroxisome proliferator activated receptor gamma (*pparg*) was upregulated by the increasing OA concentration after 24h. Simultaneously, the overall expression increased with the increasing exposure time. Concerning elongation of very long chain fatty acids 5 (*elovl5)*, its expression did not show any significant effect of OA concentration at 24 and 72h of exposure. However, after one week of exposure, *elovl5* expression showed a dose-dependent increase with the increasing OA concentration. Sterol regulatory element-binding protein 1 (*srebp1*), was downregulated by the increasing OA concentration, and its expression increased with the increasing exposure period. Fatty acid synthase (*fas*) was generally higher expressed compared to the other genes (Table 3) and it was slightly but not significantly upregulated by the increasing OA concentration. The exposure time influenced *fas* expression which was the lowest after 24h, increased at 72h and decreased again after 1 week of exposure. Fatty acid desaturase 2d5 (*fads2d5*) was downregulated by the increasing OA concentration in the medium. Such effect, together with the overall expression of the gene, increased in magnitude with longer exposure time.

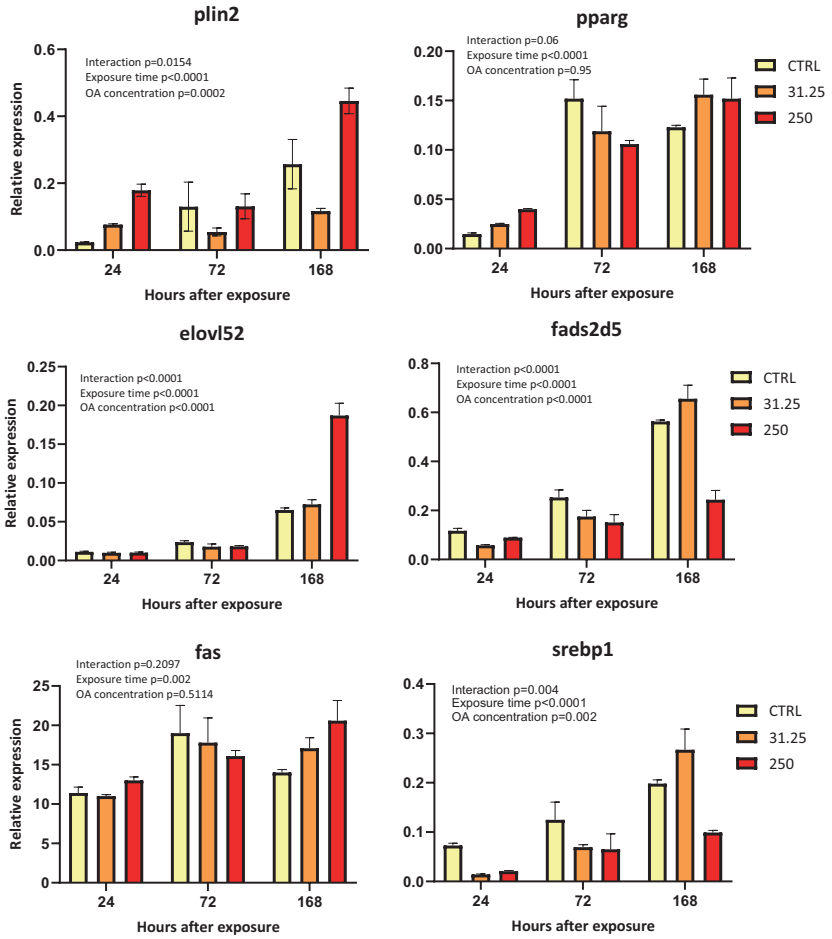


***Figure 8**. Relative expression of genes involved in LD formation, FA synthesis and metabolism. Data are mean normalised expression levels + SD. Different letters denote statistically significant differences among diet groups.*

Discussion

Lipid accumulation and metabolism in the RTdi-MI intestinal cell line

The current study founded the basis for the establishment of an in vitro lipid accumulation model able to mimic the physiological mechanisms occurring within the fish intestinal epithelium when affected by steatosis.

RTdi-MI cells exposed to OA showed abundant presence of LDs and SGs around their nuclei. Lipid droplets increased in amount and size with the increasing lipid dose and with increased exposure time, demonstrating that the cells were able to take up the lipids and that these accumulated within the cells at high and prolonged OA exposure. In parallel, the cells underwent some morphological changes. The increasing number of intracellular LDs was confirmed by the BODIPY assay results, which suggested the expansion of the cells' area as a consequence of the increased OA concentration. The cell surface area may have also increased as a consequence of the increasing number of lysosomes, as showed by the Lysotracker assay. A higher production of lysosomes has previously been associated to a higher recruitment of SGs in human cell lines [29–31].

The results obtained from the intracellular FA quantification confirmed the visual observations and clearly demonstrated that increasing OA concentration in the medium increased FA levels in all lipid fractions of the exposed cells. After 24h, intracellular FA levels were increased in cells exposed to both OA doses (31.25 and 250 μM), whereas at later time points FA levels in the cells exposed to OA at 31.25μM decreased to the level of the control samples. In contrast, the FA level in cells exposed to the high OA dose remained high throughout the time course of the experiment. These results indicate that the cells were able to metabolize the lipids when exposed to a relatively low lipid dose and return to a normal state, while exposure to a high and prolonged lipid dose resulted in FA accumulating within the cells. The RTdi-MI cells thus seem to have the capability to metabolize lipids up to a certain amount. At the same time, the increasing inclusion of OA in the medium influenced the intracellular lipid OA content. Interestingly, even in the control cells the total amount of OA increased after 168h, suggesting that the cells are able to synthetize OA via desaturation or elongation of other saturated FA, such as C16:0 or C18:0. However, since all the exposure media were formulated by the use of L-15 and 1% FBS, we cannot exclude that the FA present in the control cells are those taken up from the FBS. The intracellular OA was mainly distributed in the TAG and PL fractions, whereas low OA levels were seen in the FFA fraction. Compared to control cells, exposure to OA resulted in a shift towards higher proportion of OA distributed to the TAG fraction and the PL fraction showed correspondingly lower relative levels. This effect was stronger with the high OA dose and may be explained by the preference of storing excess lipid as TAGs rather than in the PL of

564 the cell membranes or as FFA, which are toxic in larger concentrations. For the low OA dose, this
565 shift showed a gradual reset back to control cell levels with increasing cultivation time. On the
566 other hand, cells exposed to the high OA dose showed only a slight readjustment of the OA lipid
567 fraction distribution after 168h. Taken together, these findings support the hypothesis that
568 RTdi-MI cells are able to accumulate OA in intracellular LDs. Several other studies conducted in
569 vitro in both mammalian and fish cell lines have demonstrated that OA supplementation in the
570 growth medium can promote LDs accumulation [32–38].

571 Increased use of plant ingredients in diets for Atlantic salmon have been suggested to be a
572 possible cause of the increased frequency of excessive LDs accumulation (i.e. steatosis) within
573 the intestinal enterocytes [39]. The most likely explanation for this gut disorder is deficient or
574 imbalanced supply of dietary nutrients in today's high plant diets. For instance, the study
575 conducted by Bou et al. [24] showed that an insufficient supply of long chain fatty acids can induce
576 lipid accumulation within the intestinal enterocytes. On the other hand, the studies conducted
577 by Hansen et al. and Krogdahl et al. [4,6,40] suggested that the main factor causing lipid droplet
578 accumulation in vivo is a deficiency in dietary choline, a key component of lipoproteins and
579 fundamental to allow lipid transport from the intestine to the other tissues. A recent study
580 conducted by Siciliani et al. [7] in Atlantic salmon fed choline-deficient diets confirmed the
581 relationship between dietary choline supply and steatosis symptoms. In addition, the study
582 showed a dose-response relationship between the increasing level of dietary lipid and the
583 severity of lipid accumulation within the enterocytes of the pyloric intestine. The lipid source in
584 this study was a typical mixture of rapeseed oil and fish oil in a 2:1 ratio, resulting in high OA
585 concentrations, about 31-34 % of the sum of FAs [7]. The results of the present study indicate that
586 a similar condition can be modelled in vitro by increasing the OA exposure dose to salmonid gut
587 cells. A next step could be to combine exposure of high OA doses with choline supply to assess
588 whether choline may mitigate lipid accumulation in enterocytes also in vitro.

589 To investigate mechanistical features related to LD accumulation and lipid metabolism in the
590 RTdi-MI cells, we decided to assess the expression of a set of genes involved in LD formation
591 and FA synthesis and metabolism. Our results showed, for the first time, that most of the
592 assessed genes were expressed at similar levels both in the RTdi-MI and in the rainbow trout
593 intestinal tissue. These observations are in accordance with what observed by Selvam et al. in
594 RTgutGC cells and suggest that RTdi-MI cells are provided of those metabolic compartments
595 involved in lipid metabolism and accumulation which are typically expressed in vivo. A notable
596 exception was *apoAIV*, which was highly expressed in the in vivo samples, but not in the RTdi-
597 MI cells. According to Hansen et al. [6] and Siciliani et al. [7], *apoAIV,* involved in the formation of
598 apolipoproteins, is considered as an important biomarker gene for impaired lipid transport, and

its expression has been showed to increase with increasing steatosis symptoms and dietary lipid inclusion. The lack of expression of *apoAIV* in RTdi-MI may indicate that lipoprotein formation is not a possible feature for RTdi-MI under the current cultivation conditions, an outcome which seems to disagree with the results obtained from Selvam et al. [21] on RTgutGC. However, in the study of Selvam et al, the authors observed very low expression of the major secretory lipoprotein *apob*, and although other apolipoproteins (*apoAI*, *apoAIV*, *apoC*, *apoE*, and *apoD*) were expressed, none of the transcripts in apolipoprotein synthesis were differentially expressed after FA exposure. Importantly, the cells used in the current study were seeded and grown as a 2D monolayer on regular plastic wells, whereas in Selvam et al. RTgutGC were grown in a 3D two-chambered system to a polarized cell barrier. Further studies to investigate lipid transport and metabolism in RTdi-MI will be conducted in two-chambered systems, which may induce a different morphological and functional maturation. Consequently, the obtained polarity of the cells may likely induce their capability of taking up and transporting lipids.

Another limitation to the current cultivation conditions, as well as the culture conditions used in the study by Selvam et al., may be the lack of bile components in the culture media. Human intestinal Caco-2 cells are generally unable to form chylomicrons (CM), but when supplemented with OA and taurocholate (TC), highly differentiated parent Caco-2 cells cultured on membranes are able to form and secrete CM [41]. In RTgutGC cells, we have previously demonstrated that exposure to TC, the main bile salt in salmonids, up-regulate genes involved in lipid transport and turnover, including several apolipoproteins [42].

The role of *plin2* as sensitive biomarker for LDs accumulation has been validated by several studies conducted on Atlantic salmon [4,7,6,41]. Perilipin 2 is a cytosolic protein involved in production and stabilization of intracellular LDs. In the current study, the expression of *plin2* was upregulated by the increasing OA concentration and it increased with the increasing exposure time. The study conducted by Siciliani et al [7]., which studied the effects of increasing lipid level in the diet of farmed Atlantic salmon, showed that *plin2* expression was upregulated by the increasing lipid percentage in the diet and in parallel to the increased steatosis symptoms. A scenario similar to that of *plin2* was observed for *pparg* whose expression showed a clear dose response relationship with OA concentration, especially after 24h and 1 week of exposure. A number of studies have shown that *pparg*, often referred to as the “master regulator” of adipogenesis, has an important role in inducing lipid accumulation in mammalian hepatocytes [44]. One of pparg isoforms, pparg1, has been found in many different tissues, including the intestine.

The study conducted by Schadinger et al. [45] on mouse steatotic hepatocytes observed an interesting correspondence between *pparg* expression and regulation of *fas,* involved in lipid

metabolism and de novo biosynthesis. Such result suggests that the mechanisms underlying lipid accumulation may be linked to the novo fatty acid biosynthesis and metabolism. However, while the overall expression of *pparg* increased with the increasing exposure period, that of *fas* slightly decreased 168h after exposure. Concerning the expression of *elovl5* and *fads2d5*, involved respectively in fatty acid elongation and desaturation were significantly upregulated in all samples 168h after exposure compared to 24 and 72h. Additionally, their expression followed a dose-response relationship with the OA concentration. Such outcome, together with the lipid quantification results confirms that cells can undergo lipid metabolism and synthesis even when not exposed to high concentrations of lipids.

RTdi-MI morphological and functional features after fatty acid stimulation

When Pasquariello et al. [9] derived the RTdi-MI from the distal intestine of the rainbow trout they highlighted the presence of an heterogenous cell population within the cell line. Our results seem to indicate a possibility of a stem cell population residing in the RTdi-MI which may be able to mature into adipocytes when stimulated with maturation inducing media.

As described previously, the increasing OA concentration in the culture medium increased the number of intracellular LDs, which, accumulating within the cytosol, changed the cells' morphology. 168h after exposure to the highest OA concentration, the cells were more rounded and larger in shape, and in addition the nuclei were compressed to the periphery of the cell between the LDs and the cell membrane. Such morphological characteristics have been described in several studies conducted to investigate the differentiation of Atlantic salmon preadipocytes into mature adipocytes [36,38,46,47]. According to Todorcevic et al. [44], such differentiation processes are often associated to a stress response known as unfolded protein response (UPR). The UPR is a homeostatic mechanism activated when the endoplasmic reticulum, involved in LD formation [48], is not able to respond to a too high request for LD formation. Consequently, the UPR implements the clearance of unfolded proteins derived from the endoplasmic reticulum, which are stored in SGs [49]. Our study supports this hypothesis, based on the observation that the cells exposed to high OA concentrations showed clear signs of SGs accumulation around the nuclei. The study conducted by Triana and Kaganovich [50] revealed a similar correlation between OA supplementation in the culture media and LD and SG formation in human embryonic kidney cells and human neuroblastoma cells.

Another observation supporting the hypothesis of a possible maturation of RTdi-MI into adipocytes is the increasingly higher expression of *pparg* with the increasing exposure period. According to several studies conducted on Atlantic salmon preadipocytes [36,51,52], *pparg* is responsible for the activation of adipocyte-specific genes during terminal differentiation of

preadipocytes into mature adipocytes. Such differentiation has been observed consequently to the exposure of preadipocytes to differentiation media containing high lipid concentrations. In addition, the experiment conducted by Todorcevic et al. [36] showed that the maturation of preadipocytes into mature adipocytes, accompanied by an increasing lipid droplet formation and *pparg* expression, as observed in our experiment, were only induced by exposing the cells to high concentrations of OA. Differentiation media containing either EPA or DHA, highly unsaturated fatty acids present in fish diets, lowered lipid accumulation. Interestingly, in our study the expression of *pparg* and the amount of FA products of de novo lipid biosynthesis increased with the increasing exposure period also in the control cells. Such result may suggest a possible maturation of the stem cell compartment into adipocytes even in absence of a maturation medium but only as a consequence of prolonged time in culture.

Conclusions

The results obtained from the current study demonstrate, for the first time, that RTdi-MI cells exposed to high oleic acid concentrations are able to mimic the same steatosis features observed in the enterocytes of Atlantic salmon fed to highly lipidic plant-based diets. This outcome represents the first step towards the use of in vitro platforms for the study of lipid accumulation and metabolism and relevant nutritional requirements. Further studies should be conducted to investigate the capability of RTdi-MI grown on permeable membranes to synthesize lipid droplets. In addition, the RTdi-MI exposed to the highest oleic acid concentration for the longest exposure period showed signs of maturation into preadipocytes. Although such hypothesis needs further testing, it constitutes, at present, the closest attempt for the production of an immortalized fish adipose cell line.

Author Contribution

D.S. Experimental design, laboratory work, data evaluation and interpretation, development of the first draft of the manuscript; B.R.: Experimental design, data evaluation and interpretation, manuscript review; G.L.: Experimental design, manuscript development and review; K.E.P.: Laboratory work, manuscript review; M.M.; Experimental design, laboratory work, data evaluation and interpretation, manuscript review; T.M.K.; Experimental design, data evaluation and interpretation, manuscript development and review.

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

Gene name	Gene symbol	Forward primer	Reverse primer	Amplicon Size (bp)	Annealing temperature	Efficiency	Acc. number
Target genes							
perilipin2	*plin2*	GCTACTATGTCCGTCTGGGC	TTGCCATCGGCTTCCTCATT	587	60	1.95	XM_021615226.2
elongase of very-long fatty acid 5	*elovl5*	ACATTTTGCACACCGCAACC	CTTTGTCCCCACCACACTGA	146	60	1.96	XM_036959634.1
fatty acid desaturase 2	*fads2d5*	AGATGGATCACGAGCGACAC	TATGACGGGGCATGGTAGGA	724	60	2	XM_036968515.1
sterol regulatory binding protein 1	*srebp1*	GGGCCACCCATAACAGAACA	CAGCCTCACTACAGGCCTTC	442	60	2	XM_036941593.1
fatty acid synthase	*fas*	TCATTGGCCATGTCGACTCC	TTGTGACGTAGTACCTGCCG	327	60	1.94	NM_001124339.1
peroxisome proliferator activated receptor gamma	*pparg*	CCACAGCCAGGTTCAGGAG	TGTTGAGTAGGGAAGCGGTG	154	60	2	XM_036984365.1
apolipoproteinA-IV	*apoA-IV*	GCTGATGCTGTGAGCCAGTA	AAGGCGCTCAGGTCTTTCTC		60		XM_021601985.2
Reference genes							
ribosomal protein 20	*rps20*	AGCCGCAACGTCAAGTCT	GTCTTGGTGGGCATACGG	98	60	2	NM_001124364.1
beta-actin	*actb*	CAAAGCCAACAGGGAGAAGATGA	ACCGGAGTCCATGACGATAC		60	1.82	NM_001124235
hypoxanthine phosphoribosyltransferase 1	*hprt1*	CCGCCTCAAGAGCTACTGTAAT	GTCTGGAACCTCAAACCCTATG	255	60	1.95	BT043501

Table S2a. **A**bsolute quantification of total FA represented as µg/g of cell pellet. Data are distributed between different lipid fractions i.e., phospholipids (PL), triglycerides (TAG) and free fatty acids (FFA), along the exposure period and in the control cells (CTRL) and treatments (31.25 and 250µM). The values given are means ± SD (n=3).

Time (h)	**24**			**72**			**168**		
Treatment	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**
PL (µg/g)	247.3±32	499.9±212.6	419.5±40	235.3±15.5	238±23	386.7±143	321.5±146	293±36	447±114
TAG (µg/g)	173.6±17	372±147	361±84	177±32	167±15.5	340±114	276±156	260±73	362±136
FFA (µg/g)	233.8±3	471.6±210	565,.5±280	224±10	210±20.5	362.7±127	296±134	279±57	428.5±150
Sum FA (µg/g)	654.8±39	1343.4±67.26	1346±105	636±31	625±37	1090±23	893±23	832±16	1238±44.4

Table S2b. Relative distribution of total FA represented as % of retrieved FA. Data are distributed between different lipid fractions i.e., phospholipids (PL), triglycerides (TAG) and free fatty acids (FFA), along the exposure period and in the control cells (CTRL) and treatments (31.25 and 250µM). The values given are means ± SD (n=3).

Time (h)	**24**			**72**			**168**		
Treatment	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250uM**
PL (%)	87.93±5	89.02±0.9	89.52±1.17	87.42±0.8	90.24±2.35	86.95±6.2	90.78±0.7	89.9±5.2	90.48±1.85
TAG (%)	63.72±3.8	67.88±3.7	76.9±11.7	65,62±7.7	62.9 ±0.7	78.24±1.6	77.84±15	78.42±9	70±1
FFA (%)	87.7±4.5	85.3±1.9	84.8±7	85.5±2.5	84.6±1.18	84.5±0.6	83.5±3.7	84.3±1.9	85.26±1
Sum FA (%)	79.8±14	80.7±11.2	83.7±6.3	79.5±12	79.2±14.4	83.2±4.5	84±6.5	84.2±5.7	82±10.6

Table S3a. **A**bsolute quantification of most abundant FA represented as µg/g of cell pellet within the PL fraction. Data are distributed along the exposure period and in the control cells (CTRL) and treatments (31.25 and 250µM). The values given are means ± SD (n=3).

Time (h)	24			72			168		
Treatment	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**
C 14:0	3.5 (±0.1)	5 (±3)	4 (±0.6)	4.6 (±0.3)	4 (±1)	3.2 (±0.6)	5 (±3)	5 (±2)	6 (±2)
C 16:0	36 (±3)	60 (±30)	53 (±8)	34 (±2)	31 (±5)	40 (±10)	40 (±20)	37 (±6)	40 (±10)
C 18:0	21 (±3)	40 (±20)	31 (±4)	20 (±1)	16 (±2)	23 (±9)	30 (±10)	22 (±3)	23 (±7)
Other SFA	1.4(±0.2)	3.1(±1.4)	3.3(±0.5)	2.3(±0.4)	2(±05)	2.6(±0.7)	2.9(±2.3)	3.6(±2)	3.4(±0.8)
C 16:1 n-7	6.4 (±0.3)	11 (±6)	7 (±1)	5.6 (±0.3)	4	4 (±2)	8 (±3)	5 (±1)	3 (±1)
C 16:1 n-9	8.1 (±0.6)	15 (±7)	10.2 (±0.9)	8 (±0.4)	10 (±2)	10 (±5)	11 (±4)	11 (±2)	16 (±6)
C 18:1 n-7	9 (±2)	14 (±5)	9.2 (±0.3)	7.6 (±0.7)	5.9 (±0.3)	7 (±3)	11 (±4)	8 (±1)	7 (±2)
C 18:1 n-9	60 (±10)	150 (±60)	124 (±2)	56 (±4)	71 (±4)	160 (±70)	80 (±30)	90 (±10)	190 (±60)
Other MUFA	14.8 (±1)	30 (±20)	31 (±7)	15 (±2)	15 (±1)	21 (±8)	20 (±10)	16 (±2)	25 (±7)
C 18:3 n-6	8.2 (±0.5)	18 (±9)	18 (±4)	9 (±1)	9 (±2)	13 (±4)	13 (±7)	9 (±1)	15 (±3)
C 20:3 n-6	5.5 (±0.3)	11 (±6)	11 (±3)	6 (±1)	6 (±1)	7 (±4)	8 (±4)	5.8 (±0.7)	10 (±2)
C 20:4 n-6	6.1 (±0.5)	11 (±5)	8 (±0.4)	5.1 (±0.4)	4.7 (±0.3)	8 (±3)	4 (±2)	4.7 (±0.4)	7 (±2)
C 22:4 n-6	10 (±4)	20 (±7)	21 (±5)	5 (±1)	7 (±1)	12 (±5)	12 (±6)	6 (±3)	12 (±4)

C 18:4 n-3	10.1 (±0.8)	15 (±6)	9.6 (±0.6)	9.7 (±0.9)	7.8 (±0.6)	8 (±4)	12 (±4)	12 (±2)	8 (±3)
C 22:5 n-3	4.1 (±0.9)	7 (±3)	4.6 (±0.9)	3 (±0.4)	2.7 (±0.3)	4 (±2)	3 (±1)	2.5 (±0.2)	4 (±1)
C 22:6 n-3	4.5 (±1)	8 (±3)	4.8 (±0.9)	3.4 (±0.2)	3.2 (±0.5)	5 (±2)	3 (±1)	3 (±0.3)	5 (±2)
Other FA	32.3 (±1.4)	94 (±3.5)	65 (±2)	51 (±1.4)	49 (±1.5)	82.4 (±2.8)	59.6 (±2.1)	69.4 (±2.5)	97 (±5)

Table S3b. **A**bsolute quantification of most abundant FA represented as µg/g of cell pellet within the TAG fraction. Data are distributed along the exposure period and in the control cells (CTRL) and treatments (31.25 and 250µM). The values given are means ± SD (n=3).

Time (h)	**24**			**72**			**168**		
Treatment	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**
C 14:0	3 (±1)	9 (±5)	6.7 (±0.4)	3.4 (±0.3)	3 (±0.5)	5 (±2)	5 (±3)	5 (±1)	7 (±3)
C 16:0	30 (±1)	60 (±30)	40 (±5)	31 (±4)	27 (±2)	30 (±10)	40 (±20)	34.9 (±0.4)	37 (±6)
C 18:0	12 (±2)	23 (±8)	17 (±6)	13 (±2)	11.2 (±0.8)	13 (±5)	20 (±10)	17 (±4)	20 (±7)
Other SFA	3	8(±0.2)	5(±2)	4(±0.5)	4(±0.4)	4(±0.4)	6(±2.5)	5.5(±2.5)	4(±1.7)
C 18:1 n-9	5.4 (±0.4)	50 (±20)	130 (±20)	8 (±2)	11 (±2)	160 (±60)	8 (±3)	10 (±2)	130 (±60)
Other MUFA	32(±18)	60(±33)	42(±24)	32(±19)	30(±18)	28(±16)	55(±31)	53(±30)	41.5(±23)
C 18:3 n-6	19 (±2)	40 (±10)	30 (±10)	19 (±3)	18 (±1)	16 (±7)	30 (±20)	30 (±10)	25 (±10)
C 20:3 n-6	12 (±2)	20 (±8)	15 (±7)	12 (±2)	11 (±1)	10 (±4)	20 (±10)	20 (±8)	15 (±7)
Other FA	5.8(±1)	12(±2)	10(±0.3)	7	5.6(±0.7)	8(±1)	15(±4)	6(±1.8)	10(±0.3)

Table S3c. Absolute quantification of most abundant FA represented as µg/g of cell pellet within the FFA fraction. Data are distributed along the exposure period and in the control cells (CTRL) and treatments (31.25 and 250µM). The values given are means ± SD (n=3).

Time (h)	**24**			**72**			**168**		
Treatment	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**
C 14:0	6 (±1)	14 (±6)	13 (±8)	7 (±1)	6.6 (±0.5)	11 (±3)	9 (±5)	8 (±2)	11 (±4)
C 16:0	40 (±2)	80 (±40)	90 (±50)	38 (±1)	36 (±2)	60 (±20)	50 (±20)	50 (±10)	70 (±30)
C 18:0	20 (±1)	40 (±20)	50 (±20)	17 (±1)	17 (±3)	30 (±9)	25 (±10)	40 (±30)	40 (±20)
Other SFA	5.4(±1)	12.05(±5.3)	14.5(±4)	6(±0.9)	6.16(±0.5)	9.3(±4)	7.5(±3)	6.47(±0.2)	11(±4)
C 18:1 n-9	6 (±2)	15 (±9)	19 (±9)	5.6 (±0.4)	6 (±1)	14 (±3)	8 (±3)	9 (±1)	20 (±10)
C 20:1 n-7	6 (±2)	12 (±5)	19,9	5.8 (±0.8)	6.4 (±0.5)	10 (±5)	8 (±3)	6.3 (±0.1)	11 (±3)
Other MUFA	93.65 (±3)	185(±51)	250(±71)	91(±25)	91(±25)	143(±40)	119(±33)	95(±26)	161(±44)
C 18:3 n-6	25 (±5)	50 (±20)	60 (±30)	24.4 (±0.8)	24 (±3)	40 (±10)	30 (±20)	25 (±1)	40 (±10)
C 20:3 n-6	15 (±3)	30 (±10)	40 (±20)	14.4 (±0.9)	14 (±2)	24 (±9)	20 (±10)	15.8 (±0.9)	27 (±10)
C 22:4 n-6	10 (±6)	27 (±8)	23,2	12 (±6)	8 (±0.4)	14 (±4)	12 (±6)	14 (±4)	24 (±8)
Other FA	3.3 (±1)	5 (±2.7)	8.6	3 (±5)	2.5	3.6(±1)	3.2(±2)	3.2(±0.25)	5.5(±1)

Table S4a. Relative quantification of most abundant FA represented as % of retrieved FA from the PL fraction. Data are distributed along the exposure period and in the control cells (CTRL) and treatments (31.25 and 250µM). The values given are means ± SD (n=3).

Time (h)	**24**			**72**			**168**		
Treatment	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**
C 14:0	1.2 (±0.2)	0.8 (±0.2)	0.85 (±0.05)	1.7 (±0.2)	1.6 (±0.3)	0.8 (±0.3)	1.4 (±0.2)	1.4 (±0.3)	1.2 (±0.3)
C 16:0	12.64 (±0.1)	11.1 (±0.4)	11.3 (±0.8)	12.69 (±0.08)	11.8 (±1)	8 (±1)	11.9 (±0.2)	11.5 (±0.9)	7.63 (±0.08)
C 18:0	7.4 (±0.4)	6.9 (±0.2)	6.5 (±0.4)	7.3 (±0.1)	6.1 (±0.3)	5.1 (±0.5)	7.5 (±0.1)	6.8 (±0.3)	4.6 (±0.2)
Other SFA	0.5 (±0.04)	0.5	0.7 (±0.06)	0.85 (±0.1)	0.7 (±0.04)	0.7 (±0.45)	0.76 (±0.3)	1.2 (±0.8)	0.7 (±0.07)
C 16:1 n-7	2.3 (±0.3)	1.8 (±0.3)	1.6 (±0.4)	2.1 (±0.05)	1,6	1 (±0.2)	2.4 (±0.2)	1.58 (±0.04)	0.69 (±0.09)
C 16:1 n-9	2.87 (±0.02)	2.7 (±0.1)	2.2 (±0.4)	3 (±0.1)	3.8 (±0.6)	2.3 (±0.4)	3.2 (±0.3)	3.4 (±0.1)	3.2 (±0.1)
C 18:1 n-7	3 (±0.4)	2.5 (±0.3)	2 (±0.1)	2.8 (±0.1)	2.22 (±0.09)	1.6 (±0.2)	3.1 (±0.7)	2.5 (±0.1)	1.33 (±0.03)
C 18:1 n-9	21 (±2.8)	26 (±1.3)	27 (±2.5)	20.8 (±0.5)	27.1 (±0.5)	35 (±6.8)	23 (±2.2)	28 (±1.4)	37.7 (±0.8)
Other MUFA	7.9 (±2.2)	8 (±2.7)	8.8 (±3)	8.3 (±2.5)	7.8 (±2.5)	6.7 (±2)	8.5 (±2.7)	7 (±1)	7.6 (±2)
C 18:3 n-6	2.91 (±0.04)	3.1 (±0.2)	3.8 (±0.6)	3.2 (±0.3)	3.3 (±0.5)	2.9 (±0.1)	3.5 (±0.3)	2.8 (±0.2)	3 (±0.3)
C 20:2 n-6	2 (±0.1)	1.81 (±0.04)	1.4 (±0.1)	1.86 (±0.02)	1.79 (±0.02)	1.6 (±0.2)	1.5 (±0.2)	1.8 (±0.1)	1.24 (±0.03)
C 20:3 n-6	1.95 (±0.04)	1.9 (±0.2)	2.4 (±0.4)	2.1 (±0.3)	2.1 (±0.3)	1.4 (±0.7)	2.3 (±0.1)	1.8 (±0.1)	2 (±0.2)
C 20:4 n-6	2.15 (±0.03)	2.05 (±0.08)	1.7 (±0.2)	1.9 (±0.04)	1.78 (±0.02)	1.7 (±0.1)	1.23 (±0.08)	1.5 (±0.2)	1.38 (±0.05)
C 22:4 n-6	3 (±1.1)	3.6 (±0.4)	4.3 (±0.7)	1.9 (±0.6)	2.6 (±0.6)	2.6 (±0.5)	3.3 (±0.3)	2 (±1.2)	2.4 (±0.3)
C 18:4 n-3	3.58 (±0.03)	2.7 (±0.1)	2.1 (±0.3)	3.6 (±0.2)	2.95 (±0.07)	1.8 (±0.3)	3.6 (±0.4)	3.8 (±0.2)	1.71 (±0.08)
C 22:6 n-3	1.6 (±0.2)	1.4 (±0.09)	1 (±0.2)	1.28 (±0.01)	1.2 (±0.1)	1.02 (±0.1)	0.83 (±0.04)	0.92 (±0.07)	0.9 (±0.03)

Other FA	3.5 (±0.4)	6.15 (±1.14)	6.24 (±1.5)	3.8 (±0.5)	3.9 (±0.6)	5 (±0.3)	3.14 (±0.3.6)	4.34 (±0.9)	4.48 (±1.3)

Table S4b. Relative quantification of most abundant FA represented as % of retrieved FA from the TAG fraction. Data are distributed along the exposure period and in the control cells (CTRL) and treatments (31.25 and 250µM). The values given are means ± SD (n=3).

Time (h)	**24**			**72**			**168**		
Treatment	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**
C 16:0	11.2 (±0.6)	11.1 (±0.7)	9 (±1.2)	11.4 (±0.8)	10.3 (±0.4)	6.9 (±0.3)	11 (±0.5)	11 (±1.7)	8 (±2.3)
C 18:0	4.3 (±0.6)	4.3 (±0.6)	3.7 (±0.9)	4.7 (±0.4)	4.2 (±0.3)	2.9 (±0.7)	5 (±1.1)	5.2 (±0.4)	3.8 (±0.7)
Other SFA	1 (±0.11)	1.13 (±0.18)	1 (±0.3)	1.4 (±0.14)	1.55	0.73	1.8 (±0.33)	1.65 (±0.66)	0.78
C 18:1 n-9	1.98 (±0.07)	10 (±1.3)	29 (±1.5)	2.8 (±0.5)	4 (±0.3)	36 (±2)	2.5 (±0.8)	2.9 (±0.1)	25 (±1.5)
C 20:1 n-7	1.2 (±0.1)	1.3 (±0.3)	0.9 (±0.2)	1 (±0.2)	0.9 (±0.1)	0.5 (±0.1)	2 (±1.3)	1.6 (±0.3)	0.9 (±0.2)
Other MUFA	26.7 (±4)	25 (±4)	21 (±3.4)	27 (±4)	25.6 (±3.5)	14.3 (±2)	35 (±5.2)	35 (±5)	18 (±2.6)
C 18:3 n-6	6.9 (±0.3)	6.7 (±0.9)	5 (±2)	7.1 (±0.9)	6.7 (±0.1)	3.7 (±0.4)	9 (±2.2)	9 (±1.9)	4.7 (±0.2)
C 20:3 n-6	4.5 (±0.4)	3.7 (±0.3)	3 (±1.3)	4.5 (±0.5)	4.28 (±0.07)	2.3 (±0.2)	6 (±1.4)	6 (±1.2)	2.9 (±0.3)
C 16:2 n-3	1.32 (±0.09)	1.4 (±0.3)	1.1 (±0.2)	1.3 (±0.1)	1.24 (±0.09)	0.72 (±0.08)	1.38 (±0.06)	1.3 (±0.1)	0.83 (±0.03)
Other FA	0.8 (±0.2)	0.7 (0.01)	0.95 (±0.64)	1.2 (±0.8)	0.86 (±0.2)	1 (±0.14)	1.97	0.57	0.96 (±0.17)

Table S4c. Relative quantification of most abundant FA represented as % of retrieved FA from the PL fraction. Data are distributed along the exposure period and in the control cells (CTRL) and treatments (31.25 and 250µM). The values given are means ± SD (n=3).

Time (h)	**24**			**72**			**168**		
Treatment	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**	**Control**	**31.25µM**	**250µM**
C 14:0	2.8 (±0.2)	2.4 (±0.6)	2 (±0.4)	2.5 (±0.2)	2.6 (±0.3)	2.5 (±0.3)	2.4 (±0.6)	2.8 (±0.3)	2.1 (±0.1)
C 16:0	15.4 (±0.9)	14 (±1.6)	14 (±1.5)	14.2 (±0.3)	14.3 (±0.6)	13.9 (±0.4)	13.3 (±0.2)	15.7 (±0.4)	14.5 (±0.7)
C 18:0	7.6 (±0.4)	6.8 (±0.5)	7.6 (±0.6)	6.2 (±0.3)	6.6 (±0.4)	6.9 (±0.2)	7 (±0.8)	12 (±7.5)	8 (±1.3)
Other SFA	1.87 (±0.05)	2.18 (±0.05)	2.28 (±0.3)	2.01 (±0.1)	2.48 (±0.2)	2.3 (±0.06)	2.14 (±0.3)	2 (±0.25)	2.3 (±0.2)
C 15:1	19.3 (±0.6)	21 (±2.6)	22 (±1.1)	20.3 (±0.6)	22 (±1.4)	20.8 (±0.8)	21 (±1.7)	18 (±2.2)	19 (±1.5)
C 17:1 n-7	12.8 (±0.5)	14 (±1.6)	14.9 (±0.8)	12.8 (±0.3)	13.8 (±0.7)	13.9 (±0.6)	14 (±1.2)	12 (±1.4)	12.6 (±0.4)
C 18:1 n-9	3 (±0.8)	2.1 (±1)	2.8 (±0.1)	2.8 (±1)	2.09 (±0.05)	2.9 (±0.4)	2.14 (±0.06)	2.9 (±0.6)	4 (±1.1)
C 20:1 n-7	2.1 (±0.2)	2.4 (±0.4)	2.3 (±0.06)	1.99 (±0.07)	2.51 (±0.09)	2.4 (±0.2)	2.2 (±0.2)	1.9 (±0.3)	2.3 (±0.3)
Other MUFA									
C 18:3 n-6	8.6 (±0.5)	9 (±1.4)	9.5 (±0.2)	9 (±0.7)	9.2 (±0.6)	9 (±0.5)	9.2 (±0.5)	7.5 (±0.8)	8.6 (±0.9)
C 20:3 n-6	5.4 (±0.3)	5.6 (±0.8)	5.6 (±0.5)	5.34 (±0.1)	5.6 (±0.3)	5.7 (±0.1)	6 (±0.6)	4.8 (±0.5)	5.4 (±0.2)
C 22:4 n-6	4 (±2.3)	5 (±1.2)	2 (±2.1)	6.1 (±0.9)	2.9 (±0.7)	3 (±0.3)	3.7 (±0.6)	3 (±1.1)	4.2 (±0.4)
Other FA	1 (±0.03)	1 (±0.4)	0(±0.5)	1.19 (±0.06)	1 (±0.09)	0.8 (±0.2)	1 (±0.02)	0.8 (±0.2)	1 (±0.12)

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