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

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Abstract

Foamy, whitish appearance of the pyloric caeca, reflecting elevated lipid content, histologically visible as hypervacuolation, is frequently observed in Atlantic salmon fed high-plant diets. Lipid malabsorption syndrome (LMS) is suggested as term for the phenomenon. Earlier studies have shown that insufficient supply of phospholipids may cause similar symptoms. The objective of the present study was to strengthen knowledge on the role of choline, the key component of phosphatidylcholine, in development of LMS as well as finding the dietary required choline level in Atlantic salmon. A regression design was chosen to be able to estimate the dietary requirement level of choline, if found essential for the prevention of LMS. Atlantic salmon (456 g) were fed diets supplemented with 0, 392, 785, 1177, 1569, 1962, 2354, 2746 and 3139 mg/kg choline chloride. Fish fed the lowest-choline diet had pyloric caeca with whitish foamy surface, elevated relative weight, and the enterocytes were hypervacuolated. These characteristics diminished with increasing choline level and levelled off at levels of 2850, 3593 and 2310 mg/kg, respectively. The concomitant alterations in expression of genes related to phosphatidylcholine synthesis, cholesterol biosynthesis, lipid transport and storage confirmed the importance of choline in lipid turnover in the intestine and ability to prevent LMS. Based on the observations of the present study, the lowest level of choline which prevents LMS and intestinal lipid hypervacuolation in post-smolt Atlantic salmon is 3·4 g/kg. However, the optimal level most likely depends on the feed intake and dietary lipid level.

Key words: **Choline requirements: Lipid accumulation: Gut health: Fish feed: Plant ingredients**

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

Choline is defined as an essential nutrient for mammals[5], whereas phosphatidylcholine is not, as it can be synthesised if choline is present. Regarding fish, essentiality has been established, as reported by the National Research Council (NRC)[6], but only for early stages, and the requirement differs between the species[6–12]. For salmonids, the NRC suggests a requirement of 800 mg/kg for both rainbow trout and some species of Pacific salmon[6]. However, also higher requirements about 3000 mg/kg have been indicated for Pacific salmonids[13]. Studies on juvenile rainbow trout suggest an inverse relationship between initial body weight and choline requirement. Estimated choline requirement for 0·12, 1·4, 3·2 and 3·5 g fish is 3000, 813, 714 and between 50 and 100 mg/kg diet, respectively[6,14,15]. At present, no requirement estimates are established in the scientific literature for any life stages of Atlantic salmon[6]. The available studies addressing choline requirement for early stages of fish development show that deficient supply causes symptoms

Abbreviations: AD, apparent digestibility; CH, cholesterol; DAG, diacylglycerol; DI, distal intestine; LMS, lipid malabsorption syndrome; MI, mid intestine; NMBU, Norwegian University of Life Sciences; NRC, National Research Council; OSI, organosomatic index; PI, pyloric intestine; PL, phospholipid.

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such as poor growth and low feed efficiency, fatty liver, high mortality and anorexia[11,15,16]. Lipid accumulation in the intestinal mucosa is seldom an observed endpoint in studies of choline deficiency and requirement but was observed in an early study of Japanese eel (*Anguilla japonica*) as 'white-grey coloured intestines'[17]. The symptoms appear similar to those observed in Atlantic salmon with LMS. Other studies have shown that phosphatidylcholine may prevent lipid accumulation in the intestinal mucosa and might be the limiting factor for lipid cell transport[18–24]. In these, dietary supplementation with phosphatidylcholine was observed to reduce the formation of enterocytic lipid droplets and was an important factor in lipoprotein formation[22,25–27] necessary for exporting dietary lipid from the gut[20,28,29]. The apparent disturbance in lipid metabolism of fish fed diets with high level of plant ingredients is also observed on the molecular level. Expression of genes involved in lipid metabolism seems to reflect reduced lipid export from the enterocytes[10,20–38].

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

Materials and methods

Diets

A low fishmeal, high plant diet (LF1) was used as a reference diet, containing 10 % Nordic low temperature (LT) fishmeal. The total lipid content was 70 % rapeseed oil and 30 % fish oil. The diet formulation and analysed chemical composition can be found in Table 1. The choline supplemented diets (LF2–LF9) were made by supplementing the LF1 diet with eight levels of choline chloride 70 %. Table 2 shows supplemented and analysed choline content in the experimental diets. The choline analyses indicate a higher than expected variation in the results. However, there was no systematic deviation. Therefore, the high variation should not affect the estimates of choline requirement. The diets were supplemented with standard vitamin and mineral premixes in accordance with NRC guidelines (2011) and BioMar standards to meet the requirements. Yttrium oxide (0·50 g/kg) was added as an inert marker for estimation of nutrient apparent digestibility (AD). The experimental diets were produced by extrusion (feed pellet size 6 mm) at BioMar Feed Technology Centre using a BC 45 twin screw extruder (Clextral).

Table 1. Formulation and chemical composition of the basal diet

Diets	LF1*
Ingredients (g/kg)	
Nordic LT 94 fishmeal†	100
Soya 60 % (SPC)‡	173
Maize gluten§	150
Pea protein 50‖	130
Dehulled beans‖	140
Wheat gluten**	27·5
Fish oil (standard)¶	75·5
Rapeseed oil††	176
Amino acid mix‡‡	14·5
Mineral mix§§	3·5
Monocalcium phosphate§§	24·3
Lucantin Pink CWD 10 %§§	0·4
Yttrium‖‖	0·5
Choline chloride 70 %¶¶	0
Analysed chemical composition (g/kg)	
DM	957
Protein	407
Fat	290
Starch	214

LT, low temperature; SPC, soya protein concentrate; CWD, cold water dispersible.
* Low fishmeal basal diet.
† Supplied by Norsildmel AS.
‡ Supplied by Selecta S/A.
§ Supplied by Cargill Nordic.
‖ Supplied by DLG Food Grain.
¶ Supplied by HC Handelscenter.
** Supplied by Roquette.
†† Supplied by FF Skagen.
‡‡ Supplied by Emmelev.
§§ Supplemented to meet the requirements.
‖‖ Inert marker for the evaluation of nutrient digestibility.
¶¶ Supplied by Balchem.

Experimental animals and conditions

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

Atlantic salmon (*Salmo salar* L., post-smolt, Sunndalsøra strain) with a mean initial weight of 456 (SD 65) g were pit tagged, individually weighed and randomly allocated into nine fibreglass tanks. Each tank contained 300 litres of saltwater, thirty-five fish and initial and final fish densities of 53 and 127 kg/m^3, respectively. The density was high, but within limits found compatible with good growth, health and welfare of fish under the condition that O_2 supply is sufficient[39]. The tanks were supplied with flow through seawater. Salinity ranged between 32 and 33 g/l. The water flow was increased accordingly to the increase in biomass over time and to maintain O_2 saturation above 80 %. The O_2 content of the outlet water was monitored once a week or more often in periods with larger temperature variations. The water temperature varied between 7·5 and 14·0°C during the experimental period (from July to September 2012), with an average

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

	LF1	LF2	LF3	LF4	LF5	LF6	LF7	LF8	LF9
Supplemented		392	785	1177	1569	1962	2354	2746	3139
Analysed	1340	1540	1760	2310	2600	2850	3330	3830	4020

of 10·5°C, and a constant 24-h light regimen was employed during the experimental period. Each tank was fed one experimental diet. Feed waste could not be collected and therefore feed intake was not measured. The daily amount of feed given was calculated from the expected biomass and daily growth rate and added with 15 % to secure feeding to *ad libitum*. The fish were fed using disc feeders.

Sampling

After 92 d, feeding was terminated. From each tank, ten fish were anaesthetised with tricaine methane-sulfonate (MS-222). Weight and length were recorded for all fish, and blood was sampled from the caudal vein in vacutainers with lithium heparin. The vacutainers were stored on ice prior to plasma preparation. Plasma was sampled in 2 ml aliquots and snap-frozen in liquid N_2 and stored at –80°C. Following blood sampling, the fish were killed by a sharp blow to the head and opened ventrally. The gastrointestinal tract was removed from the abdominal cavity, cleared of other organs and adipose tissue and sectioned as follows: PI: the section from the sphincter to the most distal pyloric caeca; MI from the distal end of PI and proximal to the increase in intestinal diameter; distal intestine (DI) section from the distal end of MI to the anus. The tissues of the PI and DI were collected and weighed, whereas the digesta from these two sections were split into two samples, that is, the proximal half (PI1 and DI1, respectively) and distal half (PI2 and DI2, respectively). The intestinal samples were snap-frozen in liquid N_2 and stored at –80°C. The liver was also weighed. An additional eight fish per tank were euthanised prior to sampling of the pyloric caeca for histological and gene expression analyses. The remaining fish in each tank were stripped for faeces as described by Austreng[40]. The remaining fish were then fed for one more week for an additional stripping in order to collect enough sample for analysis. The faecal samples were pooled for each tank, frozen immediately in liquid N_2 after stripping and stored at –80°C until analysis. Tissues sampled for histological examination were fixed in 10 % neutral-buffered formalin (4 % formaldehyde). Samples for gene expression analyses were rinsed in sterile saline water, submerged in RNAlater® and kept at 4°C for 24 h and subsequently kept at –40°C until analysis.

Histology

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

Gene expression

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

First-strand complementary DNA synthesis was carried out using five fish from each tank and Superscript III in 20 µl reactions (Invitrogen, ThermoFisher Scientific) using 0·8 µg total RNA and oligo $(dT)_{20}$ primers. Negative controls were performed in parallel by omitting RNA or enzyme. The obtained complementary DNA was diluted 1:10 before use and stored at –20°C. A total of twenty-six target genes with key functions in lipid and sterol metabolism were profiled by quantitative PCR. Information of gene categories and functions is provided in online Supplementary Table S1. PCR primers were obtained from the literature or designed using Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3/). Detailed information of the primers is shown in online Supplementary Table S1. PCR reaction efficiency (E) for each gene assay was determined separately using 2-fold serial dilutions of randomly pooled complementary DNA. A LightCycler 480 (Roche Diagnostics) was used for DNA amplification and analysis of the expression of individual gene targets. Each 10 µl DNA amplification reaction contained 2 µl PCR-graded water, 2 µl of 1:10 diluted complementary DNA template, 5 µl of LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0·5 µl of each forward and reverse primer. Each sample was assayed in duplicate in addition to a no template control. The three-step qPCR programme included an enzyme activation step at 95°C for 5 min followed by forty or forty-five cycles (depending on the individual gene tested) of 95°C (10 s), 58, 60 or 63°C (10 s depending on the individual gene tested) and 72°C (15 s). Quantification cycle (Cq) values were calculated using the second derivative method. The PCR products were

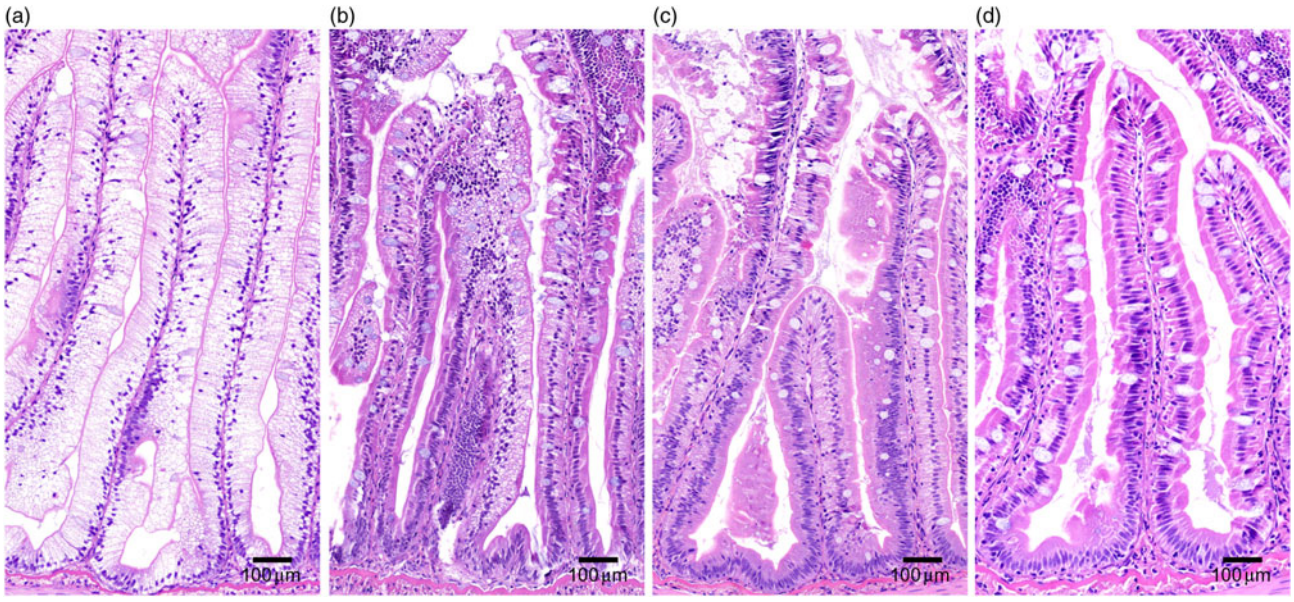

Fig. 1. Histological severity of vacuolation of the pyloric caeca tissue, representative for (a) marked, (b) moderate, (c) mild and (d) normal.

evaluated by analysis of melting curve and by agarose gel electrophoresis to confirm amplification specificity. All primer pairs gave a single-band pattern on the gel for the expected amplicon of interest in all reactions. For target gene normalisation, *actb*, *ef1a*, *gapdh*, *rnapolII* and *rps20* were evaluated for use as reference genes by ranking relative expression levels according to their stability, as described previously[42]. The *rnapolII* showed a stable expression pattern and was therefore used as a normalisation factor. Mean normalised expression of the target genes was calculated from raw Cq values by relative quantification[43].

Chemical analyses

Diets and faecal samples were analysed for DM (after heating at 105°C for 16–18 h), ash (combusted at 550°C to constant weight), crude protein (by the semi-micro-Kjeldahl method, Kjeltec-Auto System, Tecator), lipid (diethylether extraction in a Fosstec analyser (Tecator) after HCl-hydrolysis), starch (measured as glucose after hydrolysis by α-amylase (Novo Nordisk A/S) and amylo-glucosidase (Bohringer Mannheim GmbH), followed by glucose determination by the 'Glut-Dh method' (Merck Darmstadt)), gross energy (using the Parr 1271 Bomb calorimeter; Parr) and yttrium (by inductivity coupled plasma mass-spectroscopy as described by Refstie *et al.*[44]. The plasma variables, NEFA, cholesterol (CH) and total TAG, were analysed according to standard procedures at the Central Laboratory of the NMBU. Lipoprotein profile analyses (HDL, LDL and VLDL) in plasma were carried out by size exclusion chromatography and measurements of CH and TAG online using microlitre sample volumes as described by Parini *et al.*[45]. Isotop dilution MS as described by Lund *et al.*[46] was used for analysing lathosterol. 7α-Hydroxy-4-cholesten-3-one (C4) was analysed by isotope dilution and combined HPLC–MS as described by Lövgren-Sandblom *et al.*[47]. Plasma levels of oxysterols, sitosterol and camposterol were analysed by isotope dilution and combined GC–MS after hydrolysis as described by Dzeletovic *et al.*[48] for the first mentioned and by Acimovic *et al.*[49] for the last two mentioned. The pyloric caeca tissue from four fish from each of the LF1 and LF6 diets was analysed for the lipid classes NEFA, monoacylglycerol, diacylglycerol (DAG), TAG and phospholipid (PL). Four fish were considered sufficient to reveal differences in lipid characteristics of fish from these treatments. Lipid was extracted according to a modified Folch procedure[50,51]. Duplicate samples were individually applied to TLC plates to separate the lipid classes: NEFA, PL, TAG and DAG. The various lipid classes were identified by comparison with standards[52]. The respective spots were scraped into reaction vials, and the results of the further chemical[53,54] were analysed using the Hewlett-Packard Chem Station Software. In the analysis, the PL composition was related to the C23 : 0 peak, the NEFA composition to the C12 : 0 peak and the TAG and DAG compositions to the C13 : 0 peak. The combined weights of each lipid class sample thus calculated were corrected for contents of non-fatty acid material (e.g. glycerol phosphate) by multiplying each weight with appropriate factors as given by Christie[55].

Calculations

Growth of the fish was calculated as specific growth rate (percentage growth per d): specific growth rate = ((ln FBWg/ln IBWg)/D) × 100. IBW and FBW are the initial and final body weights, respectively (tank means), and D is the number of feeding days. The condition factor was calculated as: CF = FBW × 100)/Fork length cm^3). Organosomatic indices (OSI) were calculated as: (organ weight g/body weight g) × 100). Apparent digestibilities (AD) of main nutrients were estimated by using Y_2O_3[56] as an inert marker and calculated as follows: $AD_n = 100 - (100 \times (M_{feed}/M_{faeces}) \times (N_{feed}/N_{faeces}))$,

British Journal of Nutrition

where M represents the percentage of the inert marker in feed and faeces and N represents the percentage of a nutrient in feed and faeces. The 95 % confidence range for choline requirement for the selected biomarkers = the choline requirement level ± 2 × SEM.

Statistical analysis

Data were tested for normality and homogeneity of variance using Shapiro–Wilk and Brown–Forsythe tests, respectively. When necessary, data were log transformed to obtain homogenous variance (indicated by 'X' in online Supplementary Table S4). Responses of the supplemented levels of choline were evaluated using polynomial regression analyses. Visual examination of the results indicated that, for the data showing a clear relationship with dietary choline level, a second-degree function would fit the biomarkers well and be suitable for the main aim of the present study, to estimate a minimum required level of choline in salmonid diets for fish raised under conditions similar to the present. For the OSI of PI and DI, and the genes which showed significant correlation with choline level, the quadratic broken line model was applied for estimation of required level of choline[57]. Tank mean was used as the statistical unit. Effects on lipid classes and fatty acids in lipid classes were tested for significance by t test with individual fish as the statistical unit. All data are presented as mean values with their standard errors. 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 as indications of effects and mentioned as trends.

Results

Apparent nutrient digestibility and growth performance

Increasing choline inclusion did not affect apparent nutrient digestibility (AD) significantly for either protein, lipid or starch. The average AD was 90·5 (SEM 0·14) for protein, 98·0 (SEM 0·14) for lipid and 69·2 (SEM 0·95) for starch. No important relationships were observed between choline level and fatty acid digestibility (online Supplementary Table S2). Regression analysis did not reveal any significant relationship between choline inclusion level and growth (specific growth rate). However, a significant inverse relationship between CF and choline level was observed, but the R^2 for the model was very low (R^2 0·059, $P = 0{\cdot}008$; online Supplementary Table S2), and therefore, the results were not considered beneficial for the estimation of choline requirement. The mean values are presented in Table 3.

Organosomatic indices, pyloric tissue lipid content and histology

Among the OSI, those for PI and MI (OSI PI and OSI MI) decreased significantly with increasing choline levels. For the other OSI, that is, DI and liver, no significant relationship with increasing choline levels was observed (online Supplementary Table S2). Mean values are presented in Table 3. The choline (mg/kg) requirement level, estimated by a linear broken line model, was lower for OSI PI (3090 (SEM 212)) and OSI MI (2496 (SEM 538)) than estimated by the quadratic model which indicated average requirements of 3593 (SEM 226) and 3031 (SEM 195) mg/kg diet, respectively (Fig. 2).

Regression analysis revealed a significant inverse relationship also between choline level and the macroscopically observed degree of whiteness and histologically observed lipid vacuolation (online Supplementary Table S2). The results of the macroscopically and histologically examinations of the pyloric caeca showed an absence of symptoms at choline levels of 2850 and 2310 mg/kg, respectively (Figs. 3–5).

Concentration of TAG ($P = 0{\cdot}026$) and DAG ($P = 0{\cdot}039$) in the pyloric tissue, analysed only for fish fed the LF1 (1340 mg choline/kg) and LF6 (2850 mg choline/kg) diets, showed great and significant effects of choline supplementation (Fig. 6). Compared with the results for the LF1 diet, the concentrations of TAG and DAG decreased by 76 and 63 %, respectively, in fish fed the LF6 diet. No significant difference due to choline supplementation was found for NEFA or PL. Fatty acid profiles within the lipid classes TAG, DAG, NEFA and PL of the pyloric caeca tissue are presented in online Supplementary Table S3. Significant effects of choline supplementation are highlighted in Fig. 7. Choline affected a higher number of fatty acids in PL than in the other lipid classes.

Intestinal chyme DM and bile salt concentration

Increasing choline inclusion did not significantly affect the DM content of digesta nor the bile salt concentration along the intestine (Table 4 and online Supplementary Table S2).

Intestinal gene expression

The regression analysis results and the mean values of the results from the qPCR analyses of the pyloric tissue are presented in online Supplementary Tables S2 and S4, respectively. Fig. 8 illustrates that the expression of the genes showing a significant regression or a trend ($0{\cdot}05 < P > 0{\cdot}1$) and an estimated choline requirement are shown for *pcyt1a* which is involved in the phosphatidylcholine synthesis, for *apoAIV* and *apoAI*, involved in the

Table 3. Choline effects on specific growth rate (SGR), condition factor (CF) and organosomatic indices (OSI) (Mean values with their pooled standard errors)

	LF1	LF2	LF3	LF4	LF5	LF6	LF7	LF8	LF9	Pooled SEM
SGR (%/d)	0·97	0·91	0·93	0·99	1·01	0·84	0·96	0·94	0·95	0·03
CF	1·58	1·56	1·60	1·62	1·60	1·49	1·55	1·49	1·54	0·03
OSI pyloric intestine	2·31	2·15	2·09	1·85	1·75	1·82	1·57	1·68	1·68	0·090
OSI mid intestine	0·19	0·17	0·17	0·17	0·16	0·14	0·17	0·16	0·17	0·007
OSI distal intestine	0·42	0·46	0·49	0·43	0·49	0·45	0·47	0·52	0·44	0·021
OSI liver	1·15	1·28	1·20	1·18	1·23	1·24	1·23	1·31	1·11	0·057

British Journal of Nutrition

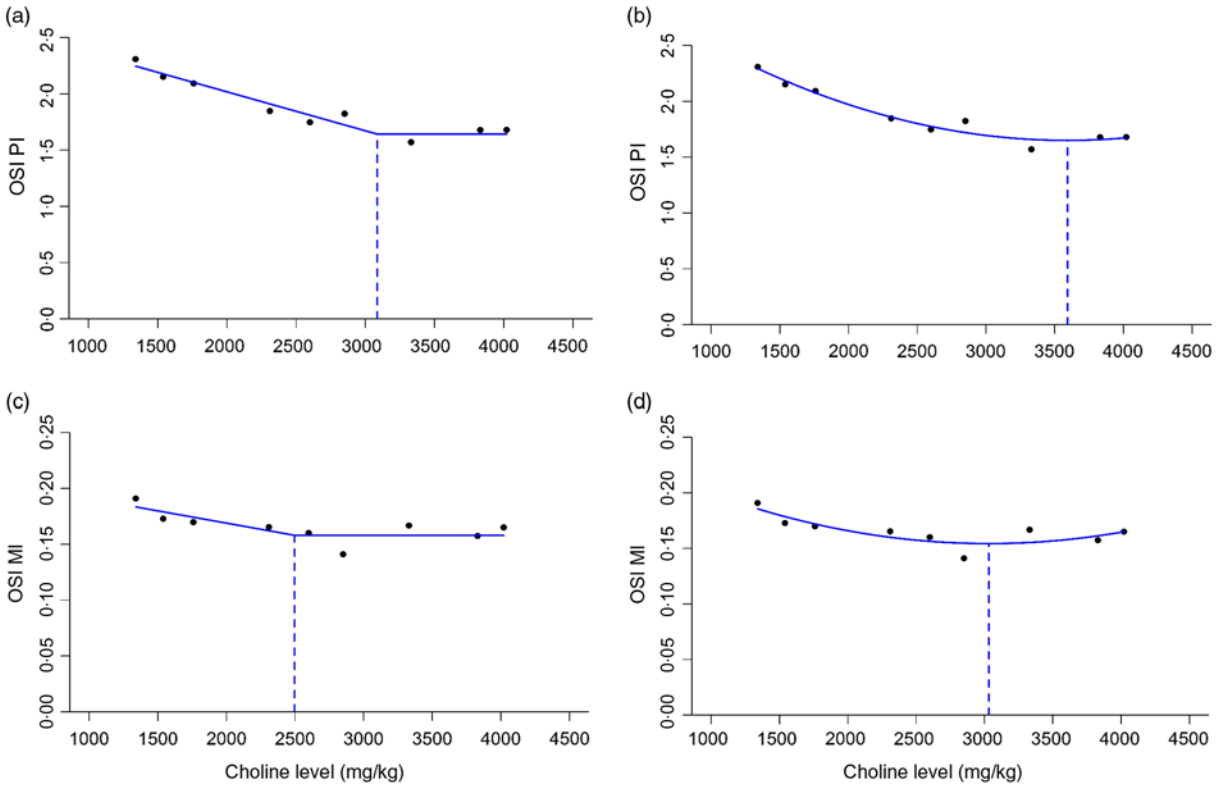

Fig. 2. Illustration of choline requirement (mg/kg) by broken line models with linear (a and c) and quadratic (b and d) portion of OSI PI (% of body weight) (a and b) and OSI MI (% of body weight) (c and d). OSI, organosomatic index; PI, pyloric intestine; MI, mid intestine.

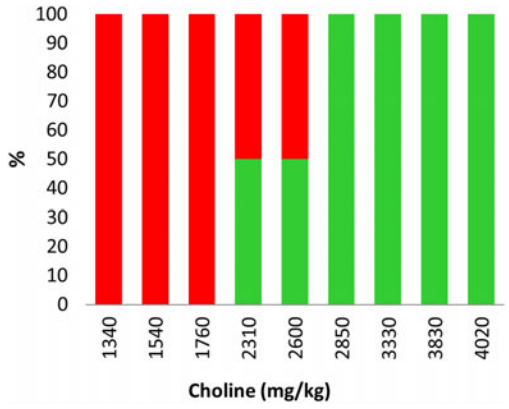

Fig. 3. Results of visual examination of the macroscopic appearance of whiteness of the pyloric intestine (*n* 8). ■, Normal; ■, pale pyloric intestine.

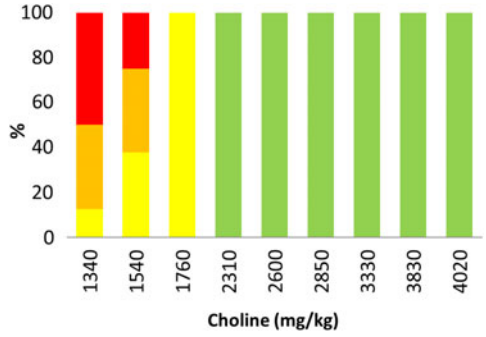

Fig. 4. Results of histological examination and evaluation of degree of vacuolation in the pyloric caeca (*n* 8). ■, Normal; ■, mild; ■, moderate; ■, marked.

lipoprotein assembly and *plin2*, the general marker for lipid load of non-adipogenic cells. Among three enzymes *chk*, *pcyt1a* and *pemt*, all involved in the pathway of phosphatidylcholine biosynthesis, *pcyt1a* decreased significantly and *chk* showed the same trend with increasing levels of choline, whereas *pemt* was not significantly affected. A significant reduction in expression by an increased level of choline was also observed for *mtp* and *fatp*, involved in lipid transport. Moreover, both *apoAIV* and *apoAI* showed a dose–response curve, increasing with increasing choline doses, levelling off at the highest inclusion level. A decreasing response was observed for *hmgcr*, an important enzyme in the regulation of the CH biosynthesis. The expression of *plin2* also decreased for then to reach a plateau at the highest choline inclusion levels. The *apoAIV* and *apoAI* revealed choline requirement levels not far from each other, 2593 (SEM 108) and 2610 (SEM 35) mg/kg, respectively. Similarities in choline requirement levels were also observed for *pcyt1a* and *plin2* (3210 (SEM 404) and 3199 (SEM 360) mg/kg, respectively; Fig. 8).

Blood plasma end points

The regression analysis revealed a significant positive relationship between choline level and LDL-cholesterol, and a similar trend was observed for total plasma CH levels (online Supplementary Table S2). Blood plasma mean values are presented in Table 5. The HDL contained most of the plasma

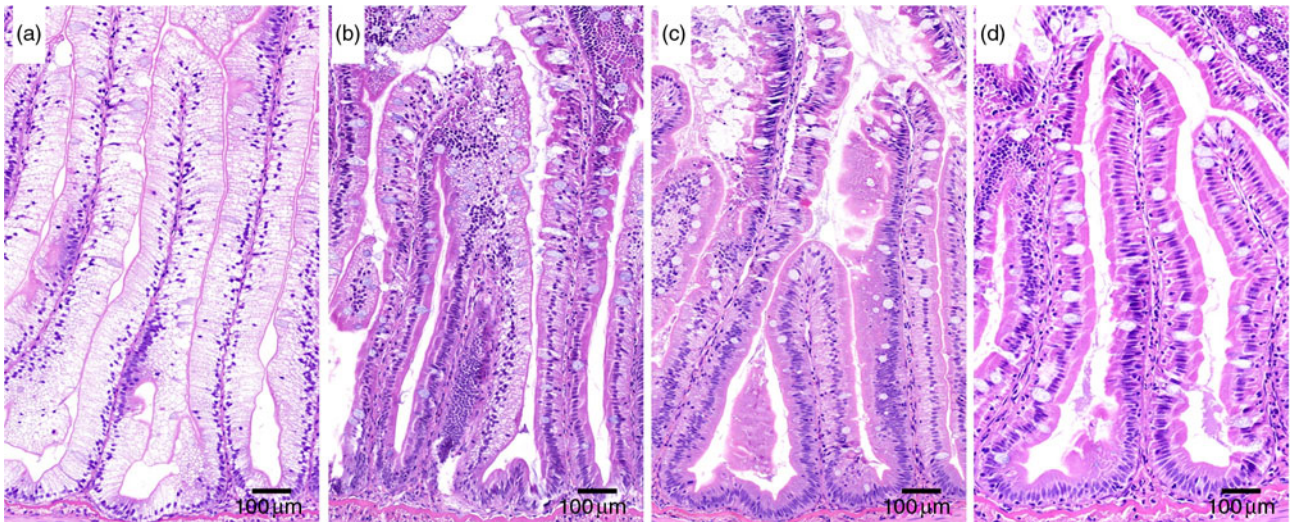

Fig. 5. Representative images of histological appearance of lipid in pyloric caeca in fish fed diets with various levels (mg/kg) of choline: (a) 1340, (b) 1540, (c) 1760 and (d) 2310.

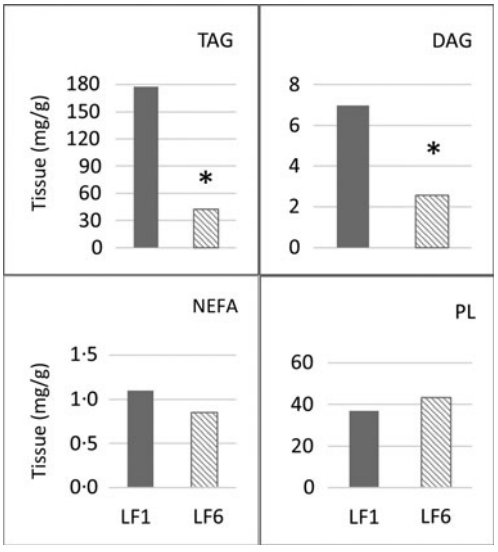

Fig. 6. Concentration (mg/g tissue) of TAG, diacylglycerol (DAG), NEFA and phospholipids (PL) in pyloric tissue from fish fed diets with 1340 mg/kg choline (LF1, *n* 4) and 2850 mg/kg choline chloride (LF6, *n* 4). Monoacylglycerol did not show measurable concentrations in the tissue. * Concentrations of TAG and DAG differed significantly ($P = 0{\cdot}026$ and $P = 0{\cdot}039$, respectively). For NEFA and PL, the differences were not significant ($P = 0{\cdot}328$ and $P = 0{\cdot}253$, respectively) (data log transformed).

CH, and there was a trend towards increasing levels with increased dietary choline levels. Lathosterol is a steroid intermediate in the CH synthesis, and the circulating level reflects CH synthesis in the liver. The circulating lathosterol levels were significantly and positively correlated with dietary choline level (online Supplementary Table S2). For the other blood plasma values, presented in online Supplementary Table S2, no significant relationship with increasing choline levels was observed.

Estimates of choline requirement level

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

Discussion

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

Estimation of choline requirement

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

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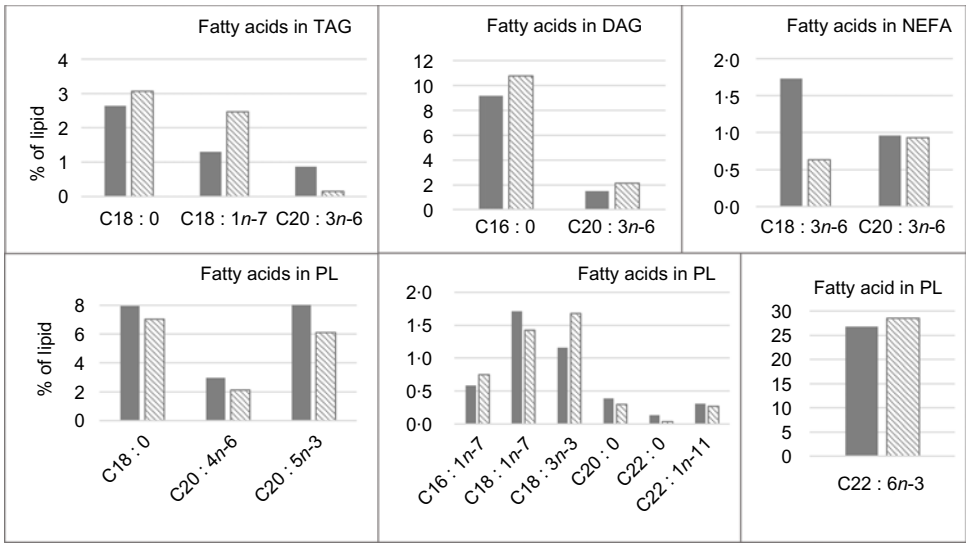

Fig. 7. Fatty acids (% of lipid) in TAG, diacylglycerol (DAG), NEFA and phospholipids (PL) of pyloric caeca tissue showing significant ($P < 0{\cdot}05$, data log transformed) effects of choline supplementation, that is, comparing samples from fish fed the diets LF1 (n 4) and LF 6 (n 4). Monoacylglycerol did not show measurable levels. ■, LF1; ▨, LF6.

Table 4. Intestinal DM and bile salt levels in digesta of Atlantic salmon (Mean values with their pooled standard errors)

	LF1	LF2	LF3	LF4	LF5	LF6	LF7	LF8	LF9	Pooled SEM
DM (%)										
PI1	13	13	11	14	14	16	13	12	15	0·92
PI2	14	15	15	16	17	16	16	14	17	0·82
MI	15	16	16	17	16	16	16	15	17	0·48
DI1	15	14	14	15	14	15	14	14	15	0·54
DI2	14	12	13	14	13	13	13	13	14	0·56
Bile salt (mg/g DM)										
PI1	110	110	110	93	114	109	105	137	123	4·0
PI2	89	88	88	85	111	104	97	93	93	2·8
MI	125	101	107	110	115	121	136	132	117	3·8
DI1	95	105	90	80	105	101	95	121	93	3·8
DI2	27	15	13	42	20	22	37	18	17	3·3

PI, pyloric intestine; MI, mid intestine; DI, distal intestine.

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

The mean requirements indicated by the biomarkers differed. Running requirement studies with fish differ from studies involving terrestrial animals since the experiments are carried out in water and this requires special considerations related to controlling the actual feed intake. The book, *Nutrient Requirements of Fish and Shrimp*[6], provides guidance on methodology and data analysis for nutrient requirement studies. There is no general agreement regarding which biomarkers give the most relevant estimates for requirements, but it is generally agreed upon that the biomarker should functionally have a close relationship with the nutrient in question and show a clear dose–response relationship with intake at levels below requirement. Although the OSI PI indicated the highest choline requirement, it may not be appropriate as a biomarker to be used for the requirement estimation. The PI, as with the other parts of the intestine, has the ability to enlarge and diminish according to needs as a natural adaptation to variation in nutritional quality of the diet[61]. The histologically observed degree of vacuolation and the macroscopically observed appearance of whiteness may be biomarkers more closely related to choline requirement and may be more relevant biomarkers, indicating overall lipid transport processes, and may therefore be the biomarkers best suited

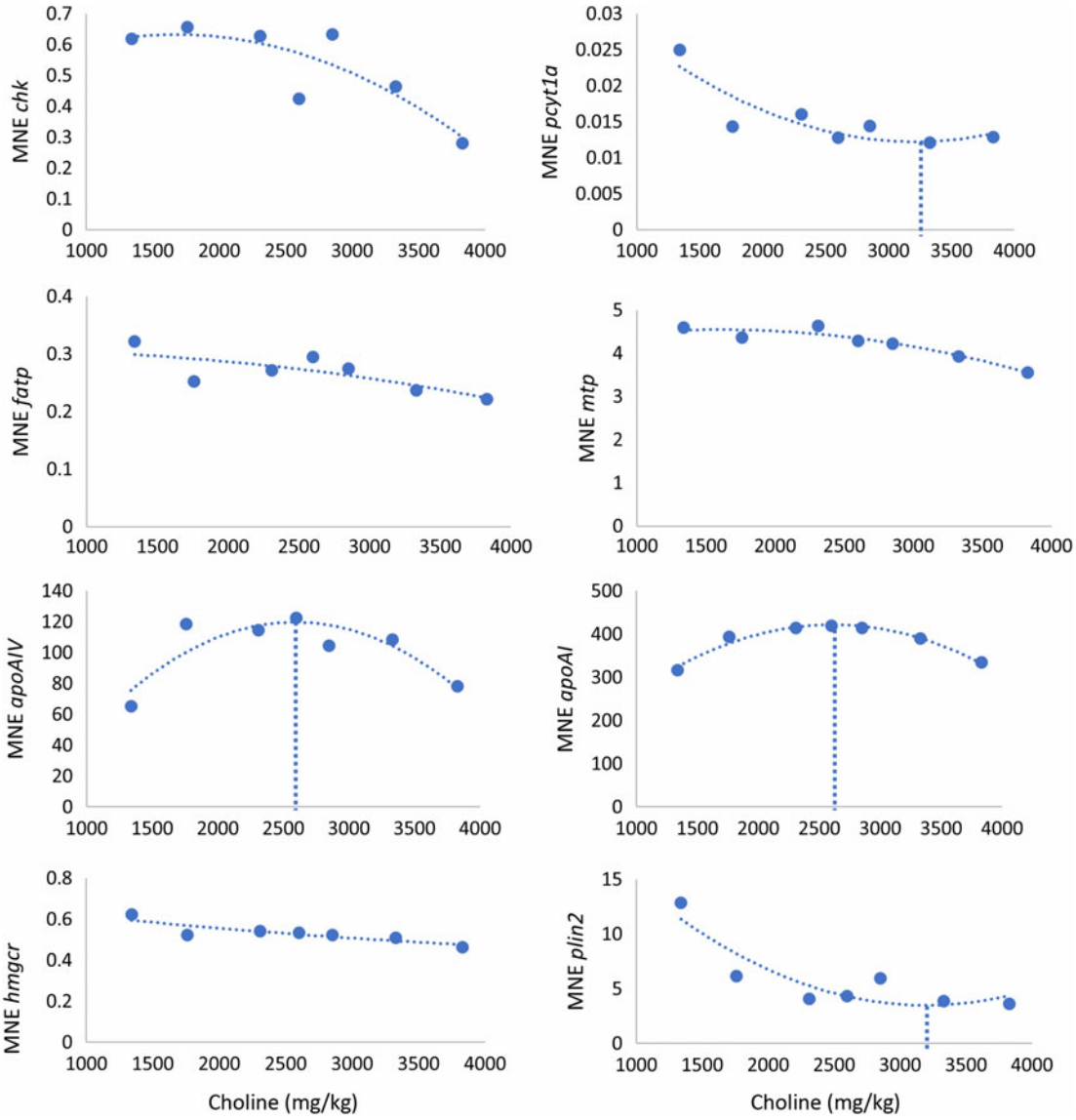

Fig. 8. Illustration of relationships between dietary choline concentration levels (mg/kg) and mean normalised expression (MNE) of *chk*, *pcyt1a*, *fatp*, *mtp*, *apoAIV*, *apoAI*, *hmgcr* and *plin2* in pyloric caeca tissue from fish fed the diets LF1 and LF3–8 (*n* 5). The curves illustrate the regression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

Table 5. Blood plasma variables (*n* 8 fish per diet)
(Mean values with their pooled standard errors)

	LF1	LF2	LF3	LF4	LF5	LF6	LF7	LF8	LF9	Pooled SEM
NEFA (mmol/l)	0·24	0·22	0·20	0·26	0·22	0·20	0·20	0·22	0·26	0·01
Total cholesterol (mmol/l)	8·89	9·97	11·1	10·7	10·6	10·7	10·0	12·3	12·1	0·35
HDL-cholesterol	7·88	8·29	9·73	9·25	8·92	8·66	8·20	10·55	10·17	0·31
LDL-cholesterol	0·72	1·19	1·08	1·07	1·29	1·39	1·36	1·16	1·53	0·08
VLDL-cholesterol	0·28	0·49	0·29	0·40	0·42	0·64	0·48	0·60	0·40	0·04
Total TAG (mmol/l)	2·04	2·41	1·99	1·78	2·13	1·92	1·51	2·12	2·07	0·08
HDL-TAG	1·21	1·68	1·51	1·34	1·15	1·32	1·09	1·53	1·13	0·07
LDL-TAG	0·35	0·34	0·28	0·22	0·37	0·34	0·27	0·20	0·46	0·03
VLDL-TAG	0·48	0·38	0·20	0·22	0·61	0·27	0·15	0·38	0·49	0·05
C4 (nmol/l)	16·9				38·6				37,3	
Sitosterol (μg/ml)	69·5	58·4	71·4	69·1	65·8	69·4	55·0	82·8	69·9	2·65
Campesterol (μg/ml)	129	116	149	143	147	140	104	171	151	6·71
Lathosterol (μg/ml)	0·80	0·86	0·94	1·47	1·54	1·69	1·75	1·13	1·78	0·13

C4, 7α-hydroxy-4-cholesten-3-one.

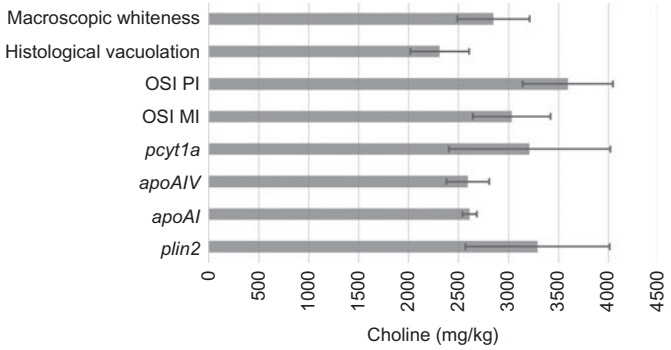

Fig. 9. Choline requirement level as indicated by biomarkers of various indicators of lipid assembly, storage and transport. Horizontal lines (⟷) indicate estimated 95 % confidence range (estimated mean ± 2SEM) for optimum choline level for each biomarker. OSI, organosomatic index; PI, pyloric intestine; MI, mid intestine.

for the estimation of choline requirement. The PI whiteness gave the highest estimate of choline requirement of the two, that is, 2850 mg/kg for whiteness and 2310 mg/kg for hypervacuolation. The difference between the two may be related to the fact that macroscopic examination summarises characteristics of the whole PI tissue, whereas the histological examination observes a very limited area of a small sample of the pyloric caeca.

Regarding the four gene expression biomarkers suitable for requirement estimation, *apoAIV* (2592 mg/kg) and *apoAI* (2610 mg/kg) suggest average choline requirements between those of whiteness (2850 mg/kg) and hypervacuolation (2310 mg/kg), whereas *pcyt1a* (3210 mg/kg), as well as *plin2* (3199 mg/kg), suggests higher requirements. The 95 % confidence range for the biomarker macroscopic whiteness overlaps with all the ranges for the other selected biomarkers for choline requirement, making it reasonable to suggest the average of the choline requirements for the biomarkers as the estimate for the present study, that is, 2936 mg/kg. It should be a goal for an optimal diet to cover the needs of 95 % of the fish, that is, 2× SEM should be added to the mean. In the present experiment, the recommended choline level, for prevention of LMS, is estimated to be 3350 mg/kg or 3·4 g/kg (Fig. 9).

With a key role in lipid transport and metabolism, it is likely that choline requirement depends on dietary lipid level and feed intake, which we may call lipid load. In the present study, the diets contained 29 % lipid, and the average specific growth rate was 0·94 % (thermal growth coefficient: 2·7). At higher growth rates, higher feed intakes and/or higher dietary lipid levels, higher choline level may be required. Further studies are needed for the characterisation of these relationships. The natural prey of wild Atlantic salmon can have a very high lipid content. Depending on the season, the lipid content in a herring from the North Sea can be as high as 50 % on a DM basis (25 % as is). There is a general lack of knowledge regarding the degree of lipid vacuolation in the intestines in a wild salmon during optimal periods where access to different foodstuff is high.

Dietary choline level effects on intestinal lipid transport

The present study showed a clear decreasing effect of choline level on the somatic index of PI, macroscopically observed degree of whiteness and histologically observed lipid vacuolation in PI. In the most serious cases of vacuolation, observed for the lowest levels of choline, the macroscopic whiteness continued from the PI and further down into the MI. Previous studies have shown that Atlantic salmon can use both PI and MI for absorption of long-chain fatty acids, for example, C18 : 1[60]. It seems possible that our observation of lipid vacuolation in the MI could be related to periods when the fish experience a lipid load higher than the capacity of the PI. These results substantiate that dietary choline increases the capacity of the PI to absorb and transport the lipid from the enterocytes[20–22,28,29]. The observed lower level of TAG in the fish given the diet with 2850 mg/kg choline compared with the un-supplemented diet with 1340 mg/kg, in combination with the absence of lipid accumulation and no significant differences in lipid digestibility, indicated an increased flux of TAG across the tissue from the gut lumen to the portal vein, as a result of choline supplementation[62]. This conclusion is consistent with other studies observing that dietary phosphatidylcholine prevents accumulation of TAG in the intestinal mucosa[21,23]. Our results also support the general understanding that TAG is the primary lipid class in lipid stores[29], also in the intestinal mucosa, in the form of lipid droplets in the enterocytes[21,28,63,64].

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

For further transport to the systemic circulation and to the peripheral tissues from the epithelial cells, the TAG must be incorporated into lipoproteins[63]. This lipoprotein production involves a series of biosynthetic processes, whereby the large hydrophobic core of the lipoprotein, containing TAG, CH esters, lipid soluble vitamins and other highly lipophilic compounds, is covered by a thin coat in which phosphatidylcholine plays an important role, in addition to apo and free CH[65,66]. Our observations of dietary choline increasing the flux of TAG across the epithelial cells and preventing accumulation of TAG in the epithelial mucosa were further supported by concomitant expression changes in genes involved in several processes of the lipid turnover. The decreased expression of *mtp*, coding for transporters facilitating the transport of TAG

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

Dietary choline level effects on plasma indicators

For the plasma indicators, the observed positive relationship between lathosterol and LDL-cholesterol and choline level indicates that choline increased the level of CH synthesis in the liver[69] and accelerated the transport of CH from the liver to peripheral tissue, seen as an increase in the LDL-cholesterol[70,71]. Similar responses to choline supplementation were also observed in our previous study[3]. Most likely, the increased plasma CH levels were also a result of the increasing capacity of the PI to assemble and transport lipoproteins, which are also responsible for the transport of CH from the intestine to the liver[66].

Dietary choline level effects on performance

The overall growth rate in the present choline dose–response study was within the range observed in fish both under experimental and commercial conditions. However, there were no apparent indications of reduced performance as a result of choline dose nor LMS, for which no significant differences on growth nor macronutrient digestibilities were observed. The present observation of choline not affecting growth is in agreement with some previous studies on adult Atlantic salmon[72], channel catfish[7] and giant grouper[73]. However, in our previous study with large Atlantic salmon[3] and in several other studies with juveniles from different species[5,10,13,74–77], increased growth with choline supplementation has been observed. The relationship between feed intake, growth and choline requirement should be addressed in future studies.

Conclusion

The results of the present study indicate that a dietary choline level of 3·4 g/kg is required for prevention of LMS and intestinal lipid hypervacuolation in 95 % of the post-smolt Atlantic salmon kept under similar conditions as in the present study. Higher levels may be required at higher feed intakes and/or higher lipid levels in the diet.

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

Co-author A. K. G. H. is employed by BioMar.

Supplementary material

For supplementary materials referred to in this article, please visit https://doi.org/10.1017/S0007114520000434

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