Aquaculture Nutrition

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Journal:	*Aquaculture Nutrition*
Manuscript ID	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Espe, Marit; NIFES, Aquaculture nutrition Andersen, Synne Veiseth-Kent, Eva; Nofima, Rønnestad, Ivar; University of Bergen, Holen, Elisabeth; NIFES, Zerrahn, Jens-Erik; Evonik Degussa, Aksnes, Anders; Ewos Innovation,
Keywords:	Fish < Aquatic Animals, Atlantic Salmon < Fish < Aquatic Animals, Amino Acids < Nutrients < Feed, Methionine < Nutrients < Feed, Requirement < Metabolism, Biochemical < Analyses

Choline supplementation increased total body lipid gain, while surplus methionine improved growth and amino acid accretion in adult Atlantic salmon (*Salmo salar*)

Espe M[1], Andersen SM[1,2], Veiset-Kent E[3], Rønnestad I[4], Holen E[1], Zerrahn J-E[5], Aksnes A[6]

[1]National Institute of Nutrition and Sea Food Research (NIFES), PO Box 2029, N-5817, Nordnes, Norway

[2]Current address: Ewos AS, Tollbodalmenningen 1b, N-5004, Bergen Norway

[3]Nofima, PO Box 210 N-1431, Ås, Norway

[4]Institute of Biology, University of Bergen, PO Box 7803, N-5020, Bergen, Norway

[5]Evonik Degussa International AG, Havneparken 2, Vejle DK-7100, Denmark

[6]Ewos Innovation AS, N-4335, Dirdal, Norway

Corresponding author: Marit Espe

e-mail address: marit.espe@nifes.no

Keywords: methionine, protein retention, choline, Atlantic salmon

Running title: Surplus methionine improves protein growth, but choline did not affect TAG transport from liver to muscle

Abbreviations:

PC	phosphatidylcholine
pemt	phospatidylethanolamine methyl transferase
PEA	phophatidylethanolamine
SAM	S adenosyl methionine
SAH	S adenosyl homocysteine
ApoB100	ApoLipoProteinB100
MAT	methionine adenine transferase
BHMT	betaine homocysteine methyl transferase
CBS	cystathionine beta synthase
CDO	cysteine dioxygenase

Abstract

Methionine choline deficient mammals are known to accumulate liver TAG probably due to PC deficiency and thus assembly of VLDL and transport of lipids from liver to peripheral organs. To assess whether supplementation of choline could spare methionine in diets almost adequate to secure a healthy liver metabolism, by reducing the endogenous phosphatidylcholine (PC) synthesis without interfering with lipid transport and distribution, Atlantic salmon with initial BW of 700g were fed adequate (1.9g Met/16gN) or surplus methionine (2.5g Met/16gN) diets of which were supplemented with choline or not for a period of 19 weeks. Fish fed the lower methionine diets had reduced growth ($p=0.013$) due to reduced protein gain ($p=0.007$), while lipid gain slightly improved in fish fed the choline supplemented diets ($p=0.047$). Also feed conversation improved when fed surplus methionine ($p<0.001$), while choline supplementation had no impact on feed conversation. No interaction between choline and methionine on growth performance or accretion existed. Phospholipid status in liver and muscle was not affected by treatments and no liver TAG accumulation occurred at the methionine levels used. Gene expression of *ApoB100* necessary for assembling VLDL or *pemt* necessary for endogenous PC synthesis were un-affected by treatments. Capacity of methylation within the liver was not affected by treatment nor was the gene expression of enzymes in liver sulfur metabolism (*MAT*, *BHMT*, *CBS* or *CDO*). Methionine status within liver was unaffected by treatments while free methionine reduced in those fish fed the lower methionine diets in muscle and plasma. Choline supplementation had no impact on sulfur amino acid metabolites in either tissues. Neither did choline supplementation improve TAG mobilization from liver to muscle as analyzed by *ApoB100* necessary for assembling VLDL. To conclude choline does not improve endogenous phospholipid synthesis or transport of TAG from liver to muscle depot when added to diets containing 1.9gMet/16gN, while surplus methionine (2.5g Met/16gN) improved growth and protein accretion, indicating that 1.9g Met/16gN is enough to support a healthy liver metabolism, but too low to support muscle protein deposition in adult salmon fed high plant protein diets for longer periods of time.

1.0 Introduction

Liver lipid accumulation is associated with increased metabolic stress, energy depletion, cytokine activation and inflammation in rodent models and human beings (Vanni et al. 2010; Vernon et al. 2011, Koek et al. 2011, Rolo et al. 2012). It is known that methionine and especially methionine choline deficient diets (MCDD) increase the liver TAG in rodent models (Chawla et al. 1988, Slow & Garrow 2006). Likewise methionine choline deficiency has been reported to increase liver TAG in Atlantic salmon (Rumsey et al. 1983, Espe et al. 2010). Supplementation of methyl group donors (betaine, SAM, folate) could not prevent apoptotic death in choline deficient hepatocytes (Shin et al. 1997). Choline is part of the phospholipid phosphatidylcholine (PC) of which is abundant in liver. PC is synthesized by two metabolic pathways within the liver either through the Kennedy pathway or through the pemt-pathway (Vance et al. 1997, Watkins et al. 2003). It has been reported that mammalian species have the capacity to synthesize almost 40% of the required choline endogenously by a three step methylation of phosphatidylethanolamine (PEA) through the enzyme phosphatidylethanolamine methyl transferase (pemt). During severe choline deficiency in rats the gene expression of *pemt* increased (Cui & Vance 1996). The endogenous choline synthesis requires three molecules of S-adenosylmethionine (SAM) as methyl donors for each choline molecule to be synthesized (Vance & Ridgeway 1988, Mato et al. 2002, Noga & Vance 2003, Stead et al. 2006). The precursors for SAM are methionine and ATP, and SAM concentration within the liver of Atlantic salmon is known to depend on methionine intake (Espe et al. 2008, 2010) as also is true in rodents (Sugiyama et al. 1998). For a healthy liver sulfur metabolism without any TAG accumulation, Atlantic salmon fed high plant protein diets requires about 2.2g Met/16gN (Espe et al. 2008, 2010). It is believed that liver TAG accumulation is due to reduced availability of PC and apolipoproteinB100 (ApoB100) of which is needed to assembly the very low density lipoproteins (VLDL) and thus transport of TAG from the liver to peripheral organs like the muscle (Vance et al. 1997, Watkins et al. 2003).

In fish methionine choline interactions have been less studied. It was reported that red drum fed choline supplemented diets increased muscle lipids and concomitantly reduced their liver lipids (Craig & Gatlin, 1997), while cobia increased muscle and liver choline concentrations as well as increased total lipid in the muscle following choline supplementation (Mai et al. 2009). In both of these studies, dietary methionine was equal in the diets used and present at requirement. Choline supplementation to diets containing low sulfur amino acids (1.6g

TSAA/16g N) on the other hand, improved growth performance in tilapia (Kasper et al. 2000). Juvenile Atlantic salmon fed low methionine diets (1.6g Met/16gN) of which was supplemented with choline only showed a tendency of increased muscle choline and TAG following choline supplementation, while liver choline and TAG were unaffected by treatments (Espe et al. 2015b). This may be due to the relative short period of feeding (8 weeks) and/or the fact that juveniles are fed less lipids and therefore incorporate less lipids in muscle as compared to adult salmon. Neither was gene expression of either *ApoB100* or *pemt* affected by choline supplementation in the juvenile salmon (Espe et al. 2015b). Even though the juvenile Atlantic salmon fed the low methionine diets did not accumulate TAG they increased the concentration of PC in liver and white trunk muscle when the low methionine diet was supplemented with choline implying that choline may be beneficial on the phospholipid status in fish fed the low methionine diets. Therefore the current study aimed to test if adult Atlantic salmon, known to accumulate more lipid in muscle and liver as compared to the juveniles, fed diets containing methionine at approximately the requirement of adult salmon to attenuate liver TAG accumulation (1.9g Met/16gN, requirement 2.2g Met/16gN, Espe et al. 2008; 2010) would benefit from choline supplementation and possibly spare liver methionine to be used for other methylation reactions. For comparison diets surplus in methionine (2.5g Met/16gN) also were tested for any benefits of choline supplementation on muscle protein and lipid gain.

2.0 Material and Methods

2.1 Experimental diets

Four experimental diets were prepared being slightly deficient in methionine to attenuate liver TAG accumulation (Espe et al. 2008; 2010) or added DL-methionine to a concentration well above established requirement (NRC 2011). Diets were mainly based on soy and pea protein concentrates and contained 1.9 or 2.5 g Met/16gN, respectively. The low and high methionine diets were either added 2.8g choline chloride/kg diet or not added any to give choline concentrations of approximately 1 and 3g choline/kg diet in the un-supplemented and supplemented diets, respectively. Diets were produced at the facilities at EWOS Innovation AS, Dirdal, Norway. All diets were extruded and a pellet size of 6 mm was produced. Dietary composition and chemical analyses of the diets are given in Tables 1 and 2.

2.2 Fish experiment

The feeding trial was carried out the facilities of EWOS Innovation AS located in Dirdal, Norway. The diets were randomly assigned to triplicate tanks, each containing 40 fish with an initial BW of 729±5 g. Each tank was supplied with running seawater (salinity 33 g L^{-1}) with mean temperature 8.1±0.2°C at a flow rate of 1.5 L kg^{-1} biomass min^{-1}. A 24 hour constant light regime was applied to maximize the growth potential. The fish were fed three times daily using belt feeders and collection of uneaten feed as described (Espe et al. 2006) during a period of 19 weeks. Individual weights and lengths were recorded at the start and end of the experimental period. BW, length, liver and gastro-intestinal weights were recorded from 10 fish from each tank at the end of the experiment to allow calculation of representative tank mean condition factor, hepatosomatic and viscerosomatic indexes. Further, 10 fish were collected at the start of the experimental period and 10 fish per tank were collected at the end and analyzed for protein, fat and amino acids to allow calculation of mean tank accretion of proteins, lipids and amino acid retention. Liver, head kidney and muscle were sampled individually from 6 fish from each tank 5 hours post-prandial, flash frozen in liquid N_2 and stored at -80°C until analyzed for gene expression. Heparinized blood was drawn from the caudal vein of 10 fish per tank, pooled to one sample per tank before stored at -80°C until analyzed for N-metabolites. The body weight of all fish were measured and included in calculation of mean tank growth performance. Before handling fish were anaesthetized with chlorobutanol (0.4 g L^{-1}). The experiments complied with the guidelines of the Norwegian Regulation on Animal Experimentation and European Community Directive 86/609/EEC.

2.3 Chemical analyses

Diets were analyzed for crude composition of protein, lipids, ash and energy as described (Espe et al. 2006). Dietary amino acid composition was analyzed after hydrolyzation for 22h in 6M HCl using the UPLC as described (Espe et al., 2014). Free amino acids were analyzed in de-proteinized plasma, liver and muscle samples using the Biochrome (30+ Biochrome, UK) with post column derivatization with ninhydrin as described (Espe et al. 2006). Samples of liver, muscle and feed were extracted in 4 volumes 10mM TRIS buffer (pH 7.6) as described (Espe et al. 2014) and stored at -80°C until de-proteinized and analyzed for choline. Choline was analyzed by a commercial kit (BioVision Mountain View, CA, USA, #K615-100) following the instructions given by the supplier. The lipid classes (TAG, phosphatidylethanolamine (PE), PC, total phospholipids (PL), non-esterified fatty acids (NEFA), total cholesterol) in liver and muscle were analysed

after lipid extraction with 2:1 chloroform: methanol (v:v) as described (Bell et al. 1993, Liaset et al. 2003). Plasma total phospholipids, TAG, total cholesterol, NEFA and total bile acids were analyzed as described (Espe et al. 2010) using commercial kits (Diagnostic Laboratories).

2.4 RNA extraction and gene expression

mRNA was extracted from liver, muscle and head kidney of 6 fish per tank using the EZ1 BioRobot and the RNA Universal Tissue Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. The quality and quantity of the RNA was assessed using NanoDrop ND-1000 UV and Agilent 2100Bioanalyzer and RNA integrity assessed using the RNA 6000Nano LabChip® kit. A two-step real time qPCR assay was run as described (Torstensen et al. 2011). The PCR primer sequences used were delivered by Invitrogen using the primers acidic ribosomal protein (ARP) and elongation factor 1A (EF1A, Hevrøy et al. 2007), Apo lipoprotein 100B (ApoB100, Torstensen et al. 2011), carnitine palmitoyl transferase1 (CPT-1, Kennedy et al. 2006), fatty acid synthase (FAS, Castro et al. 2013), pemt1 (forward: GCCTAGGCACCCTCATCATC reverse: AGGTCCCAGTGAATCCGAGA). Primers for liver sulfur metabolic enzymes (methionine adenosine tyransferase (MAT) forward: GAAACAGGACCCAGATGCCA reverse: ATCTCTCCACACAGCAGCAC, Betaine homocysteine methyl transferase (BHMT) forward: ATCAGGGCTGTAGCTGAGGA reverse: CATGGAGGGACACTTGGGAC, Cystathionine beta synthetase (CBS) forward: TCGGCCTCAAGTGTGAAGTC reverse: TGGTTTCAGATGTCCTGCCC and taurine production through cysteine dioxygenase (CDO) forward: GGAACCTGGTGGATGAAGGG reverse: CAGTGGGAGTCTGTGTGGTC). In addition markers for apoptosis and mitochondrial myogenesis (caspase-3, P-38 Mitogen activating phosphokinase (p38MAPK) and PPARγ coactivator 1a (PGC1α), Holen et al. 2014, Castro et al. 2013) were addressed in the liver. Pro-inflammatory interleukins and cytokines (IL-1b, IL-6, IL-8, TNFα, Holen et al. 2014) were analyzed in head kidneys. While muscle was analyzed for markers for ubiquination (muscle atrophy Fbox (Mafbx), ring finger proteins 1b and 1, (Murf1b and Murf1, Bower et al. 2009) and anabolic markers (Myosin light chain 2 (MLC2, Bower et al. 2008) and insulin growth factor-1 (IGF-1, Hevrøy et al. 2006) and mammalian target of rapamycin (mTOR Olsvik et al. 2013). Normalized gene expression was calculated using the two reference genes, ARP and EF1A, as described and verified by Olsvik and co-workers (2005).

2.5 Statistical analyses

All results are reported as the tank means±SE (n=3) and all tank values were based on pooled samples of 10 fish with the exception of gene expression data that was analyzed individually in 6 fish and the tank mean values calculated after analyzed. According to the design a two way ANOVA followed by Tukeys test was used to assess any differences by treatments (dietary methionine*choline supplementation). P-values being less than 0.05 were accepted as statistically different. Levenes test was used to assess homogeneity in variation. Statistical analyses were done using the statistical program Statistica (Stat. Inc. Version 12.0).

3.0 Results

3.1 Growth performance

The growth performance and accretion is listed in Table 3. Fish fed the near lower methionine diets had lower specific growth rate than did those fish fed the methionine surplus diets (p=0.013), but choline supplementation had no impact on growth performance. This was supported with an increased amino acid retention following surplus methionine. Likewise, the fish fed the low methionine diets had worse feed conversion (p<0.001), but choline supplementation had no impact on feed conversion. Mean lipid gain on the other hand was slightly higher in fish fed the diets supplemented with choline (p=0.047), but the dietary methionine inclusion had no impact on lipid gain. Protein gain was reduced in the fish fed the low methionine diets (p=0.007), but supplementation with choline had no effect on protein gain. No interactions between choline and methionine were present in growth performance, feed utilization or accretion. The reduced protein gain was supported with the retention of total methionine in whole body, being high for methionine in fish fed the lower methionine diet and lower in those fed the high methionine diets, while retentions of other amino acids being opposite (Table 3).

3.2 Phospholipids in plasma, liver and muscle

Phospholipids, TAG, NEFA, total cholesterol and total bile acids in plasma, liver and white trunk muscle are listed in Table 4. Plasma total phospholipids decreased by surplus methionine supplementation (p=0.032) and increased following choline supplementation (p<0.001). Likewise

total cholesterol decreased following surplus methionine supplementation (p=0.004) and increased following choline supplementation (p=0.002). Total plasma bile acids increased with methionine supplementation (p=0.026) while choline supplementation had no effect on plasma bile acids. Neither TAG nor NEFA were affected by treatments. There were no interactions between methionine and choline supplementation in plasma lipid classes or total bile acids. Liver lipid classes was not affected by treatments. Neither were white trunk phospholipids, cholesterol or TAG affected by treatments.

3.3 Non protein nitrogen compounds in plasma, liver and muscle

Non protein nitrogen metabolites in plasma liver and muscle as affected by treatments are listed in Table 5. Plasma free methionine (p=0.004), cysteine (p=0.05), cystathionine (p=0.004) and taurine (p=0.016) all increased following surplus methionine supplementation. Plasma PEA was not affected by treatments, while ethanolamine tended to increase (p=0.05) following methionine supplementation. In liver neither choline nor free methionine was affected by treatment, but liver taurine (p=0.009) and cystathionine (p=0.01) increased by surplus methionine. Neither liver PEA nor ethanolamine were affected by treatments. Liver SAM and SAH were un-affected by treatments. There were no interactions between methionine and choline supplementation on non-protein nitrogen compounds in the liver.

Muscle choline increased (p<0.001) following choline supplementation, while muscle free methionine (p=0.01) and taurine (p=0.02) increased following surplus methionine supplementation, but there were no interactions between choline and methionine supplementation. Muscle cysteine, cystathionine and ethanolamine were below detectable limits, and there was no treatments effect on PEA. There were no interactions between methionine and choline on muscle non protein nitrogen components.

3.4 Gene expression in liver

To assess endogenous choline synthesis and assembly of VLDL in liver as affected by treatment gene expression of *pemt* and *ApoB100*, respectively were analyzed. Even though mean *pemt* expression increased in fish fed the lower methionine diets not added any choline, the differences were not significant. Neither was *ApoB100* expression different between treatments (results not shown). To assess possible differences in lipid metabolism liver *FAS* and *CPT-1* expression were analyzed for lipogenesis or lipolysis, but none of these were different between treatments (not shown). To

cheque for the liver health status and mitochondrial myogenesis, the gene expression of caspase-3, p38MAPK and PGC1a were analyzed, however the treatments had no impact on these genes (not shown). Further, were the gene expression of enzymes associated with SAM synthesis from methionine and its transmethylation addressed (i.e. *MAT, BHMT*) together with cystathionine synthase expression (*CBS*) and taurine synthesis (*CDO*) but none of these were affected by treatments (not shown).

3.5 Gene expression in muscle

Genes associated with muscle protein synthesis and accretion (*mTOR*, *IGF-1*, *MHC*, *MLC2*) or some marker genes for protein degradation through ubiquination (*Murf1*, *mafbx-a*, *Murf1b*) were all unaffected by treatments. Only a slight tendency towards increased expression of *Murf1* was present in muscle of fish fed the lower methionine diets (p=0.06, Figure 1).

3.6 Gene expression in head kidneys

To address whether choline supplementation to lower or surplus methionine diets might be beneficial on inflammation, head kidneys were analyzed for pro-inflammatory interleukins (*IL-1b, IL-6, IL-8*) and cytokine (*TNFα*), but neither of these pro-inflammatory marker genes were affected by treatments (not shown).

4.0 Discussion

We previously showed that 1.6g Met/16g N were less than required for a healthy liver sulfur metabolism in juvenile salmon and resulted in reallocation of free methionine from muscle stores to liver keeping the liver sulfur amino acid metabolism similar to those fed adequate methionine (Espe et al. 2014; 2015a), while adult Atlantic salmon required about 2.2g Met/16g N to secure a healthy liver sulfur metabolism without TAG accumulation (Espe et al. 2008; 2010). The current study thus should be regarded as containing slightly suboptimal or surplus methionine as the methionine concentrations used were 1.9 and 2.5g Met/16gN (corresponding to 7.8 and 9.8g methionine per kilo diet, Table 2).The current study aimed to test whether supplementation of choline to suboptimal methionine diets fed to adult Atlantic salmon would spare methionine to be utilized for other metabolic pathways within liver and or affect transport of liver TAG to peripheral organ as the white trunk muscle preventing development of a fatty liver. Further, to test whether slightly suboptimal

methionine diets would decrease protein and amino acid retention in fish as indicated in the juvenile salmon (i.e. muscle protein gain). In that context it was interesting that surplus methionine increased growth due to increased protein accretion, and increased body amino acid retention as compared to those fed the slightly suboptimal methionine diets. Previously when the juvenile Atlantic salmon were fed low methionine diets (1.6g Met/16gN) or adequate (2.2g Met/16gN) of which were supplemented with choline or not, reduced growth due to reduced protein gain were present, but the juveniles fed the low methionine diets did not accumulate liver TAG (Espe et al. 2014, 2015a). In the current study no liver TAG accumulation occurred independent of diets were supplemented with choline or not. Even though the mean TAG in muscle increased following choline supplementation, while mean liver TAG decreased, as expected, the variation between tanks were too high to reach a statistical value. We showed previously that juveniles fed low methionine diets (containing 1.6gMet/16g N) had reduced phospholipids in both muscle and liver of which improved when the diets were supplemented with choline (Espe et al. 2014), while salmon at similar body weights as used in the current study fed diets containing 1.6gMet/16g N had increased liver TAG, but phospholipids were unaffected as compared to those fed diets containing 2.2 g Met /16gN (Espe et al. 2010). Recently, Belghit and co-workers (2014) reported that rainbow trout fed low (1.4gMet/16gN) adequate (2.1g Met/16gN) or surplus (3.1g Met/16gN) methionine diets to rainbow trout, only reduced growth when fed the low methionine diet were present, while those fed the intermediary methionine showed equal growth and accretion to those fed the surplus methionine diet. Their observations was supported by increased gene expression and abundance of proteolytic enzymes and reduced anabolic markers in those fish fed the low methionine diet. Our lower methionine diet is only slightly lower than the adequate methionine diet used by Belghit et al. (2014), but growth and protein accretion is significantly less than in salmon fed surplus methionine diets. Even though we were unable to measure any difference in gene expression in muscle, the tendency to better performance in the fish fed surplus methionine was present (less ubiquination and better IGF-1, Figure 1). Unfortunately no markers for activation of protein synthesis could be analyzed as no muscle samples were collected to be analyzed for activation protein synthesis or degradation as analyzed by western blots.

Neither did the fish seem to be under any increased metabolic stress as there were not any differences in gene expression of cytokines or pro-inflammatory interleukins in the head kidneys. Juvenile Atlantic salmon fed lower dietary methionine (1.6 g Met/16gN) than used in the current study, had elevated gene expression of liver TNFα (Espe et al. 2014) as compared to fish fed adequate methionine. Implying that sulfur amino acids availability may interact with inflammation if fish as also shown in obese mammalian models (Lin et al. 2013, Rosa et al. 2014). However, the

minimum amount of sulfur amino acid necessary to prevent interactions with inflammation still needs to be addressed, as it seems to be well below 1.9g met/16gN as used in the current study.

Liver sulfur metabolism was more dependent on methionine than on choline as assessed by the metabolites and in line with previous reports using lower and slightly higher dietary methionine as compared to the current study (Espe et al. 2010, 2015a). Using the dietary methionine is adopted in the current study, the gene expression of enzymes involved in liver sulfur amino acid metabolism was unaffected by treatments. Thus indicating that feeding diets with 1.9g Met/16g N seemingly is enough to support a healthy liver sulfur amino acid metabolism, but definitely not enough to concomitantly also support growth and muscle protein deposition during longer term feeding studies as used in the current study. Even though taurine increased in those fed the surplus methionine diet, the gene expression of *CDO* was unaffected. This is opposite to values reported for juvenile turbot where methionine supplementation decreased (Gaylord et al. 2007) or increased (Wang et al. 2014) gene expression of *CDO*. However, as activities of enzymes in liver sulfur amino acid metabolism were not addressed one cannot rule out that there were differences between treatments. There were no differences between liver SAM or SAH concentration between treatments, again pointing to that liver sulfur metabolism seems to be unaffected by dietary methionine used in the current study, while transsulfuration probably was reduced as validated by the reduced cystahionine and taurine concentrations in both plasma and muscle in the fish fed the lower methionine diets. Our study thus confirms that 1.9 g Met/16N is enough to support a healthy liver sulfur amino acid metabolism and prevents liver TAG accumulation as previously reported when fed low methionine diets (Espe et al., 2010), but it does not support maximum protein accretion within muscle. Choline supplementation only had very limiting effects on liver sulfur amino acid metabolism in the current study, but did not improve phospholipid synthesis at dietary methionine concentrations of 1.9g Met/16gN and probably is not necessary to supplement when diets are around 2g Met/16gN. However, the minimum methionine necessary to maximize protein accretion and growth of Atlantic salmon during the seawater out growing phase still needs to be determined during long time feeding experiments of which probably lies between 2.2 (Espe et al. 2008; 2010) and 2.5g Met/16gN (the current study).

In conclusion choline supplementation did not increase endogenous PC synthesis when dietary methionine was 1.9g Met/16g. Neither was any TAG accumulation present. But as protein accretion and growth improved when Atlantic salmon were fed surplus methionine, the methionine supplementation to maximize protein and amino acid retention and growth when using high plant protein inclusions might be higher than the values recommended by NRC (2011) and especially so

when fish are fed for longer periods than the 90days usually adopted determining requirements in fish. Thus a regression determining the methionine necessary to fulfill the protein synthesis and accretion in adult Atlantic salmon fed low fishmeal diets still needs to be accurately determined including analysis of activation of signals stimulating protein synthesis and accretion in fish muscle.

Acknowledgement

This work was supported through the project "Integrated Amino Acid Requirement in Fish" financed by Research Council of Norway (project no 208352/E-40) and EWOS Innovation AS. Elisabeth Eie at EWOS Innovation AS is thanked for taking care of the experimental fish. Technical support from Anita Birkenes and Eva Mykkeltvedt at NIFES is highly appreciated.

Author contribution

AA, J-EZ and ME plan the study, while AA ran the study. All authors collected and analyzed the samples. ME was the main responsible for writing the manuscript, all co-authors contributed and approved the final manuscript. There are no conflicts of interests to report.

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Table 1. Composition and crude chemical analysis (g/kg) of the diets used.

Diets	1	2	3	4
Fishmeal	70	70	70	70
Wheat gluten	60	60	60	60
Pea protein concentrate	100	100	100	100
Soy protein concentrate	235	235	235	235
Corn gluten	25	25	25	25
Sunflowermeal	30	30	30	30
Raw wheat	127	124.2	125	122.2
L-lysine	1	1	1	1
DL-methionine	1	1	3	3
Choline Cl	0	2.8	0	2.8
Fish oil	317	317	317	317
Mineral and vitamin mixtures	34	34	34	34
Chemical analysis				
Dry matter	948.5	945.5	959.9	957.2
Crude protein	388.1	389.4	395.6	403.7
Crude lipids	284.5	315.9	307.3	290.5
Energy (MJ kg^{-1})	23.5	23.7	24.2	24.1

All diets were added the same mineral and vitamin mixture to support requirement (NRC 2011).

Table 2. Dietary amino acid profiles plus choline and taurine (g/kg feed) or as (g/16gN) in parenthesis.

Diets no	1		2		3		4	
Methionine (M)	L		L		H		H	
Choline (C)	L		H		L		H	
Met	7.9	(2.0)	7.6	(1.9)	9.8	(2.5)	9.7	(2.4)
Cys	5.8	(1.5)	5.6	(1.4)	5.7	(1.4)	5.7	(1.4)
TSAA	13.7	(3.5)	13.2	(3.4)	15.5	(3.9)	15.4	(3.8)
Lys	24.1	(6.2)	23.6	(6.1)	23.6	(6.0)	23.8	(5.9)
Thr	15.6	(4.0)	15.0	(3.9)	15.0	(3.8)	15.1	(3.7)
Arg	27.4	(7.1)	26.6	(6.8)	26.9	(6.8)	26.5	(6.6)
Ile	18.8	(4.8)	18.4	(4.7)	18.5	(4.7)	18.5	(4.6)
Leu	36.7	(9.4)	32.8	(8.4)	33.1	(8.4)	29.5	(7.3)
Val	20.3	(5.2)	20.0	(5.1)	20.1	(5.1)	20.0	(4.9)
His	9.9	(2.5)	9.6	(2.5)	9.8	(2.5)	9.7	(2.4)
Phe	21.5	(5.5)	21.0	(5.4)	21.1	(5.3)	20.9	(5.2)
Gly	18.2	(4.7)	17.7	(4.6)	17.8	(4.5)	17.7	(4.4)
Ser	20.4	(5.2)	19.5	(5.0)	19.5	(4.9)	19.8	(4.9)
Pro	25.1	(6.5)	25.1	(6.4)	24.7	(6.2)	24.5	(6.1)
Ala	18.8	(4.8)	18.3	(4.7)	18.4	(4.6)	18.3	(4.5)
Asp	40.5	(10.4)	39.2	(10.1)	39.2	(9.9)	39.2	(9.7)
Glu	84.1	(21.6)	81.7	(21.0)	82.3	(20.8)	82.2	(20.4)
Choline	0.9	-	2.9	-	1.3	-	3.0	-

Taurine	0.7	-	0.6	-	0.6	-	0.6	-

TSAA is the sum of methionine and cysteine

Table 3. Growth performance and mean protein and lipid gain. Also the mean amino acid retention (% retained of consumed) is listed. Values are mean of three tanks±SE (ANOVA followed by Tukey p<0.05)

Diets no	1	2	3	4	p-values		
Methionine (M)	L	L	H	H	M	C	M*C
Choline (C)	L	H	L	H			
IBW (g/fish)	732±2	723±16	724±11	724±7	0.77	0.67	0.69
FBW (g/fish)	1915±34	1951±24	1998±37	2024±35	**0.045**	0.40	0.88
SGR	0.90±0.01	0.93±0.01	0.95±0.00	0.96±0.01	**0.013**	0.20	0.60
MFI (g/fish/day)	8.4±0.1	8.5±0.1	8.5±0.2	8.7±0.2	0.38	0.28	0.63
FCR	0.95±0.01	0.93±0.00	0.90±0.01	0.90±0.01	**0.0005**	0.27	0.11
CF	1.50±0.02	1.52±0.02	1.54±0.01	1.51±0.01	0.26	0.88	0.21
HSI	1.29±0.07	1.38±0.09	1.30±0.05	1.30±0.03	0.64	0.52	0.48
VSI	11.7±0.3	11.5±0.3	11.7±0.3	10.9±0.2	0.29	0.09	0.38
Protein gain (g/fish)	191.2±5.2	190.5±4.6	210.1±2.0	207.5±7.5	**0.007**	0.75	0.85
Lipid gain (g/fish)	296.2±4.8	308.5±3.9	287.2±4.1	301.2±8.4	0.19	**0.047**	0.89
AA-retention:							
Met	76.9±4.4	74.2±1.5	67.7±1.9	68.0±0.1	**0.01**	0.65	0.58
Cys	39.5±2.4	37.3±1.0	45.2±1.6	43.5±0.8	**0.006**	0.26	0.87
Thr	62.7±2.6	58.3±0.9	68.7±1.3	68.6±0.6	**0.006**	0.19	0.21
Arg	46.2±1.5	44.6±0.5	48.1±0.8	49.0±0.4	**0.01**	0.70	0.19
Ile	48.2±3.1	46.3±1.5	54.4±2.0	52.1±0.8	**0.02**	0.32	0.92
Leu	46.4±3.0	43.6±1.1	50.8±1.0	50.3±0.8	**0.02**	0.36	0.53
Val	52.4±3.4	49.8±1.5	58.4±1.7	56.2±0.9	**0.02**	0.29	0.92
His	54.6±3.5	51.2±1.4	60.5±1.3	59.6±0.2	**0.008**	0.31	0.55
Phe	40.0±2.4	37.5±0.9	43.6±0.8	43.6±0.5	**0.02**	0.39	0.39
Gly	71.7±1.9	73.8±3.6	69.3±2.7	74.4±4.0	0.78	0.29	0.65

Ser	42.4±1.9	39.9±0.7	45.0±1.2	45.9±0.2	**0.007**	0.51	0.20
Pro	34.0±0.6	34.2±0.6	35.3±0.5	35.5±0.9	0.09	0.80	0.96
Ala	68.1±2.5	65.2±1.25	71.3±1.4	72.6±0.4	**0.01**	0.62	0.22
Asp	49.5±2.7	46.8±0.9	54.3±1.0	54.0±0.4	**0.01**	0.35	0.45
Glu	32.2±1.7	30.6±0.6	34.6±0.6	34.6±0.2	**0.02**	0.43	0.44

Thr, Arg, Ile, Leu, Phe, Asp & Glu as affected by dietary methionine were assessed by Kruskall Wallis.

SGR=[(ln final tank body weight-ln initial tank body weight)/days of feeding]*100

FCR=(consumed feed*final biomass)/body mass increase

HSI and VSI are mean tank relative liver weight and viscera weight to body weight, respectively

Gained nutrients (lipid and protein)=[(deposited nutrient/consumed nutrients (as is)]/number of fish per tank.

AA retention= (deposited amino acid as percentage of consumed amino acid during the experimental period)*100

Table 4. Lipid classes in plasma (mmol/L, bile acids given as µmol/L), liver (mg/g liver) and muscle (mg/g muscle) in fish fed diets reduced or surplus methionine of which are supplemented or not with choline. Values are tank means±SE, n=3 (Tukey, p<0.05) ….

Diets no	1	2	3	4	p-values		
Methionine (M)	L	L	H	H	M	C	M*C
Choline (C)	L	H	L	H			
Plasma							
Total PL	10.8±0.1	12.3±0.2	10.0±0.3	11.8±0.3	**0.032**	**<0.001**	0.66
TAG	3.3±0.1	2.7±0.1	3.0±0.1	3.3±0.7	0.11	0.29	0.90
Total Cholesterol	9.4±0.3	10.7±0.1	8.7±0.2	9.6±0.2	**0.004**	**0.002**	0.43
NEFA	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.23	0.17	0.35
Total bile acids	11.7±2.7	20.7±3.7	23.6±2.3	24.9±3.0	**0.026**	0.12	0.23
Liver							
PC	*27.3*±1.9	*26.3*±2.7	*29.7*±2.1	*24.3*±3.6	*0.94*	*0.26*	*0.42*
PE	*8.7*±0.8	*7.7*±0.7	*9.3*±0.1	*7.2*±0.9	*0.98*	0.05	*0.45*
PC:PE ratio	*3.2*±0.1	*3.4*±0.0	*3.2*±0.2	*3.4*±0.1	*0.95*	*0.14*	*0.75*
Total PL	*44.7*±3.3	*42.2*±4.3	*48.6*±2.7	*38.9*±5.7	*0.95*	*0.18*	*0.41*
TAG	*13.0*±3.5	*11.4*±3.6	*15.2*±2.3	*17.0*±7.3	*0.59*	*0.12*	*0.25*
Cholesterol	*3.0*±0.5	2.7±0.5	*3.4*±0.3	*2.8*±0.7	*0.68*	*0.45*	*0.77*
NEFA	*0.4*±0.0	*0.5*±0.2	*0.4*±0.1	*0.4*±0.1	*0.65*	*0.88*	*0.65*
Muscle							
PC	8.5±0.3	8.9±0.4	8.6±0.3	8.7±0.1	0.77	0.39	0.60
PE	3.0±0.2	3.0±0.2	2.9±0.1	2.8±0.1	0.47	0.60	0.75
PC:PE ratio	2.9±0.1	3.0±0.1	2.9±0.1	3.1±0.0	0.47	0.15	0.97
Total PL	12.0±0.5	12.3±0.6	12.1±0.3	12.0±0.2	0.70	0.76	0.64

Cholesterol	0.6±0	0.6±0.0	0.6±0.0	0.6±0.0	0.58	0.58	0.58
TAG	80.6±13.3	103.9±16.5	83.2±22.9	94.1±16.2	0.84	0.36	0.73
NEFA	nd	nd	nd	nd	-	-	-

Table 5. N-metabolites in plasma, liver and muscle (µmol/100ml or µmol/100g tissue). SAM and SAH (µmol/100g liver) were analyzed in liver only. Values are tank means±SE, n=3 (Tukey p<0.05). Choline was not addressed in plasma.

Diets no	1	2	3	4	p-values		
Methionine (M)	L	L	H	H	M	C	M*C
Choline (C)	L	H	L	H			
Plasma							
Met*	11.2±0.4	10.6±0.9	29.6±1.9	29.2±3.3	**0.004**	0.81	0.97
Cys	0.4±0.0	0.4±0.0	0.6±0.1	0.6±0.2	**0.051**	0.82	0.86
Taurine*	20.6±1.0	23.4±1.5	27.9±4.3	35.3±3.7	**0.016**	0.12	0.46
Cystathionine*	0.4±0.0	0.3±0.0	1.2±0.1	1.3±0.2	**0.004**	0.70	0.30
PEA	2.5±0.1	2.6±0.3	2.8±0.4	2.8±0.3	0.41	0.86	0.99
Ethanolamine	1.5±0.2	1.4±0.1	1.2±0.2	1.2±0.1	0.050	0.70	0.67
Liver							
Choline	*1725*±293	*1914*±57	*1726*±204	*1542*±61	*0.34*	*0.99*	*0.34*
Met	*32±3*	*29±2*	*33±1*	*35±2*	*0.15*	*0.91*	*0.23*
Cys	*17±2*	*12±1*	*15±2*	*14±3*	*0.90*	*0.25*	*0.41*
Taurine	*645±104*	*759±161*	*1090±158*	*1175±46*	***0.009***	*0.45*	*0.91*
*Cystathionine**	*5±0*	*6±0*	*24±4*	*23±6*	***0.01***	*0.95*	*0.82*
PEA	*28±1*	*26±1*	*30±2*	*30±2*	*0.09*	*0.54*	*0.47*
Ethanolamine	13*±2*	13*±1*	14*±1*	13*±0*	0.79	0.64	0.42
SAM	*5.7±0.2*	*5.7±0.4*	*6.5±0.2*	*5.4±0.5*	*0.43*	*0.12*	*0.14*
SAH	*3.2±0.3*	*3.3±0.2*	*3.7±0.1*	*3.4±0.1*	*0.16*	*0.54*	*0.36*
SAM:SAH	*1.8±0.1*	*1.8±0.2*	*1.8±0.1*	*1.4±0.2*	*0.31*	*0.29*	*0.33*
Muscle							

Choline	228±28	447±52	200±23	467±50	0.92	**<0.001**	0.57
*Met**	6*±1*	5*±0*	16*±2*	15*±1*	**0.01**	0.47	0.97
Taurine	22*±1*	29*±4*	38*±5*	38*±5*	**0.02**	0.43	0.48
Cystathionine	nd	5*±2*	8*±1*	10*±1*	-	-	-
PEA	5*±0*	5*±0*	6*±1*	6*±0*	0.20	0.60	0.88

PEA is phosphatidyl ethanolamine

Figure 1

Mean normalized gene expression (MNE) of two anabolic markers (*IGF-1*, *mTOR*) and two ubiquination markers (*murf1*, *murf1b*) in muscle. Only *Murf1* tended to be higher in fish fed the low methionine diet but did not reach a statistical difference (p=0.06), while none of the other genes tested differed between treatments (p>0.05 Tukeys). Values are tank means±SE, n=3, where LL is low methionine, low choline, LH is low methionine high choline, HL is high methionine, low choline and HH is high methionine, high choline.