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ORIGINAL ARTICLE

Methionine limitation results in increased hepatic FAS activity, higher liver 18:1 to 18:0 fatty acid ratio and hepatic TAG accumulation in Atlantic salmon, *Salmo salar*

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Abstract The current experiment aimed to study whether interactions with lipid metabolism possibly might explain the relative increased liver weight obtained in fish fed sub-optimal methionine levels. A basal diet based on a blend of plant proteins which is low in methionine (1.6 g Met/16 g N) was compared to a methionine adequate diet (2.2 g Met/16 g N) prepared by adding DL-methionine (2.4 g/kg) to the basal diet in the expense of wheat grain. Fish oil was used as the lipid source. The diets were balanced in all nutrients except methionine. The diets were fed to Atlantic salmon (500 g BW) for a period of 3 months. Feed intake did not differ, rendering the intake of all nutrients except methionine equal. Fish fed the low methionine diet had an increased liver size relative to body weight, indicating fat deposition in the liver. Fish given the sub-optimal methionine diet showed about six times higher fatty acid synthase (FAS) activity as compared to the fish fed the adequate methionine diet, indicating a higher de novo lipogenesis. A significant rise in the liver 18:1 to 18:0 fatty acid ratios also supported storage of lipids over fatty acid oxidation. Indeed, methionine limitation resulted in significantly higher TAG concentrations in the liver. Sub-optimal dietary methionine also resulted in lower hepatic taurine concentrations and the total bile acids concentrations were reduced in faeces and tended to be reduced in plasma. Taken together, our data show that salmon fed sub-optimal methionine levels had increased relative liver weight and developed signs commonly described in the early stage of non-alcoholic fatty liver disease in rodent models (increased FAS activity, changed fatty acid ratios and TAG accumulation).

Keywords Methionine deficiency · Fatty liver development · TAG accumulation · Taurine depletion · Bile acids · NAFLD · Atlantic salmon

Abbreviations

IAAs	Indispensable amino acids
DAAs	Dispensable amino acids
PC	Phosphocholine
PE	Phosphoethanolamine
SAH	*S*-Adenosylhomocysteine
SAM	*S*-Adenosylmethionine
tHcy	Total homocysteine
TAG	Triacylglycerol
NAFLD	Non-alcoholic fatty liver disease
FAS	Fatty acid synthase

Introduction

The expansion in the aquaculture industry in combination with variable availability and prices of fishmeal, are underlying factors for the aquaculture feed producers to

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include more plant proteins in the aquaculture feeds (Tacon 1995; FAO 2005). However, the amino acid profiles in fishmeal and plant proteins are not identical, and the exchange of fishmeal with plant proteins in aquaculture diets therefore might cause metabolic alterations in the farmed fish. Indeed, feeding farmed fish diets with high levels of alternative protein sources have been reported to cause reduced growth (Gomes et al. 1995; Kaushik et al. 1995; Gomez-Requeni et al. 2004; Robaina et al. 1995; Dias et al. 2005). Soy protein, which is commonly used as a substitute for fishmeal in aquaculture feeds, contains a low amount of the indispensable amino acid methionine. Sub-optimal dietary methionine levels might be one reason for the reduced growth performance reported in long-term studies with fish fed soy protein-rich diets (Mambrini et al. 1999; Dias et al. 2005).

Methionine is the precursor for *S*-adenosylmethionine (SAM) an important methyl-donor in a wide variety of substrates. Upon delivery of the methyl-group, SAM is converted to *S*-adenosylhomocysteine (SAH), which is further converted to homocysteine. Therefore, the methylation activity will affect the total homocysteine production. The liver is the organ with the highest capacity to perform methylation reactions as well as trans-sulfuration reactions while minor activities also are present in the kidney (Mato et al. 2002 and references therein). Previously, we have shown that in Atlantic salmon the plasma homocysteine level increased linearly with increasing methionine intake (Espe et al. 2008), and this also has been shown in mammals (Hirche et al. 2006). In rodents the methionine-induced increase in homocysteine is reported to be prevented by addition of glycine and serine, probably due to these amino acids ability to participate in the remethylation of homocysteine to methionine (Fukada et al. 2006; Hirche et al. 2006). Thus, the methionine load might determine plasma homocysteine levels as well as the availability of activated methionine for methylation reactions important for the metabolism and health of the animal (Cantoni and Chiang 1980; Cantoni 1982; Mato et al. 2002; Rowling et al. 2002).

A limited capacity to perform methylation reactions might also play an important role in the lipid metabolism. Administration of SAM to the diabetic rat has been reported to reduce body weight gain, i.e. lipid gain (Jin et al. 2007) indicating interactions with lipid accumulation in the body. Furthermore, increasing the dietary methionine content to rats fed choline-deficient diets reduced the fatty liver development, even though methionine supplementation could not fully compensate for the choline deficiency (Slow and Garrow 2006). Together, these studies indicate that methionine limitations might affect lipid storage pattern through reduced methylation capacity.

Animals do synthesise choline upon delivery of methyl groups from SAM through the alternative phosphatidyl ethanolamine transferase (pemt) pathway (Noga and Vance 2003). When rats were fed a diet deficient in both betaine and methionine, administration of exogenous SAM was not efficient to produce sufficient amounts of choline (Chawla et al. 1998). Recently it was found that the hepatic triacylglycerol (TAG) accumulation commonly present in the obese rat can be reduced by a moderate increase in carnitine palmitoyl transferase-1 activity (Stefanovic-Racic et al. 2008). In a poultry study, dietary supplementation with methionine and betaine was reported to increase creatine and free carnitine levels in the liver, to reduce abdominal fat content and increase the breast muscle yield (Zhan et al. 2006), these results further support a role for methylation reactions in the lipid metabolism. Whether methylation reactions also can influence on lipid metabolism in fish has not been extensively studied. However, feeding the European seabass (*Dicentrarchus labrax*) diets with high amounts of soy protein or corn gluten has been reported to affect lipid metabolism. Both groups of fish fed the plant protein ingredients had reduced plasma concentrations of TAG, cholesterol and cholesterol esters when compared to the fishmeal-fed control group (Dias et al. 2005). Liver TAG levels were also lower in fish given the plant protein-based diets. Dias et al. (2005) did not balance the dietary amino acid profiles of the test diets towards the fishmeal control, and the observed effects could be related to amino acid imbalance in the diets and a reduced feed intake in the fish fed the plant protein diets.

The end-product of methionine methylation and trans-sulfuration reactions is taurine (2-amino ethanesulfionic acid) which is mainly produced by the liver. Taurine is the main free intracellular amino acid in several different tissues and has been found to be a conditionally indispensable nutrient in juvenile fish (Kim et al. 2005; Matsunari et al. 2005; Gaylord et al. 2006; Takagi et al. 2006). In the seawater on-growing phase (BW 0.5 kg) we reported a linear relationship between methionine intake and hepatic taurine concentration (Espe et al. 2008). Taurine is involved in lipid metabolism through its conjugation with bile acids in liver and proper bile acid conjugation is important for the absorption of nutrients (Yokogoshi and Oda 2002). Exogenous taurine administration to rodent diets has been found to have a hypocholesterolemic effect when high cholesterol diets are fed (Sugiyama et al. 1998; Nanami et al. 1996; Chen et al. 2004; Choi et al. 2006). In addition, taurine by itself also might enhance transformation of cholesterol to bile acids and thus have an impact on cholesterol clearance (Yokogoshi et al. 1999; Yokogoshi and Oda 2002; Murakami et al. 2002; Kishida et al. 2003; Chen et al. 2004). Recently, we showed that rats given a taurine-rich diet had elevated plasma bile acid levels, reduced liver lipids, lower plasma TAG concentrations and decreased abdominal obesity (Liaset et al. 2009). Gaylord

et al. (2007) added both methionine and taurine to juvenile rainbow trout diets and reported that taurine addition improved growth, but only methionine affected the lipid deposition. Thus, it is likely that methionine availability might influence lipid metabolism both through methylation reactions and as a precursor for taurine synthesis. In keeping with this, the present experiment aimed to study whether methionine limitations affected parameters related to hepatic lipid metabolism that could explain the increased liver size observed in fish as the response to methionine limitations (Rumsey et al. 1983; Rodehutscord et al. 1997; Espe et al. 2008). In order to standardise the metabolic status (i.e. feeding status) of the salmon all samples were collected 5 h after feeding. In the present study, we used wheat as the main protein source. Wheat is low in methionine and deficient in taurine, and in one diet we kept the methionine low, whereas in the control diet we supplemented methionine to the established methionine requirement in Atlantic salmon.

Materials and methods

Diets and sampling procedures

Diets were based on plant proteins containing only 5% fish meal, and the methionine levels were either sub-optimal or at the requirement of Atlantic salmon (1.6 or 2.2 g Met/16 g N, respectively). To the diets we added 5% fish protein concentrate (FPC) and 3% squid hydrolysate to maximise feed intake and thus growth performance in this specie (Espe et al. 2006, 2007). The methionine-sufficient diet was based on the methionine requirement of Atlantic salmon (Sveier et al. 2001; Espe et al. 2008). The rest of the protein source was blended plant proteins thus both diets had low animal protein content. In the low methionine diet no DL-methionine was added, while the adequate amount of 2.4 g/kg diet was added at the expense of wheat grain. The dietary amino acid (AA) profile was balanced according to requirement by adding smaller amounts of crystalline amino acids not to jeopardise absorption and utilisation (Espe et al. 1993, 1999). The dietary lipid source was fish oil. To allow calculation of digestibility both diets were added yttrium oxide. The diets were extruded and 6 mm pellets produced as previously described (Espe et al. 2006). The dietary compositions as well as the dietary AA profiles are given in Tables 1 and 2.

Triplicate tanks of Atlantic salmon with mean BW of 495 g were fed the respective diets for a period of 12 weeks, during which period the body weight more than doubled (117–127% of initial BW). The fish experiment was performed as previously described with controlled feed intake (Espe et al. 2006). In short each tank (water volume of 0.5 m^3) containing 60 fish was supplied by running seawater (salinity 33 g/L, temperature 8 ± 1°C and water flow of 0.8 L/kg biomass per min). Fish were fed three times daily and feed leftovers were collected. A 24-h light regime was adopted. Samples of plasma and liver were collected 5 h after the last meal and the samples were immediately flash frozen in liquid N as described (Espe et al. 2007). In addition faeces samples were collected by stripping and whole fish sampled at the start and end of the experiment as described (Espe et al. 2006) to allow calculation of gain and digestibility. Before handling, the fish were anaesthetised with chlorobutanol (0.4 g L^{-1}). The experimental protocol was approved by the Norwegian Board of Experiments with Living Animal.

Table 1 The diet composition (g/kg)

	Low methionine	Adequate methionine
Diets		
Fish meal	50	50
Fish soluble CPSP	50	50
Wheat gluten	238	238
Soy protein concentrate	11	11
Squid hydrolysate	30	30
Amino acid mix[a]	68	68
Wheat grain	229.4	227
Yttrium oxide	0.1	0.1
Micronutrients	43.5	43.5
DL-methionine	–	2.4
Fish oil	280	280
Chemical composition		
Dry matter	926	933
Crude protein	420	434
Crude fat	302	294
Ash	46	67
Energy (MJ/kg)	24	24
DP/DE (MJ/kg)	18.8	19.5

[a] AA mixture (% of mixture): Biolysine60 25.74, L-Trp 1.76, L-Ile 6.47, L-His 5.88, L-Val 14.71, L-Leu 20.44, L-Arg 11.76, L-Thr 7.35, L-Phe 5.88. Biolysine60 is composed of 47.3% Lys, 0.15% Trp, 0.5% Ile, 1.2% Val, 0.8% Leu and 1% Arg. Micronutrients contain minerals and vitamins to fulfil the requirement of Atlantic salmon

Chemical methods

Crude chemical analyses and dietary and faecal AAs were done as previously described (Espe et al. 2006). AAs in deproteinised liver samples was determined on an amino acid analyser Biochrom 20 plus Amino Acid Analyzer (Amersham Pharmacia Biotech, Sweden) equipped with a lithium column using post-column derivatisation with ninhydrin as previously described (Espe et al. 2006). SAM

Table 2 Dietary AA's (g/16 g N) and the mean amino acid intake (mg/fish per day) in fish fed deficient or adequate methionine

Diets	Dietary AA		Mean AA intake	
	Low methionine	Adequate methionine	Low methionine	Adequate methionine
Met*	1.6	2.2	41 ± 3[b]	64 ± 5[a]
Cys	1.4	1.4	32 ± 2	37 ± 3
Lys*	4.9	4.9	113 ± 7	128 ± 10
Thr*	3.8	3.8	88 ± 5	100 ± 8
Arg*	5.3	5.4	122 ± 8	142 ± 12
Ile*	3.8	3.9	88 ± 5	102 ± 8
Leu*	8.6	8.4	199 ± 12	220 ± 18
Val*	5.4	5.4	125 ± 8	142 ± 12
His*	2.4	2.4	55 ± 3	63 ± 5
Phe*	4.6	4.5	106 ± 7	118 ± 10
Gly	3.8	3.9	88 ± 6	102 ± 8
Ser	3.9	3.9	90 ± 6	102 ± 8
Ala	3.3	3.3	76 ± 5	86 ± 7
Asp	4.8	4.9	111 ± 7	128 ± 10
Glu	22.3	22.3	516 ± 32	585 ± 48
IAA:DAA	1.0	1.0	0.9	0.9
Sum AA	79.9	80.6	1,705 ± 107	1,952 ± 161
Non AA-N	20.1	19.4	–	–

AA's followed by * is considered indispensable for Atlantic salmon. Tyr, OH-pro and Trp* were not analysed. Non AA-N is nitrogen not accounted for in the analysed AAs

Means followed by different letters differ significantly ($p < 0.05$)

and SAH were determined on a reverse phase HPLC after deproteinisation in 0.4 M $HClO_4$ as described by Wang et al. (2001). The concentration of SAM and SAH were quantified using standards of the respective metabolites (Sigma). Plasma total homocysteine (tHcy) was determined on the Biochrom 20 plus Amino Acid Analyzer as described above after being reduced by addition of 12% DDT (1:0.1, v:v) for 2 min and deproteinised for 1 h at room temperature with addition of 10% sulfosalicylic acid 1:1 (v:v). Norleucine was used as internal standard and tHcy was determined using a standard of homocysteine (Sigma). Plasma total cholesterol, HDL-cholesterol, total phospholipids, TAG and total bile acids were determined using commercial kits (Diagnostic Laboratories) following the instructions from the supplier. The lipid classes in hepatic samples were analysed after lipid extraction with 2:1 chloroform: methanol (v:v) as described previously (Bell et al. 1993; Liaset et al. 2003). Carnitine was estimated according to the method described by Arakawa et al. (1989) analysing the Coenzyme A (CoA) produced after treatment with carnitine acyl transferase. The CoA produced was separated on a HPLC equipped with a UnisilQC8 column and detected at 254 nm. Carnitine content was calculated assuming 1 mol of CoA was equal to 1 mol carnitine. The carnitine level was calculated as micromole per gram of tissue, but presented relative to the level present in the adequate methionine-fed group of which was set to 1. Fatty acid composition in hepatic samples was determined as previously described using 19:0 as internal standard (Liaset et al. 2003). Hepatic FAS activity was determined as described in Hsu et al. (1965) and the activity calculated as the amount of protein required to catalyse the incorporation of 1 μmol 1-^{14}C labelled AcCoA into fatty acids per minute per milligram of protein. The result is presented relative to the adequate methionine-fed fish of which was set equal to 1. Total fatty acid oxidation capacity in the liver was measured using homogenate as described in Madsen et al. (1999) and the activity calculated as the amount of main oxidation products of [1-^{14}C] palmitoyl-CoA (representing ketone bodies, acetylcarnitine, and Krebs cycle intermediates) per hour per milligram of tissue protein. Faecal bile acids were extracted as described by Suckling et al. (1991) and total bile acid present determined using a commercial kit (Diagnostic Laboratories) following the instructions from the supplier. The protein concentration in tissue homogenates was determined colorimetrically using BSA as a standard (Pierce).

Statistical methods

Data are given as tank means ± SE ($n = 3$); t test was used to evaluate any treatment differences. Statistical analyses were done using the statistical program of CSS Statistica™, Stat Soft, Inc., USA (1999). p values less than 0.05 was accepted as statistically different.

Results

Fish performed well and no significant differences occurred in either mean daily voluntary feed intake, growth performance or the mean protein and lipid gains (Table 3). Fish fed the methionine-limiting diet had significantly lower ($p = 0.02$) mean daily methionine intake as compared to those fed the adequate diet (consuming 41 or 64 mg methionine/day, respectively). As the diets were balanced in all amino acids except the methionine and the fact that the mean voluntary feed intake did not differ, no differences in intake of any of the other amino acids were present (Table 2). Fish fed the sub-optimal methionine diet had lower plasma concentrations of free methionine, tHcy and taurine than the fish fed the adequate methionine diet, while plasma total- and HDL-cholesterol, total phospholipids and TAG were unaffected by treatment as analysed 5 h postprandial (Table 4). Serine and glycine are both used in methylation and trans-sulphuration reactions; however, the plasma free serine and glycine were not different between treatment groups (Table 4). Fish fed the sub-optimal methionine diet had an increased liver size relative to body weight as compared to fish fed the adequate methionine diet (Table 5). Further, fish fed the methionine-limiting diet tended to have less SAM ($\sim$33%, $p = 0.095$) and SAH ($\sim$15%, $p = 0.063$) in liver samples than the fish fed the sufficient methionine diet. Hepatic taurine was reduced ($p = 0.012$) when fish were fed the sub-optimal methionine diet. Liver, free glycine was unaffected (glycine $\sim$5% elevated $p = 0.32$) whereas the liver free serine was higher (serine $\sim$ 31%, $p = 0.006$) in the salmon fed the low methionine diet (Table 5). Plasma levels of asparagine amino transferase (ASAT) or alanine amino transferase (ALAT) activities and plasma protein concentrations were unaffected by treatments (values not shown).

To evaluate whether lipid metabolism was affected by methionine limitation, liver samples were analysed for lipid classes. Liver TAG concentration was significantly higher (38% higher, $p = 0.030$) in the fish fed the sub-optimal methionine diet as compared to those fed the methionine-sufficient diet (Table 5). Neither sterol esters nor free fatty acids were affected by the different methionine intake ($p = 0.13$ and $p = 0.16$, respectively). The liver phospholipids levels were unaffected by dietary treatments in the current study (Table 5).

Table 3 Final body weight (BW, g), mean weight gain (MWG, g/fish), mean feed intake (MFI, g/fish per day), mean lipid and protein gain (g/fish)

Diets	Low methionine	Adequate methionine	*p* Value
BW end	1,056 ± 49	1,125 ± 46	0.41
MWG	566 ± 28	630 ± 50	0.31
MFI	5.5 ± 0.3	6.0 ± 0.5	0.42
Lipid gain	122 ± 9	131 ± 15	0.64
Protein gain	96 ± 6	99 ± 9	0.72

Values are tank means ± SE

Table 4 Plasma free methionine, serine, glycine and taurine (μmol/dl), total homocysteine (tHcy) and taurine (μmol/dl), plasma cholesterol, HDL-cholesterol, phospholipids (PL) and TAG (mmol/L) as occurring 5 h postprandial in fish fed low or adequate methionine

Diets	Low methionine	Adequate methionine	*p* Value
Methionine	10 ± 2	31 ± 2	**0.004**
Serine	17 ± 2	16 ± 2	0.60
Glycine	45 ± 3	37 ± 3	0.17
Taurine	77 ± 1	92 ± 1	**0.004**
tHcy	1.9 ± 0.3	5.4 ± 0.1	**0.004**
Cholesterol	10.0 ± 0.2	10.1 ± 0.9	0.93
HDL-Cholesterol	7.7 ± 0.1	7.9 ± 0.3	0.43
PL	13.3 ± 1.3	13.3 ± 1.4	0.99
TAG	2.0 ± 0.2	2.1 ± 0.3	0.93

Values are mean of tanks ± SE

The *p* values being significant are given in bold letters

Table 5 Relative hepatic size (% of body weight) and gram liver, hepatic free methionine, glycine and serine (μmol/g), SAM (nmol/g), SAH (nmol/g), hepatic taurine (μmol/g), hepatic TAG (mg/g liver), phosphatidylethanol (PE, mg/g liver), phosphatidylcholine (PC, mg/g liver), PC:PE ratio, total phospholipids (PL's, mg/g liver), cholesterol esters and cholesterol (mg/g liver) as occurring 5 h postprandial in fish fed diet with low or adequate methionine

Diets	Low methionine	Adequate methionine	*p* Value
Relative hepatic size	1.74 ± 0.05	1.48 ± 0.02	**0.025**
Gram liver	18.4 ± 1.2	16.6 ± 0.9	0.31
Met	0.58 ± 0.03	0.55 ± 0.02	0.58
Gly	3.07 ± 0.13	2.93 ± 0.13	0.32
Ser	3.05 ± 0.10	2.10 ± 0.10	**0.006**
SAM	31.66 ± 4.90	47.46 ± 2.35	**0.095**
SAH	13.21 ± 0.32	15.54 ± 0.92	**0.063**
SAM:SAH	1.9 ± 0.6	3.1 ± 0.6	0.29
Taurine	9.17 ± 0.58	15.79 ± 1.27	**0.012**
TAG	23.0 ± 1.7	14.3 ± 0.9	**0.030**
PE	7.7 ± 0.1	8.9 ± 0.8	0.145
PC	18.4 ± 0.6	20.9 ± 1.7	0.19
PC:PE	2.4 ± 0.1	2.4 ± 0.02	0.74
PL	36.3 ± 1.2	37.2 ± 3.4	0.80
Free FA	4.8 ± 0.1	3.7 ± 0.6	0.16
Sterol esters	1.15 ± 0.13	0.79 ± 0.07	0.13
Cholesterol	3.8 ± 0.1	3.8 ± 0.5	0.89

Values are means ± SE

The *p* values being significant are given in bold letters

An increased synthesis of lipid, a decreased fatty acid oxidation or lower transport of lipids from the liver might contribute to the increased relative liver size and hepatic TAG accumulation. In order to elucidate whether methionine limitation influenced the de novo lipogenesis, we measured fatty acid synthase (FAS) capacity in the liver. Fish fed the methionine-limiting diet had almost six times higher FAS activity in crude liver extracts as compared to the activity present in the methionine adequate-fed fish (Fig. 1a, $p = 0.016$).

Next, we measured the fatty acid compositions which are known to change during fatty liver development. The fatty acid profile in liver was unaffected by methionine limitations (Table 6). A general trend towards more n-6 fatty acids in fish fed methionine-limiting diet as compared to those fed adequate methionine resulted in a tendency towards an elevated n-6 to n-3 fatty acid ratio (Fig. 1b, $p = 0.077$). Interestingly the ratio of 18:1 to 18:0 was significantly higher in the liver of those fish fed the methionine-limiting diet (Fig. 1c, $p = 0.03$). In the liver, saturated fatty acids can be desaturated and used in the synthesis of TAG or sterol esters for storage in lipid compartments, or the saturated fatty acids might be directed for oxidation. In the latter case, the fatty acids are coupled to carnitine in a reaction catalysed by the mitochondrial carnitine palmitoyl transferase. As SAM is the methyl-donor in the carnitine synthesis, we hypothesised that sub-optimal dietary methionine levels could reduce the carnitine concentration, and that this might be a limiting factor for fatty acid oxidation. However, liver carnitine levels were unaffected by the dietary methionine levels used in the present study (Fig. 2a) and the total fatty acid oxidation did not differ between treatments (Fig. 2b).

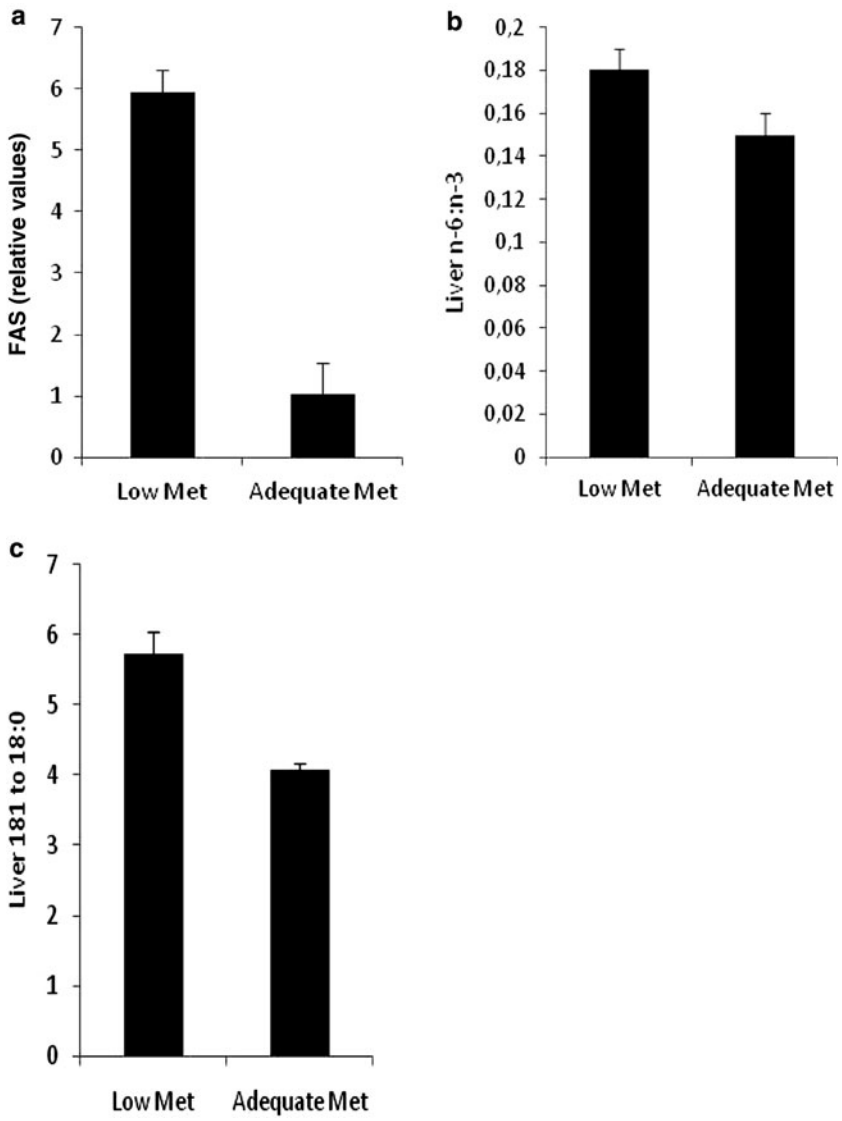

Fig. 1 Fish fed the low methionine diet had higher relative hepatic FAS activity (**a**, $p = 0.016$), a tendency to higher ratio of n-6 to n-3 fatty acids (**b**, $p = 0.077$) and a higher ratio of 18:1–18:0 fatty acid (**c**, $p = 0.03$)

Table 6 Fatty acid profile in crude liver extract (mg/g tissue) of fish fed the low or the adequate methionine diets

Fatty acids	Low methionine	Adequate methionine	*p* Value
14:0	0.85 ± 0.07	0.65 ± 0.08	0.15
16:0	5.12 ± 0.17	4.99 ± 0.33	0.70
18:0	1.69 ± 0.10	1.81 ± 0.23	0.62
Σsaturated	7.95 ± 0.36	7.69 ± 0.67	0.72
16:1[a]	1.92 ± 0.19	1.44 ± 0.18	0.18
18:1[a]	9.73 ± 1.09	7.33 ± 0.77	0.21
20:1[a]	3.20 ± 0.37	2.40 ± 0.29	0.22
22:1[a]	1.61 ± 0.16	1.22 ± 0.25	0.25
Σmonoenic	16.47 ± 1.79	12.39 ± 1.47	0.21
18:2n-6	1.13 ± 0.09	0.84 ± 0.09	0.13
20:2n-6	0.31 ± 0.03	0.23 ± 0.02	0.16
20:3n-6	0.11 ± 0.01	0.10 ± 0.00	0.40
20:4n-6	0.69 ± 0.03	0.64 ± 0.04	0.41
Σn-6	2.24 ± 0.16	1.81 ± 0.15	0.16
18:3n-3	0.22 ± 0.02	0.15 ± 0.02	0.10
18:4n-3	0.09 ± 0.01	0.08 ± 0.01	0.27
20:4n-3	0.46 ± 0.04	0.38 ± 0.03	0.24
20:5n-3	2.93 ± 0.13	2.94 ± 0.17	0.98
22:5n-3	0.91 ± 0.04	0.89 ± 0.06	0.79
22:6n-3	8.12 ± 0.23	7.93 ± 0.38	0.68
Σn-3	12.74 ± 0.46	12.37 ± 0.68	0.67
FA concentration (mg/g)	39.4 ± 2.8	34.3 ± 2.9	0.31
Hepatic FA (mg)	732 ± 94	572 ± 80	0.32

Values are tank means ± SE

[a] Sum of isomers (n-7, n-9 and n-11)

Methionine is a precursor for endogenous taurine synthesis and taurine is known to affect the cholesterol metabolism and thus might affect the production of bile acids. Therefore, we measured total bile acids in plasma and in faeces. In plasma the mean total bile acid concentration measured 5 h postprandial tended to be reduced (about 58%) in fish fed the low methionine diet as compared to those fed the methionine-sufficient diet (Fig. 3a, $p = 0.084$) of which might indicate less export from liver to plasma or reduced synthesis from cholesterol. Faecal total bile acid concentration on the other hand was significantly reduced ($p = 0.037$) in fish fed the methionine-limiting diet (Fig. 3b). Thus, the salmon fed the low methionine diet had reduced taurine concentrations in plasma and liver, as well as altered bile acid metabolism.

Discussion

The present study was undertaken to investigate whether a sub-optimal dietary methionine level affected the liver lipid metabolism and possibly increase the lipid stores which might contribute to our understanding of factors contributing to the increased relative hepatic size previously observed in fish fed diets with methionine limitations (Rumsey et al. 1983; Rodehutscord et al. 1997; Espe et al. 2008). There are several intersections between hepatic methionine metabolism and lipid metabolism, including metabolic pathways involved in lipoprotein secretion, bile acid metabolism and in the de novo lipogenesis.

In the present study, the salmon given the low methionine diet had significantly higher relative liver weight and elevated liver TAG concentrations, whereas liver levels of phospholipids and cholesterol were not significantly altered. Our results are not in agreement with the findings of Dias et al. (2005) who reported reduced hepatic size and lower TAG concentrations in liver of European seabass fed soy protein as compared to fishmeal-fed controls. Dias et al. (2005) also reported elevated liver levels of phospholipids and cholesterol in the fish fed the soy protein-based diets. They postulated that the results obtained probably was due to a combination of reduced voluntary feed intake in fish fed the plant protein diets and the imbalanced amino acid profiles in the plant protein-based diets. It is known that a methionine and choline-deficient diet causes fatty liver in rodents (Chawla et al. 1998) which pinpoints a role for methionine in the development of fatty liver. Knockout MAT1A mice do not express the gene encoding for the liver-specific isoform of methionine adenosine transferase, MAT1A. These mice have a strongly reduced capacity for conversion of methionine to SAM that leads to a dramatically increased plasma methionine concentration (776%) and a reduced hepatic SAM concentration (−74%, Lu et al. 2001). Concomitantly, the MAT1A$^{-/-}$ mice develop fatty livers and have 40% higher relative liver weights (Lu et al. 2001). Thus, sufficient hepatic methylation capacity might be important in the protection against increased liver lipids. One mechanism that has been linked to hepatic TAG accumulation is insufficient synthesis of phosphatidylcholine (PC) in the rat liver causing a lower secretion of lipoproteins (Noga and Vance 2003; Dolinsky et al. 2004; Vance 2008), In the liver, synthesis of PC can either take place through the classic Kennedy pathway, or by three successive methylation reactions of phosphatidylethanolamine (PE) catalysed by the enzyme pemt (Noga and Vance 2003; Shields et al. 2005; Stead et al. 2006; Vance 2008). Previously it has been found that the synthesis of PC from PE consumes almost 50% of SAM present in the liver (Jacobs et al. 2005; Shields et al. 2005). Sugiyama et al. (1998) reported increased PC/PE ratio, due to higher PC concentrations, and elevated plasma cholesterol in rats fed increasing levels of methionine. In the current study the liver PC/PE ratio and the plasma TAG, total- and

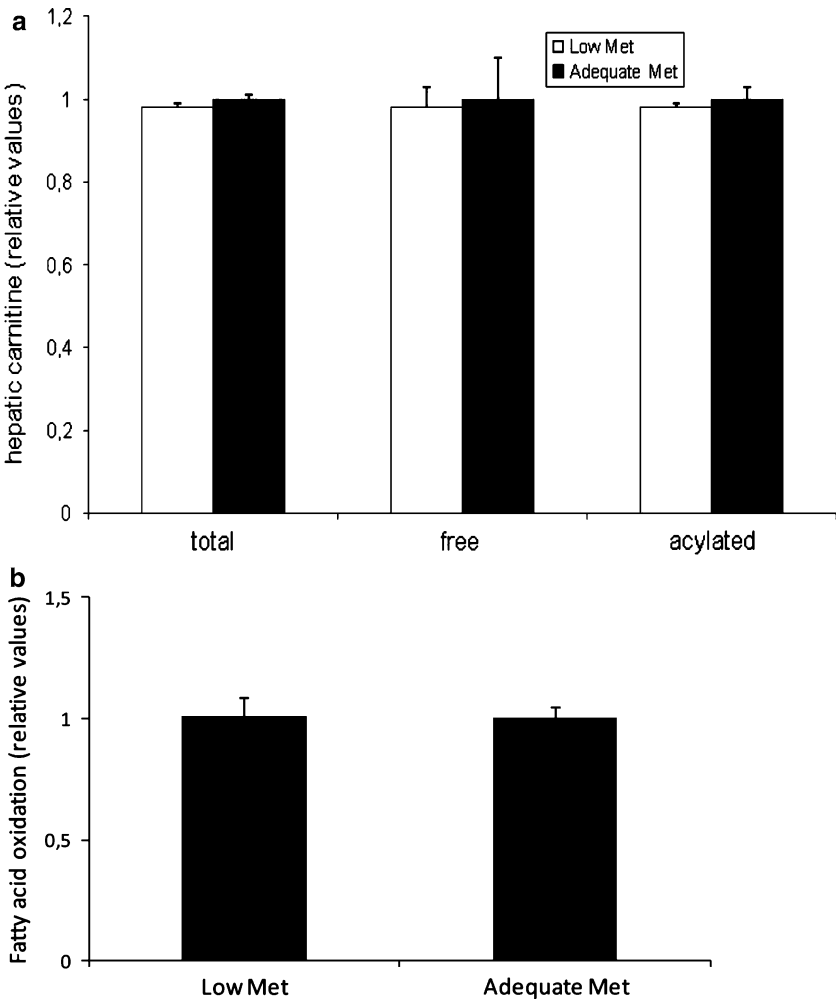

Fig. 2 Hepatic carnitine (**a**) and total fatty acid oxidation capacity (**b**) were unaffected by methionine limitation. Values are relative to the adequate methionine-fed group. Values are means ± SE

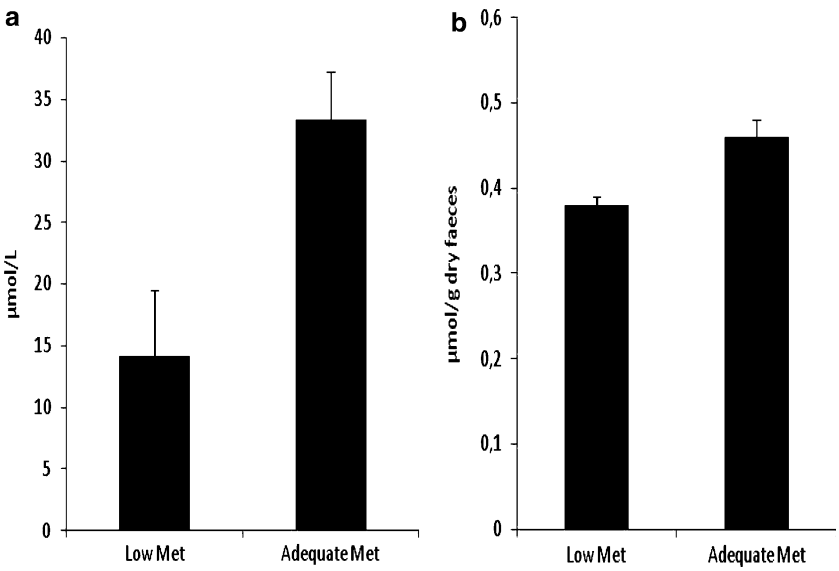

Fig. 3 Plasma (**a**, µmol/L, $p = 0.084$) and faecal total bile acids (**b**, µmol/g dry faeces, $p = 0.037$). Values are means ± SE

HDL-cholesterol levels were unaffected by methionine intake. It is possible that our model did not provide a sufficiently low methionine intake or that a feeding period of 3 months was too short to affect the phospholipids significantly. Although a correlation between methionine intake and the hepatic PC ($r = 0.89$, $p = 0.044$) existed

and the SAM tended to be reduced in the fish fed the low methionine diet ($r = 0.81$, $p = 0.10$). Therefore, to assess the methionine limitations on the endogenous PC synthesis as described in rodents (Cui and Vance 1996; Vance and Walkey 1998; Walkey et al. 1998) and the capacity of methylation (Sugiyama et al. 1998) following low methionine administration in fish should be addressed in forthcoming studies addressing the effect of methionine/choline limitations in fish feeds.

Another mechanism that could have been impaired by reduced methylation capacity was the carnitine synthesis, important for efficient mitochondrial fatty acid oxidation. However, no difference was found in either liver carnitine concentrations or total fatty acid oxidation capacity between the salmon fed the sub-optimal and adequate methionine levels. Thus, it is not likely that reduced hepatic methylation capacity in the salmon fed the low methionine diet was the underlying mechanism that led to higher liver TAG concentrations in the current experiment.

Methionine is a precursor of endogenous taurine synthesis, and the protein sources in the present diets were mainly based on wheat, that is devoid of taurine, whereas only a small fraction of the feed ingredients contained taurine. Therefore, the salmon in the present study needed to support dietary taurine with endogenous taurine synthesis. We previously showed that taurine in liver of Atlantic salmon increased when methionine intake increased (Espe et al. 2008). In the present study, the salmon fed sub-optimal methionine levels had significantly lower liver and plasma taurine concentrations, relative to those fed adequate methionine levels. In the liver, taurine might be conjugated to bile acids, a process that increases the bile acids' solubility and enhances their biliary secretion (Vessey et al. 1983). Indeed, taurine deficiency has been reported to decrease total bile acid excretion in faeces (Chen et al. 2004). Furthermore, we recently showed that feeding a taurine-rich fish protein hydrolysate diet to rats increased faecal and plasma bile acid concentrations, as compared to those fed the taurine-free casein diet (Liaset et al. 2009). By feeding Atlantic salmon a fish meal-based diet, thus containing higher taurine levels than the current experiment, fish fed the diet added crystalline methionine had higher taurine concentration in the liver, but the increased relative liver size present in the current study did not occur. Neither was TAG in liver affected (Espe et al., unpublished results). In the present study, the salmon receiving the sub-optimal methionine diet had lower liver taurine concentrations, and these fish also had significantly decreased faecal bile acid concentrations and tended to have decreased plasma bile acid concentrations. Thus, decreased taurine availability might have caused this reduction in bile acid levels in the salmon fed sub-optimal methionine diets.

Bile acids are endogenous activators of the nuclear receptor farnesoid X receptor (FXR) (Makishima et al. 1999; Parks et al. 1999). Increasing the bile acid concentration by cholic acid feeding has been shown to decrease liver and plasma TAG concentrations in mice (Watanabe et al. 2004). The reduction in hepatic TAG concentrations was explained by a FXR-dependent suppression of the transcription factor sterol response element binding protein 1c (SREBP-1c) activity (Watanabe et al. 2004). The de novo lipogenesis is strongly promoted by increased SREBP-1 activity, as this transcription factor induces transcription of the genes encoding for acetyl-coenzyme A carboxylase (ACC-1), fatty acid synthase (FAS) and stearoyl-coenzyme A desaturase (SCD), all involved in fatty acid synthesis (Horton et al. 2003). Therefore, the elevated hepatic TAG levels found in the salmon fed sub-optimal methionine levels in the present study might have been caused by the lower bile acid concentrations promoting a higher SREBP-1 activity and increased lipogenesis. Unfortunately the codon for SREBP-1c in salmonids has not been published yet (Zheng et al. 2009). However, the hepatic FAS capacity was dramatically increased in the salmon given low methionine diets and this indicates that these fish had increased hepatic lipogenesis. In further support of an increased SERBP-1 activity, the significantly increased hepatic 18:1 to 18:0 fatty acid ratio in the fish given low methionine diets. The conversion of saturated to monounsaturated fatty acids are catalysed by SCDs, and the increase in 18:1 to 18:0 indicates a higher SCD activity in these fish. The increased TAG accumulation and lipogenetic enzyme activity, followed by the altered fatty acid ratios as a result of methionine limitations in Atlantic salmon coincide with the changes occurring in the non-alcoholic fatty liver disease (NAFLD) described in mammalian species fed diets low in methionine or fed diets that causes a reduced betaine choline pathway (Sugiyama et al. 1998; Anstee and Goldin 2006; El-Badry et al. 2007; Puri et al. 2007; Kohjima et al. 2007; Allard et al. 2008; Kwon et al. 2009). The fatty liver disease might if allowed to continue untreated result in inflammation, apoptosis and necrosis and might become detrimental for the general health of the farmed fish.

In conclusion, salmon fed sub-optimal methionine levels had an increased relative liver weight, elevated hepatic FAS capacity, increased 18:1 to 18:0 fatty acid ratio and higher liver TAG concentrations. It is not likely that the methylation capacity in the liver was sufficiently reduced to cause the observed alterations in lipid metabolism. Rather, our data suggests that the lower available taurine resulted in decreased bile acid concentrations, and that this reduction might have facilitated a higher lipogenesis leading to hepatic TAG accumulation.

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