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RESEARCH ARTICLE

Early feeding of rainbow trout (*Oncorhynchus mykiss*) with methionine-deficient diet over a 2 week period: consequences for liver mitochondria in juveniles

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ABSTRACT

Methionine is a key factor in modulating the cellular availability of the main biological methyl donor *S*-adenosylmethionine (SAM), which is required for all biological methylation reactions including DNA and histone methylation. As such, it represents a potential critical factor in nutritional programming. Here, we investigated whether early methionine restriction at first feeding could have long-term programmed metabolic consequences in rainbow trout. For this purpose, trout fry were fed with either a control diet (C) or a methionine-deficient diet (MD) for 2 weeks from the first exogenous feeding. Next, fish were subjected to a 5 month growth trial with a standard diet followed by a 2 week challenge (with the MD or C diet) to test the programming effect of the early methionine restriction. The results showed that, whatever the dietary treatment of fry, the 2 week challenge with the MD diet led to a general mitochondrial defect associated with an increase in endoplasmic reticulum stress, mitophagy and apoptosis, highlighting the existence of complex cross-talk between these different functions. Moreover, for the first time, we also observed that fish fed the MD diet at the first meal later exhibited an increase in several critical factors of mitophagy, hinting that the early nutritional stimulus with methionine deficiency resulted in long-term programming of this cell function. Together, these data extend our understanding of the role of dietary methionine and emphasize the potential for this amino acid in the application of new feeding strategies, such as nutritional programming, to optimize the nutrition and health of farmed fish.

KEY WORDS: Fish, Mitophagy, Mitochondria, ER stress, DNA and histone methylation, Nutritional programming

INTRODUCTION

For more than 20 years, it has been widely accepted that nutritional stimuli (quantity or quality of nutrients) experienced at critical periods of an organism's life can result in permanent changes in postnatal growth potential, health and metabolic status in animals (Burdge and Lillycrop, 2010; Lucas, 1991, 1998), referred to as nutritional programming. The concept of nutritional programming is increasingly studied in aquaculture because it opens new areas of research and opportunities toward the optimization of the nutrition and health of farmed animals with obvious economic implications (Panserat et al., 2019). Thus, although still limited, information available today stems from pioneering studies that explored the possibility of altering the functioning of long-chain fatty acid desaturation in European seabass (Vagner et al., 2009) or the use of dietary carbohydrates in rainbow trout (Geurden et al., 2007, 2014) and zebrafish (Fang et al., 2014). In the same trend, we reported the positive impact of the early feeding of a plant-based diet on its future acceptance and utilization in rainbow trout (Balasubramanian et al., 2016; Geurden et al., 2013), raising the interesting possibility of directing specific metabolic pathways or functions in juvenile fish to improve the use of substitutes for fish meal and oil, and hence to promote sustainability in aquaculture.

One possible biological process for imprinting the nutritional programming event until adulthood is the epigenetic regulation of gene expression, which has received considerable attention in recent years (Block and El-Osta, 2017; Lillycrop and Burdge, 2012). Epigenetics is defined as heritable changes in gene expression (transmitted from cell to cell or from generation to generation) that are not caused by alterations in the DNA sequence. Molecular mechanisms that mediate epigenetic regulation include DNA and histone methylation. These covalent modifications of DNA or histones can influence the structure of chromatin and, therefore, gene expression from the early stages of development and throughout life.

Interestingly, dietary methionine emerged as a key factor in modulating the cellular availability of the main biological methyl donor *S*-adenosylmethionine (SAM) needed for all biological methylation reactions including DNA and histone methylation (Anderson et al., 2012; Niculescu and Zeisel, 2002; Waterland, 2006). As such, methionine potentially represents a critical factor in nutritional programming. In this regard, we recently reported that feeding a methionine-deficient diet to rainbow trout broodstock for 6 months affected the activation and/or expression of several key metabolic factors in offspring through DNA methylation (Veron et al., 2018), confirming the possibility of nutritional programming in fish through parental methionine nutrition (Fontagné-Dicharry et al., 2017; Seiliez et al., 2017).

In order to deepen our knowledge of the nutritional programming potential of methionine, in the present study we investigated whether early methionine restriction at first feeding could have programmed metabolic consequences in rainbow trout. For this purpose, fry were fed with either a control (C) diet or a methionine-deficient (MD) diet for 2 weeks from the first exogenous feeding. Next, fish were subjected to an 18 week growth trial with a commercial diet followed by a 2 week challenge with the MD diet (compared with the C diet). Recently, we showed that a dietary

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List of abbreviations

asns	*asparagine synthetase*
C	control (diet)
CII	succinate dehydrogenase
CIV	cytochrome *c* oxidase
ddit3	*DNA-damage inducible transcript 3*
DM	dry matter
eef1α1	*eukaryotic translation elongation factor 1α1*
eIF2α	eukaryotic translation initiation factor 2α
ER	endoplasmic reticulum
GCN2	general control non-depressible 2
HSI	hepatosomatic index
MD	methionine deficient (diet)
MFN2	mitofusin2
mtDNA	mitochondrial DNA
mTOR	mechanistic target of rapamycin (serine/threonine kinase)
PARKIN	parkin RBR E3 ubiquitin protein ligase
PARP	poly (ADP-ribose) polymerase
p-S65-Ub	phospho-ubiquitin (Ser65)
S6	ribosomal protein S6
SAH	*S*-adenosyl-L-homocysteine
SAM	*S*-adenosylmethionine
TG	triglyceride
TIM23	translocase of inner mitochondrial membrane 23
UPR	unfolded protein response
xbp1	*x-box binding protein 1*

methionine deficiency caused a general hepatic mitochondrial defect, associated with the induction of mitochondrial degradation through mitophagy (Séité et al., 2018). Here, we therefore focused on hepatic mitochondrial integrity, function and degradation through mitophagy and investigated the associated mechanisms.

MATERIALS AND METHODS

Ethics

The experiments were conducted in compliance with the legal frameworks of France and the EU. They respect directive 2010/63/EU relating to the protection of animals used for scientific purposes, and decree no. 2013-118, 1 February 2013 of the French legislation governing the ethical treatment of animals. The protocol was approved by the French National Consultative Ethics Committee under reference number APAFIS8222-2017041016141425-v4.

Feeding trial and rearing

Swim-up fry of rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792), with a mean initial mass of 0.1 g were reared in the INRA experimental facilities (Donzacq, France) at a constant water temperature (~17°C) under natural photoperiod (June to December). The fish were randomly distributed into 6 tanks (17 l, 220 fish per tank). The first feeding fry were fed for 2 weeks with one of the two iso-nitrogenous (41% crude protein) and isoenergetic (23 kJ g^{-1} dry matter, DM) extruded diets (n=3 tanks per diet) manufactured at the INRA experimental facilities (Donzacq) (Table 1). The two experimental diets were formulated to meet nutrient requirements of rainbow trout (NRC 2011, AMINOSalmonid®), except for the methionine content, which was adequate (0.91% diet, as-fed basis) in the C diet and restricted (0.39% diet, as-fed basis) in the MD diet (Table 1). The cysteine (Cys) level was kept constant at 0.43±0.1% diet. On day 14, 16 h after the last meal, 15 fish per tank were terminally anaesthetized by bath immersion in benzocaine (30 mg l^{-1} water), and snap-frozen in liquid nitrogen, then stored at −80°C until further mRNA analyses.

Table 1. Ingredients and analytical composition of the control (C) and methionine-deficient (MD) diets

Ingredients and analytical composition	C	MD	Amino acids	C	MD
Fish protein concentrate[a]	5	5	Cysteine	0.43	0.44
Faba bean protein concentrate[b]	17.5	17.5	Histidine	1.18	1.20
Soy protein concentrate[c]	17.5	17.5	Isoleucine	1.90	1.91
White lupin meal[d]	12	12	Lysine	2.61	2.68
Dehulled pea meal[e]	6	6	**Methionine**	**0.91**	**0.39**
Fish oil[f]	15	15	Phenylalanine	2.13	2.20
Gelatinized starch[g]	10	10	Threonine	1.89	1.95
$CaHPO_4{\cdot}2H_2O$ (18% P)	3	3	Valine	2.18	2.21
Mineral premix, INR1[h]	2	2	Alanine	2.40	2.55
Vitamin premix, INRA[i]	2	2	Aspartic acid	3.54	3.70
Free amino acid	10*	10**	Glutamic acid	4.98	5.08
Dry matter	96.94	96.73	Glycine	2.71	2.89
Carbohydrate	13.85	13.91	Proline	1.91	1.96
Crude protein	41.42	41.66	Serine	1.90	2.01
Lipid (% DM)	18.04	18.15			
Gross energy (% DM)	23.21	23.21			

Percentages are given for each diet; analytical composition [dry matter (DM), carbohydrate, crude protein, lipid and gross energy] and amino acid content data are percentage as fed unless noted otherwise. [a]CPSP-G (Sopropêche, Wimille, France); [b]Fabaqua 55 (Sotexpro); [c]Estrilvo (CP 70, Sopropêche); [d]Farilup500 (Terrena); [e]Aquatex (Sotexpro); [f]southern hemisphere (Sopropêche); [g]Roquette (Lestrem, France); [h]mineral premix (g or mg kg^{-1} diet): 4.3 g calcium carbonate (40% Ca), 2.48 g magnesium oxide (60% Mg), 0.4 g ferric citrate (21% Fe), 0.8 mg potassium iodide (76% I), 0.08 g zinc sulfate (36% Zn), 0.6 g copper sulfate (25% Cu), 0.06 g manganese sulfate (33% Mn), 10 g dibasic calcium phosphate (23% Ca, 18% P), 0.4 mg cobalt sulfate, 0.6 mg sodium selenite (46% Se), 1.8 g KCl and 0.8 g NaCl; [i]vitamin premix (IU or mass kg^{-1} diet): 120 IU DL-a tocopherol acetate, 10 mg sodium menadione bisulfate, 30,000 IU retinyl acetate, 6000 IU DL-cholecalciferol, 30 mg thiamine, 60 mg riboflavin, 30 mg pyridoxine, 0.1 mg B12, 350 mg nicotinic acid, 1 g folic acid, 2 g inositol, 5 mg biotin, 0.1 g calcium pantothenate and 4 g choline chloride. *Free amino acid (% of the diet): 0.07% arginine, 0% cysteine, 0.42% histidine, 0.30% isoleucine, 0.77% leucine, 1.36% lysine, 0.55% DL-methionine, 0.5% phenylalanine, 0.81% threonine, 0.07% tryptophan, 0.45% tyrosine, 0.54% valine, 0.97% alanine, 0.51% aspartic acid, 0.45% glutamic acid, 1.20% glycine, 0.52% proline and 0.5% serine (Evonik). **Free amino acid (% of the diet): 0.07% arginine, 0% cysteine, 0.42% histidine, 0.30% isoleucine, 0.77% leucine, 1.36% lysine, 0.0% DL-methionine, 0.5% phenylalanine, 0.81% threonine, 0.07% tryptophan, 0.45% tyrosine, 0.54% valine, 1.10% alanine, 0.58% aspartic acid, 0.51% glutamic acid, 1.35% glycine, 0.58% proline and 0.57% serine.

The remaining fish (n=205 per tank) were subjected to an 18 week growth trial with a common standard diet (Table 2) manufactured at the INRA experimental facilities (Donzacq). Subsequently, rainbow trout from each of the two groups were randomly distributed into 6 tanks (130 l, 45 fish per tank) and subjected to a 2 week challenge with the MD diet or the C diet (Fig. 1). At the end of the final challenge and 16 h after the last meal, blood and liver were collected from 3 fish per tank as follows. Fish were anaesthetized with benzocaine (30 mg l^{-1-1}) and killed by a sharp blow to the head. Blood samples from the caudal vein (3 fish per tank) were collected into heparinized syringes and centrifuged at 3000 ***g*** for 5 min; the recovered plasma was immediately frozen and kept at −20°C. Livers were collected, dissected, weighed and immediately frozen in liquid nitrogen and kept at −80°C. Fish were counted and weighed at the beginning and end (n=6 randomly selected fish per tank, 3 tanks per group) of the feeding trial to follow fish growth and food utilization. Individual body mass, daily growth index, daily food intake and food efficiency were calculated as described by Belghit et al. (2014).

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Table 2. Ingredients and analytical composition of the standard (S) diet

Ingredients and analytical composition	S	Amino acids	S
Fish meal 70[a]	32	Cysteine	0.71
Corn gluten meal[b]	9	Histidine	1.04
Wheat gluten meal[c]	10	Isoleucine	2.18
Soybean meal[d]	2.5	Lysine	2.77
Soyabean protein concentrate[e]	8.5	Methionine	1.09
White lupin meal[f]	2.5	Phenylalanine	2.40
Dehulled pea meal[g]	1.5	Threonine	1.75
Rapeseed meal[h]	2	Valine	2.40
Whole wheat[i]	14	Alanine	2.70
Soyabean lecithin[j]	1	Aspartic acid	3.86
Mineral premix[k]	1	Glutamic acid	9.48
Vitamin premix[l]	1	Glycine	2.40
Fish oil[m]	15	Proline	3.18
Dry matter	96.59	Serine	2.17
Carbohydrate	11.27		
Crude protein (% DM)	48.51		
Lipids (% DM)	20.42		
Gross energy (% DM)	24.01		

Percentages are given for each diet; analytical composition [dry matter (DM), carbohydrate, crude protein, lipid and gross energy] and amino acid content data are percentage as fed unless noted otherwise. [a]Sopropêche (Wimille, France); [b]Soal; [c]Roquette (Lestrem, France); [d]CP 48 (Inzo, France); [e]Estrilvo CP 70 (Sopropêche); [f]Farilup500 (Terrena); [g]Aquatex (Sotexpro); [h]Primor 00 (Sud Ouest Aliment, France); [i]Sud Ouest Aliment, France; [j]Louis François (Croissy-Beaubourg, France); [k]mineral mixture (g kg^{-1} premix): 500 $CaHPO_4{\cdot}2H_2O$, 215 $CaCO_3$, 124 $Mg(OH)_2$, 90 KCl, 40 NaCl, 20 $FeSO_4{\cdot}7H_2O$, 4 $ZnSO_4{\cdot}7H_2O$, 3 $MnSO_4{\cdot}H_2O$, 3 $CuSO_4{\cdot}5H_2O$, 10 NaF, 0.04 KI, 0.03 Na_2SeO_3 and 0.02 $CoCl_2{\cdot}6H_2O$ (all ingredients were diluted with α-cellulose); [l]vitamin premix (IU or mass kg^{-1} premix): 500,000 IU retinyl acetate, 250,000 IU cholecalciferol, 5000 IU DL-α-tocopheryl acetate, 1 g sodium menadione bisulfate, 0.1 g thiamin-HCl, 0.4 g riboflavin, 1 g niacin, 2 g D-calcium pantothenate, 0.3 g pyridoxine-HCl, 20 mg D-biotin, 0.1 g folic acid, 1 mg cyanocobalamin, 5 g L-ascorbyl-2-polyphosphate, 30 g *myo*-inositol and 100 g choline (all ingredients were diluted with α-cellulose); [m]Sopropêche.

Fry liver area

On day 14, 16 h after the last meal, 3 fry per condition were killed by bath immersion in benzocaine (30 mg l^{-1} water), and the livers were sampled and photographed (at the same time) to measure their area. Measurements were performed using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). The data are expressed as a percentage of the control condition, which correspond to: (area of liver×100)/control group mean.

Isolation of mitochondria

At the end of the final challenge, 3 fish per tank were killed directly by a sharp blow to the head and livers were quickly collected, minced and homogenized with a Potter homogenizer in 5 ml of ice-cold mitochondrial isolation buffer (MIB, 275 mmol l^{-1} sucrose, 20 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EGTA, 1 mg ml^{-1} BSA, pH 7.2). Mitochondria were purified by differential centrifugation (1000 ***g*** for 10 min at 4°C) and supernatants were then centrifuged at 10,000 ***g*** for 10 min at 4°C. Finally, the crude mitochondrial pellet was resuspended in an appropriate volume of MIB. Mitochondrial protein concentration was determined using the Bradford reagent method.

Chemical composition of the diets

DM, crude fat and gross energy content of the diets were determined following procedures previously outlined (Belghit et al., 2014). Crude protein content was measured as N×6.25 by the Kjeldahl method after acid digestion (ISO 937:1978), using an FP-2000 analyser (Leco Corp., St Joseph, MI, USA). Starch content was measured by an enzymatic method (InVivo Labs). Dietary amino acid concentration was obtained by wet chemistry at Evonik-Degussa Laboratory (Hanau, Germany) by ion-exchange chromatography with post-column derivatization with ninhydrin. Amino acids were oxidized with performic acid, and neutralized with sodium metabisulfite (Llames and Fontaine, 1994; Commission Directive 1998). Amino acids were liberated from the protein by hydrolysis with 6 mol l^{-1} HCl for 24 h at 110°C and quantified with the internal standard method by measuring the absorption of reaction products with ninhydrin at 570 nm.

Western blot analysis

At the end of the final challenge, livers sampled 16 h after the last meal (*N*=6 per condition) were homogenized and analysed as

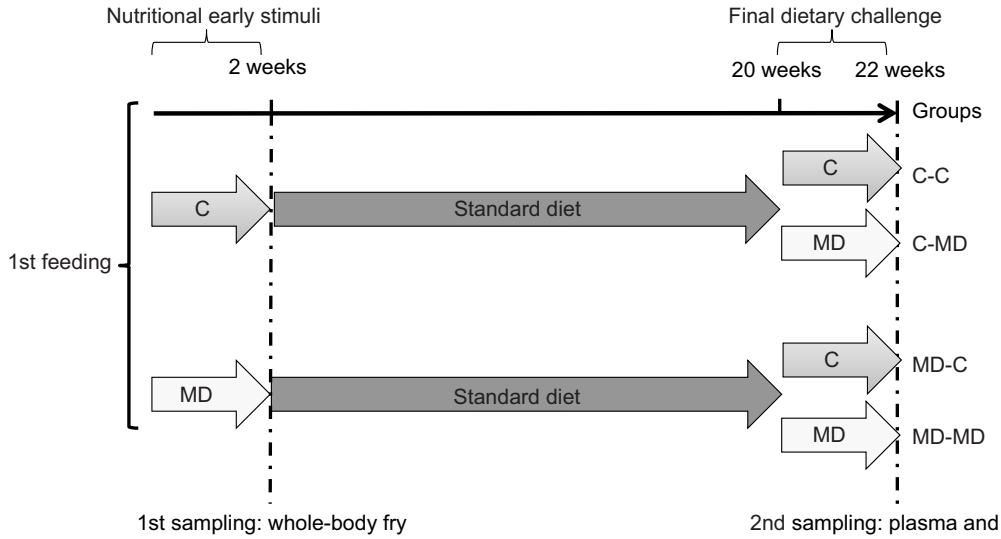

Fig. 1. Experimental set-up. At first feeding, fry were fed with either a control diet (C) containing 0.91% methionine or a methionine-deficient diet (MD) containing 0.39% methionine for 2 weeks. Fish were then subjected to an 18 week growth trial with a standard diet, followed by a 2 week challenge test with the MD diet or the C diet in order to test the existence of nutritional programming. Resulting groups are indicated on the right. Samples were taken at the indicated times, 16 h after the last meal.

previously detailed (Belghit et al., 2014) using the following antibodies: anti-phospho-ribosomal protein S6 (S6 Ser235/Ser236, 4856, Cell Signaling Technologies); anti-carboxyl terminal S6 (2217, Cell Signaling Technologies); anti-phospho-eukaryotic translation initiation factor 2α (eIF2α Ser51, 9721, Cell Signaling Technology); anti-carboxyl terminal eIF2α (9722, Cell Signaling Technology); anti-β-tubulin (2146, Cell Signaling Technology); anti-translocase of inner mitochondrial membrane 23 (TIM23, 611222, BD Transduction Laboratories™), anti-mitofusin2 (MFN2, ab56889, Abcam); anti-parkin RBR E3 ubiquitin protein ligase (PARKIN, ab15954, Abcam); anti-phospho-ubiquitin (Ser65) (Ser65, ABS1513-I, EMD Millipore); anti-total ubiquitin (MAB1510, EMD Millipore); and anti-cleaved poly (ADP-ribose) polymerase (PARP, 95425, Cell Signaling Technology). All of these antibodies (except anti-cleaved PARP) have already been validated in rainbow trout (Belghit et al., 2014; Seiliez et al., 2016; Séité et al., 2018). For cleaved PARP, the amino sequence of the corresponding protein was monitored in the SIGENAE database (http://www.sigenae.org) to check for the conservation of the antigen sequence with the corresponding sequence from mammals, ensuring a good specificity of the mammalian antibody used in the analysis of our samples. In order to measure the global levels of selected histone modification (H3K4me3, H3K9me3 and H3K36me3), histones from livers were extracted according to a previously detailed protocol (Liu et al., 2017). Histone isolation was used for western blot analysis as described above and using the appropriate antibodies: anti-H3K4me3 (C15410003, Diagenode); anti-H3K9me3 (C15410056, Diagenode); anti-H3K36me3 (15410192, Diagenode); and anti-H3 (ab1791, Abcam).

Enzyme activity

Mitochondrial proteins (10–30 µg) were diluted in phosphate buffer (50 mmol l^{-1} KH_2PO_4, pH 7.5), and then subjected to spectrophotometric analysis to measure enzymatic activity of citrate synthase, succinate dehydrogenase (complex II) and cytochrome *c* oxidase (complex IV) at 37°C. Citrate synthase activity was measured at 412 nm (extinction coefficient ε=13,600 l mol^{-1} cm^{-1}) after the addition of 0.1 mmol l^{-1} acetyl-CoA, 0.1 mmol l^{-1} oxaloacetate and 0.1 mmol l^{-1} 5,5-dithiobis-2-nitrobenzoic acid (DTNB). Complex II activity was measured at 600 nm (ε=21 l mol^{-1} cm^{-1}) after the addition of 0.1 mmol l^{-1} phenazine methosulfate (PMS), 40 mmol l^{-1} succinate, 0.1 mmol l^{-1} dichlorophenolindophenol (DCPIP) and 40 mmol l^{-1} malonate. Complex IV activity was determined at 550 nm (ε=18,500 l mol^{-1} cm^{-1}) after the addition of 12.5 µg ml^{-1} antimycin and 100 µmol l^{-1} cytochrome *c*. The specific complex IV activity was defined as the flux difference before and after the addition of 1 mmol l^{-1} KCN (inhibitor of complex IV).

Reverse transcription-quantitative PCR analysis

Reverse transcription-quantitative PCR (RT-qPCR) analysis was performed on whole fry sampled 16 h after the last meal during the first sampling (*N*=6 per diet) and on the liver of fish sampled 16 h after the feeding trial (second sampling, *N*=6 per condition) The protocol conditions for sample preparation and RT-qPCR were as previously described (Fontagné-Dicharry et al., 2015; Seiliez et al., 2016). The primers used for qPCR assays were also as described in previous studies (Seiliez et al., 2016; Séité et al., 2018; Séité et al., 2019). For the expression analysis, relative quantification of target gene expression was done using the ΔCT method (Pfaffl et al., 2002). The relative gene expression of *eukaryotic translation elongation factor 1α1* (*eef1α1*) was used for the normalization of the measured expression values of the target mRNA, and it was found not to change significantly among dietary treatments (data not shown).

Determination of mitochondrial DNA (mtDNA) copy number

DNA isolation was performed on fish liver sampled 16 h after the last meal (second sampling, *N*=6 per condition) following a previously detailed protocol (Liu et al., 2017). mtDNA copy number was measured using qPCR as described above. mtDNA copy number (mitochondrially encoded tRNA leucine 1UUA/G: forward primer: AAAACAGACAAGGGGGCACA, reverse primer: AGGGTGAG-GAAAGCAACTGC) was normalized to β-actin genomic DNA in the sample (forward primer: GATGGGCCAGAAAGACAGCTA, reverse primer: TCGTCCCAGTTGGTGACGAT).

Methionine and related metabolites in liver

At the end of the final challenge, livers sampled 16 h after the last meal (second sampling, *N*=6 per condition) were homogenized in 10 volumes of phosphate buffer (20 mmol l^{-1}; 1 mmol l^{-1} EDTA, pH 6.5) using an ULTRA-TURRAX homogenizer (IMLAB sarl). Homogenates were centrifuged at 4°C for 15 min at 10,000 ***g***. A sample of the supernatant was used for protein quantification using the Lowry method. Another sample of the supernatant was deproteinized with an equal volume of meta-phosphoric acid (MPA 2.5%) and centrifuged for 5 min at 2000 ***g***. The supernatant was collected for HPLC analysis. Hepatic methionine was analysed according to Cohen and Michaud (1993). The liver aliquot was derivatized using an AccQ·Fluor™ reagent kit (Waters®, Milford, MA, USA) according to the manufacturer's instructions and separated using an AccQ·Tag™ column (3.9 mm×150 mm i.d. 4 µm; Waters®). The column was operated at 37°C. The injection volume was 20 µl and the flow rate was set at 1 ml min^{-1}. The eluate was monitored with fluorescence detection (excitation at 250 nm, emission at 395 nm) for 45 min using the manufacturer's recommended elution gradient. The standard L-methionine was purchased from Waters®.

SAM and *S*-adenosyl-L-homocysteine (SAH) in the liver were analysed according to the modified protocol of Cook et al. (1989). Chromatographic separation was achieved on a Resolve C18 column (3.9 mm×150 mm i.d. 5 µm; Waters®). The column was operated at 40°C. The injection volume was 50 µl and the flow rate was set at 0.7 ml min^{-1}. The eluate was monitored with absorbance detection at 258 nm. A binary solvent system was used, consisting of (A) phosphate buffer 20 mmol l^{-1} pH 7.2 (TFA) with OSA 8 mmol l^{-1}; and (B) methanol. The following gradient elution was employed: 0–10 min: 95% A, 5% B; 20 min: 30% A, 70% B; 35–40 min (column equilibration): 95% A, 5% B. The standards SAM and SAH were purchased from Sigma-Aldrich®.

Protein, triglyceride (TG) and glycogen levels in liver

Liver soluble-protein fraction was determined with the Bradford reagent method (Bradford, 1976). For TG determination, tissues were first homogenized in 5% Igepal (Sigma-Aldrich). The samples were then slowly heated from room temperature to 90°C in a dry bath for 2–5 min, and then cooled down to room temperature (this step was repeated once). After centrifugation at 5000 ***g*** for 5 min at 4°C, TG levels were measured from the supernatant using the triglycerides LQ GPO-POD method (Sobioda, Montbonnot, France). Glycogen content was determined by a previously described hydrolysis technique (Good et al., 1933). Each sample was ground in 1 mol l^{-1} HCl (VWR, Radnor, PA, USA). An aliquot was saved at this stage and neutralized with 5 mol l^{-1} KOH (VWR) to measure free glucose content in samples. After 5 min centrifugation at 5000 ***g*** at 4°C, free

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glucose was measured from supernatant using a plasma glucose kit (Glucose RTU, BioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Remaining ground tissue was boiled at 100°C for 2.5 h and then neutralized with 5 mol l^{-1} KOH (VWR). After 5 min centrifugation at 5000 ***g*** at 4°C, total glucose (free glucose+glucose obtained from glycogen hydrolysis) was measured from supernatant using the same kit as before. Glycogen content was evaluated by subtracting free glucose levels.

Global DNA methylation

DNA isolation was performed on fish liver sampled 16 h after the last meal (second sampling, *N*=6 per condition) using a DNA and RNA kit (80204, Qiagen). DNA was quantified by Qubit (k32854, ThermoFisher Scientific) and the quality (integrity of DNA) was verified on a 1% agarose gel. Global DNA methylation was assessed at the Epigenomics core facility at the Paris Diderot University by the LUminometric Methylation Assay (LUMA) as previously described (Karimi et al., 2006).

Statistics

Data are expressed as means±s.d. Normality was assessed using the Shapiro–Wilk test, while the equality of variances was determined using Levene's test. When the normality and/or equal variances of data were respected, a *t*-test or a two-way ANOVA was used to detect significant differences. When the normality and/or equal variances of data were not respected, PERMANOVA was used. Following two-way ANOVA or PERMANOVA analysis, the Tukey test was used for *post hoc* analysis. For all statistical analyses, the level of significance was set at $P<0.05$.

RESULTS

Expression of genes related to methionine deficiency in fry

We first aimed to verify whether the fry showed a response to the 2 week dietary methionine deficiency at the first meal. For this purpose, we measured the mRNA levels of two genes, *asparagine synthetase* (*asns*) and *DNA-damage inducible transcript 3* (*ddit3*), known to be overexpressed upon amino acid deficiency. As shown in Fig. 2A, fry fed the MD diet exhibited significantly higher *asns* and *ddit3* mRNA levels. Interestingly, this effect was accompanied by an increase in the size of the liver of MD-fed fry (Fig. 2B). Overall, these data indicate that the 2 week dietary methionine deficiency was long enough to cause a change in the fry and to potentially induce long-term effects.

Direct and programmed effects of dietary methionine deficiency on survival and growth parameters

We then sought to determine the effect of the 2 week dietary methionine deficiency on the survival and growth performance in both the short term (direct effect) and the long term (programming effect). Early methionine deficiency did not show any effects on survival and growth parameters in fry, or in juveniles fed the standard diet for 18 weeks (Table 3). In contrast, after the 2 week final challenge, although the survival of the fish was not impacted, fish fed the MD diet at the first meal (MD-C and MD-MD) presented significantly higher mass than those fed the C diet at the first meal (C-C and C-MD) (Table 3). We also measured the hepatosomatic index (HSI), i.e. the liver to body mass ratio, as a marker of methionine deficiency stress (Table 3). The results indicated that, whatever the first feeding of the fry, the 2 week final challenge with the MD diet affected the HSI of the fish. However, for the fish fed the MD diet (C-MD and MD-MD), those stimulated with the MD diet at the first feeding (MD-MD) exhibited a significantly higher HSI than their control counterparts (C-MD). Together, these results clearly show that a short (2 week) early nutritional stimulus may have physiological consequences in the long term.

Direct and programmed effects of dietary methionine deficiency on liver composition

In order to clarify and understand the mechanisms behind the observed direct and programmed physiological effects of dietary methionine deficiency, we first analysed the levels of methionine and some related metabolites in the liver of sampled fish at the end of the feeding challenge. The results showed that whatever the first feeding, the levels of methionine and SAM as well as the SAM/SAH ratio were lower in fish fed the MD diet during the final challenge compared with their respective controls (Fig. 3A,B). However, the level of SAH appeared to be significantly impacted by

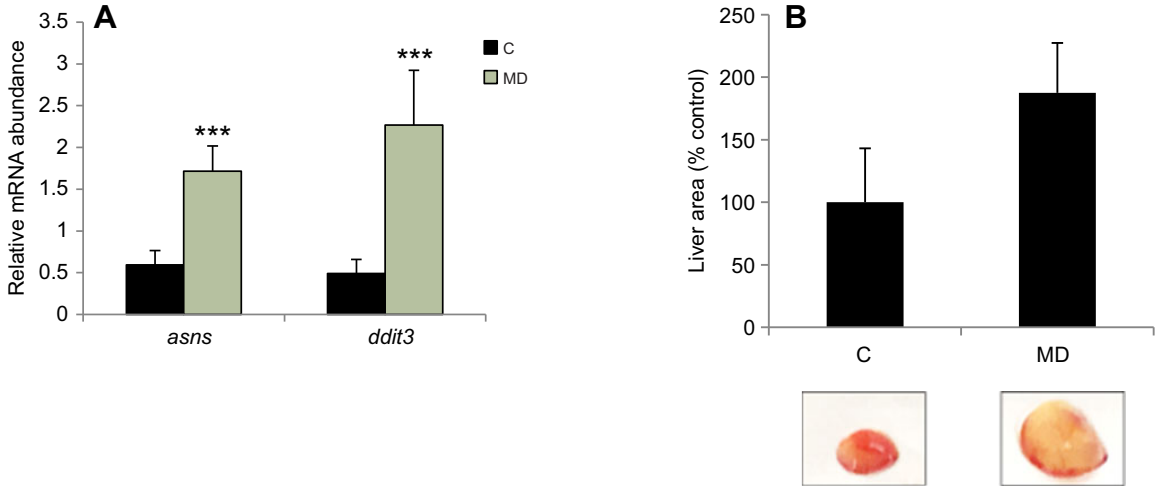

Fig. 2. Fry respond to dietary methionine deficiency. (A) The mRNA levels of *asparagine synthase* (*asns*) and *DNA-damage inductible transcript 3* (*ddit3*) were measured using reverse transcription-quantitative PCR (RT-qPCR) assays in liver of fry sampled 16 h after the last meal. Expression values were normalized to expression of *eukaryotic translation elongation factor 1α1* (*eef1α1ef1*) mRNA. Values are means+s.d. (*n*=6). Asterisks indicate significant differences between the two dietary groups (***$P<0.05$; *t*-test). (B) Graph of liver area (% of control) of 3 fish per condition. Images below show livers of fry fed the C or MD diet.

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Table 3. Direct and programmed effects of methionine restriction on survival, mass, food intake and efficiency, and hepatosomatic index (HSI) in rainbow trout

	Weeks 0–20		Weeks 20–22			
	C	MD	C-C	C-MD	MD-C	MD-MD
Survival (%)	98.61±1.86	98.05±2.38	80.74±2.56	81.48±1.28	82.96±2.56	82.96±1.28
Final body mass (g)	56.12±1.45	59.56±2.15	81.26±11.79[a]	80.08±17.18[a]	89.21±18.16[b]	91.16±19.64[b]
Food intake (% body mass day^{-1})	2.38±0.82	1.92±0.20				
Food efficiency	1.18±0.03	1.24±0.04	1.61±0.31[a]	2.26±0.50[b]	1.56±0.28[a]	2.59±0.41[c]

Weeks 0–20 corresponds to first stimuli and growth trial; weeks 20–22 corresponds to the final challenge. C, fish fed the control diet for the first 2 weeks (first meal) and the standard diet for the subsequent 18 weeks; MD, fish fed the methionine-deficient diet for the first 2 weeks (first meal) and the standard diet for the subsequent 18 weeks; C-C, fish fed the C diet during the first meal and during the final challenge; C-MD, fish fed the C diet during the first meal and the MD diet during the final challenge; MD-C, fish fed the MD diet during the first meal and the C diet during the final challenge; and MD-MD, fish fed the MD diet during the first meal and during the final challenge. Survival (%)=100×final fish number/initial fish number. Food intake=100×total amount of ingested food (kg) divided by the mean biomass over the experimental period [(initial biomass+final biomass)/2, expressed as kg wet mass] and the number of days. Food efficiency=gain in total biomass (final biomass−initial biomass in kg wet mass) divided by the amount of ingested dry matter (kg DM). HSI=(liver mass/total body mass)×100. Mean values with unlike superscript letters were significantly different among the two dietary groups (P<0.05; t-test).

methionine deficiency at the last meal only in fish from methionine-deprived fry (Fig. 3B). We then analysed the protein, TG and glycogen levels in the livers of fish. The results show that, regardless of the first meal that the fry were fed, the 2 week challenge of juveniles with the MD diet decreased the hepatic levels of both proteins and TG (Fig. 3C,D). In contrast, this dietary treatment increased hepatic glycogen levels with a major effect on fish from fry fed the MD diet at the first meal (MD-MD) (Fig. 3E). Collectively, these data reveal that dietary methionine deficiency induces strong liver metabolic perturbations in both the short term (direct effect of the diet) and long term (programming effect).

Direct and programmed sensing of dietary methionine deficiency

We then sought to determine whether the modifications in liver composition observed in the present study were also accompanied by significant changes in the phosphorylation of two key factors of the critical nutrient-sensing pathways general control non-depressible 2 (GCN2)/eIF2α and mechanistic target of rapamycin complex 1 (mTORC1). As shown in Fig. 3F,G, whatever the first feeding of the fry, the 2 week challenge of juveniles with the MD diet increased the phosphorylation of eIF2α and decreased that of S6 in the liver. However, we also observed that fish from MD diet-treated fry exhibited higher phosphorylation of eIF2α and lower phosphorylation of S6 compared with fish from control fry (Fig. 3F,G).

Methionine deficiency has direct effects on mitochondrial function

In order to determine whether mitochondria were affected under these nutritional conditions, we measured several mitochondrial markers in the liver of trout sampled at the end of the dietary challenge: the relative mtDNA copy number (reflecting the mitochondrial content), the level of TIM23 (a protein located in the mitochondrial inner membrane) and the activity of complexes II and IV [involved in the oxidative phosphorylation (OXPHOS) system]. The results showed no direct effect of methionine deficiency on either the relative mtDNA copy number or the activity of complexes II and IV (Fig. 4A,C,D). In contrast, they revealed that fish fed the MD diet at the last dietary challenge (C-MD and MD-MD) exhibited lower levels of TIM23 (involved in mitochondrial protein transport) compared with the control groups (C-C and MD-C) (Fig. 4B). Together, these data support a direct (but not programmed) effect of methionine deficiency on liver mitochondrial function while not impairing mitochondrial content.

Methionine deficiency has a direct and programmed effect on mitophagy

Considering that dietary methionine deficiency directly impacted mitochondrial function, we measured the level of some markers related to autophagy-dependent mitochondrial degradation (termed mitophagy) in our samples. The pink1 (PTEN-induced putative kinase 1)/PARKIN (parkin RBR E3 ubiquitin protein ligase) axis is considered a major pathway of mitophagy regulation. Induction of this pathway has been shown to correlate with an increase of ubiquitin phosphorylation at Ser65 (p-S65-Ub) and a decrease in the level of the PARKIN-target protein MFN2. Here, we found that methionine deficiency at the last dietary challenge increased the levels of PARKIN (regardless of the first meal of fry) and of p-S65-Ub (at least in fish from fry fed the control diet; Fig. 5A,B). Furthermore, we also observed that fish fed the MD diet at the first meal (MD-C and MD-MD) exhibited an increase of PARKIN and p-S65-Ub as well as a decrease of MFN2 in comparison with fish from fry fed the C diet (C-C and C-MD) (Fig. 5). Thus, these results demonstrate that dietary methionine deficiency-induced direct defects of mitochondria were accompanied by induction of mitophagy. Moreover, although mitochondrial integrity does not appear to be affected by the first meal of the fry, we also observed an increase in markers of mitophagy in the livers of fish fed the MD diet at the first meal, suggesting other mechanisms at play in this programmed effect.

Underlying mechanisms involved in the observed direct effect of methionine deficiency on mitophagy: possible role of endoplasmic reticulum (ER) stress

Several studies have demonstrated that mitophagy is induced upon severe ER stress, through the unfolded protein response (UPR), in order to clear stress-damaged mitochondria and to protect cells from apoptosis (Eisenberg-Lerner et al., 2009; Maiuri et al., 2007; Zhang et al., 2014). We therefore measured the mRNA levels of three main target genes of the UPR [namely *asns*, *ddit3* and *x-box binding protein 1* (*xbp1*)] in our samples, as well as levels of cleaved PARP, the prominent marker of apoptosis. Whatever the nutritional past of fry, the 2 week challenge of juveniles with the MD diet increased mRNA levels of *asns*, *ddit3* and *xbp1*, supporting the induction of the ER stress response in the livers of these fish (Fig. 6A). Surprisingly, the levels of cleaved PARP also increased in those fish, revealing apoptosis induction (Fig. 6B). Overall these results associated the short-term (direct) dietary methionine deficiency-mediated induction of mitophagy to ER stress, but also suggested

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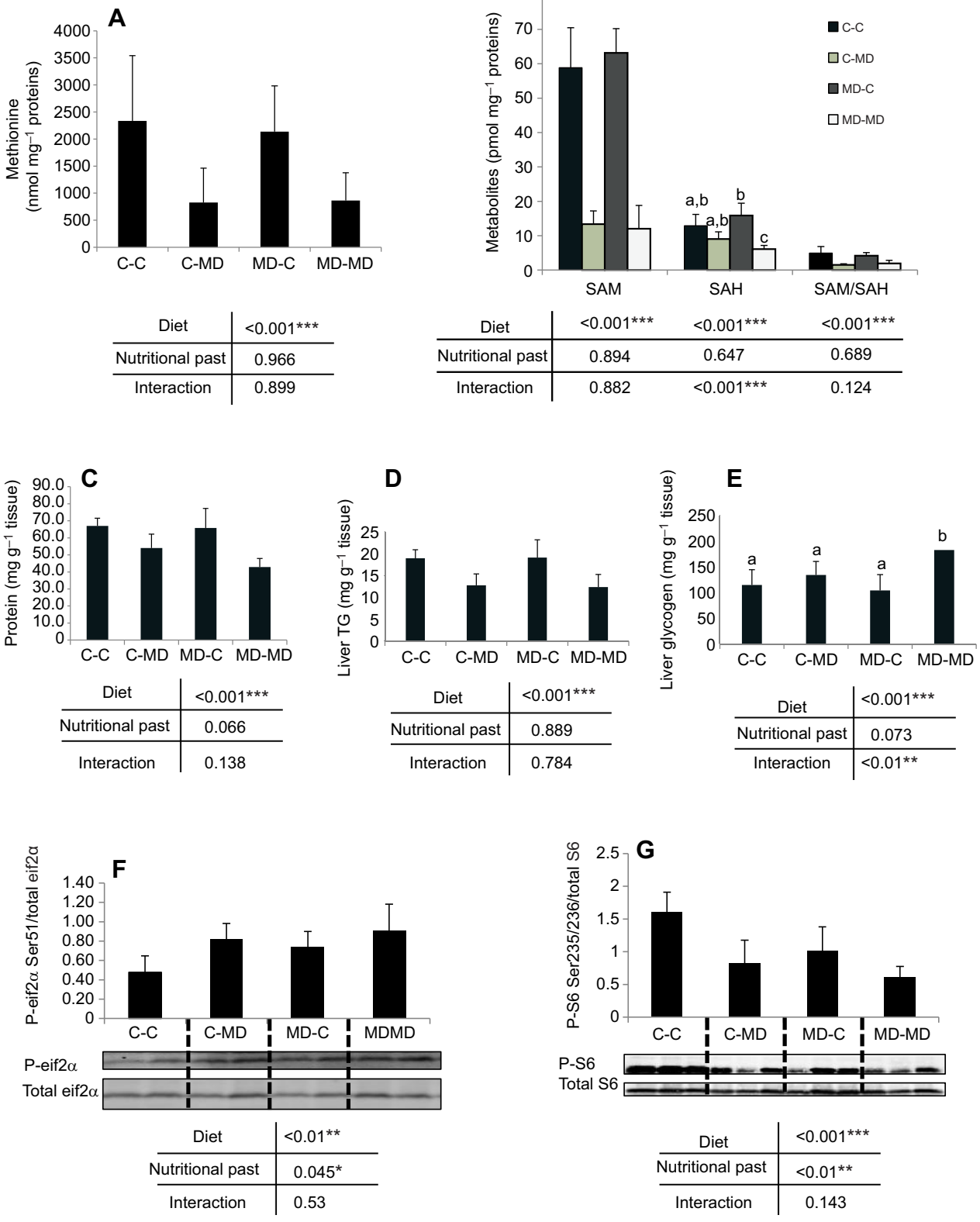

Fig. 3. Direct and programmed effects of dietary methionine deficiency on the liver. (A) Methionine, (B) methionine-related metabolites, (C) protein, (D) triglycerides and (E) glycogen. (F,G) Phosphorylation of key factors of the mTOR (F) and GCN2/eIF2α (G) signalling pathways in the liver of trout sampled 16 h after the last meal. Western blot analyses in F and G were carried out on six individual samples per treatment; a representative blot is given and the graphs show the ratio of the phosphorylated form to the total targeted protein. Values are means+s.d. (n=7–9 for liver composition and n=6 for western blot analyses), and were analysed using two-way ANOVA or PERMANOVA, followed by Tukey's *post hoc* test for multiple comparisons (see individual table for P-values). Where the interaction between diet and nutritional past is significant (asterisks), lowercase letters in the graphs represent statistically significant differences (P<0.05, Tukey's HSD). SAM, *S*-adenosylmethionine; SAH, *S*-adenosyl-L-homocysteine.

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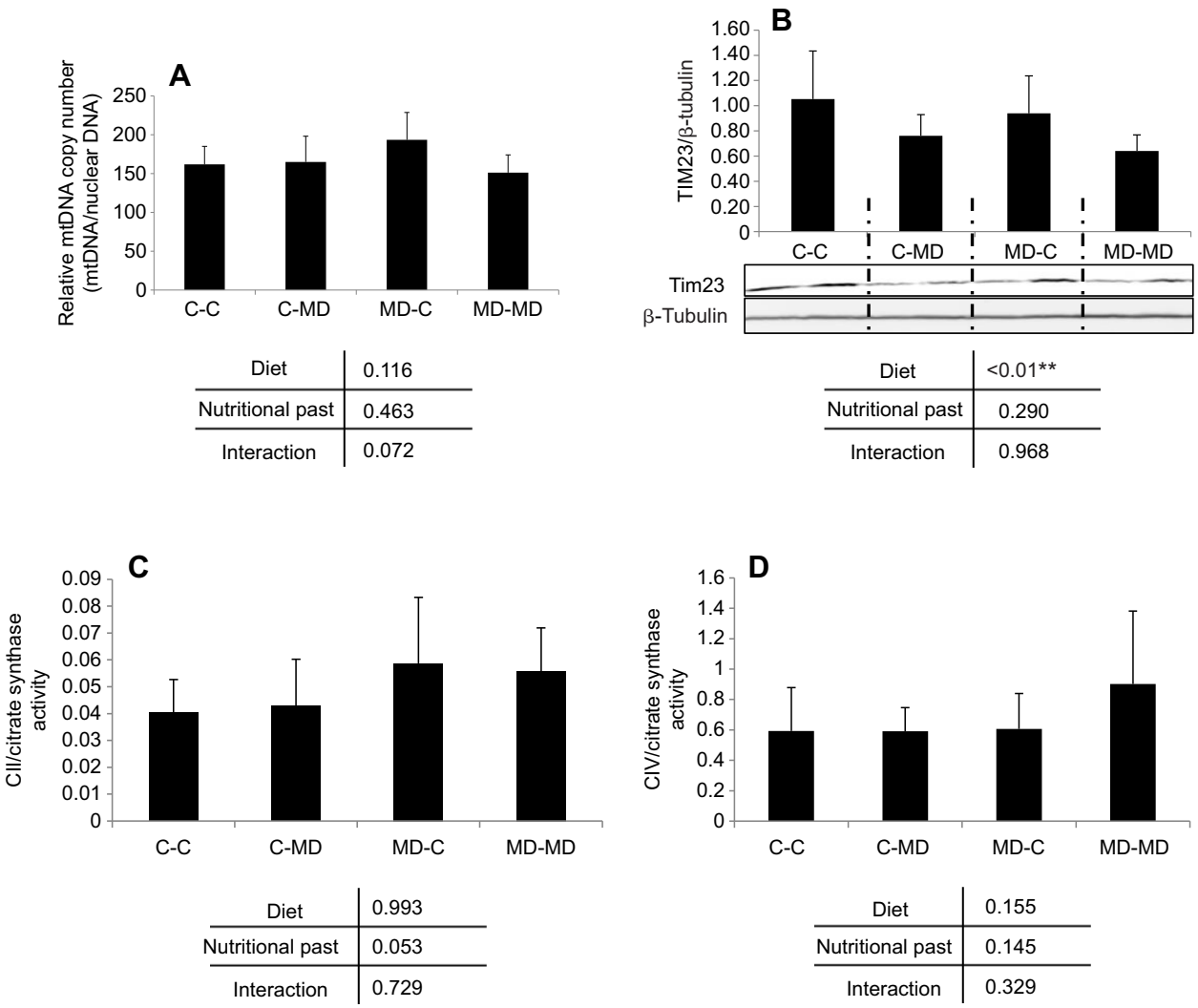

Fig. 4. Methionine deficiency and mitochondrial integrity. (A) Relative mitochondrial DNA (mtDNA) copy number in the liver of experimental trout sampled 16 h after the last meal. (B) TIM23 levels in the liver of trout of C-C, C-MD, MD-C or MD-MD groups sampled 16 h after the last meal. Western blot analysis was carried out on six individual samples per treatment; a representative blot is given and the graphs show the ratio of the amount of TIM23 to β-tubulin used as a loading control. (C,D) Activity of complex II (CII; C) and complex IV (CIV; D) in hepatic mitochondria isolated from trout from the C-C, C-MD, MD-C or MD-MD group sampled 16 h after the last meal and expressed relative to citrate synthase activity. Analysis was carried out on six individual samples per treatment. Values are means+s.d. (n=6) and were analysed using two-way ANOVA or PERMANOVA, followed by Tukey's *post hoc* test for multiple comparisons. Where the interaction between diet and nutritional past is significant (asterisks), lowercase letters in the graphs represent statistically significant differences (P<0.05, Tukey's HSD).

that this induction of mitophagy does not prevent cells turning on apoptosis.

Underlying mechanisms involved in the programmed effect of methionine deficiency on mitophagy: possible role of histone methylation

One of the critical mechanisms involved in nutritional programming, and therefore one that may explain the observed programmed effect of methionine deficiency on mitophagy, is epigenetics. Here, we assessed whether methionine deficiency at the first meal affected global epigenome modifications by targeting DNA methylation and histone marks previously reported to be affected in mitochondrial stress conditions (permissive H3K4me3 and H3K36me3, and repressive H3K9me3). The results showed that the global DNA methylation and the level of H3K9me3 were not different among the four dietary groups (Fig. 7A,B). In contrast, fish fed the MD diet at the first meal (MD-C and MD-MD) exhibited higher H3K36me3 in comparison to fish fed the C diet at the first meal (C-C and C-MD) (Fig. 7C). Moreover, we also observed that the levels of H3K4me3 significantly increased in fish fed the MD diet at the first meal and during the final challenge (MD-MD) compared with fish fed the C diet at the first meal (C-C and C-MD) (Fig. 7D). Overall, these data demonstrate that methionine deficiency at the first feeding affects the hepatic epigenetic landscape of fish at later juvenile stages, suggesting that epigenetic events may play a role in the observed nutritional programming effect of methionine deficiency on mitophagy.

DISCUSSION

In addition to its role as a building block for protein synthesis, methionine has recently emerged as a key factor in modulating several cell signalling pathways (Belghit et al., 2014; Séité et al., 2018; Skiba-Cassy et al., 2016), the antioxidant defence system (Andersen et al., 2016; Fontagné-Dicharry et al., 2015; Séité et al., 2018; Tesseraud et al., 2009) and the epigenetic processes of histone and DNA methylation (Veron et al., 2018; Waterland, 2006), and

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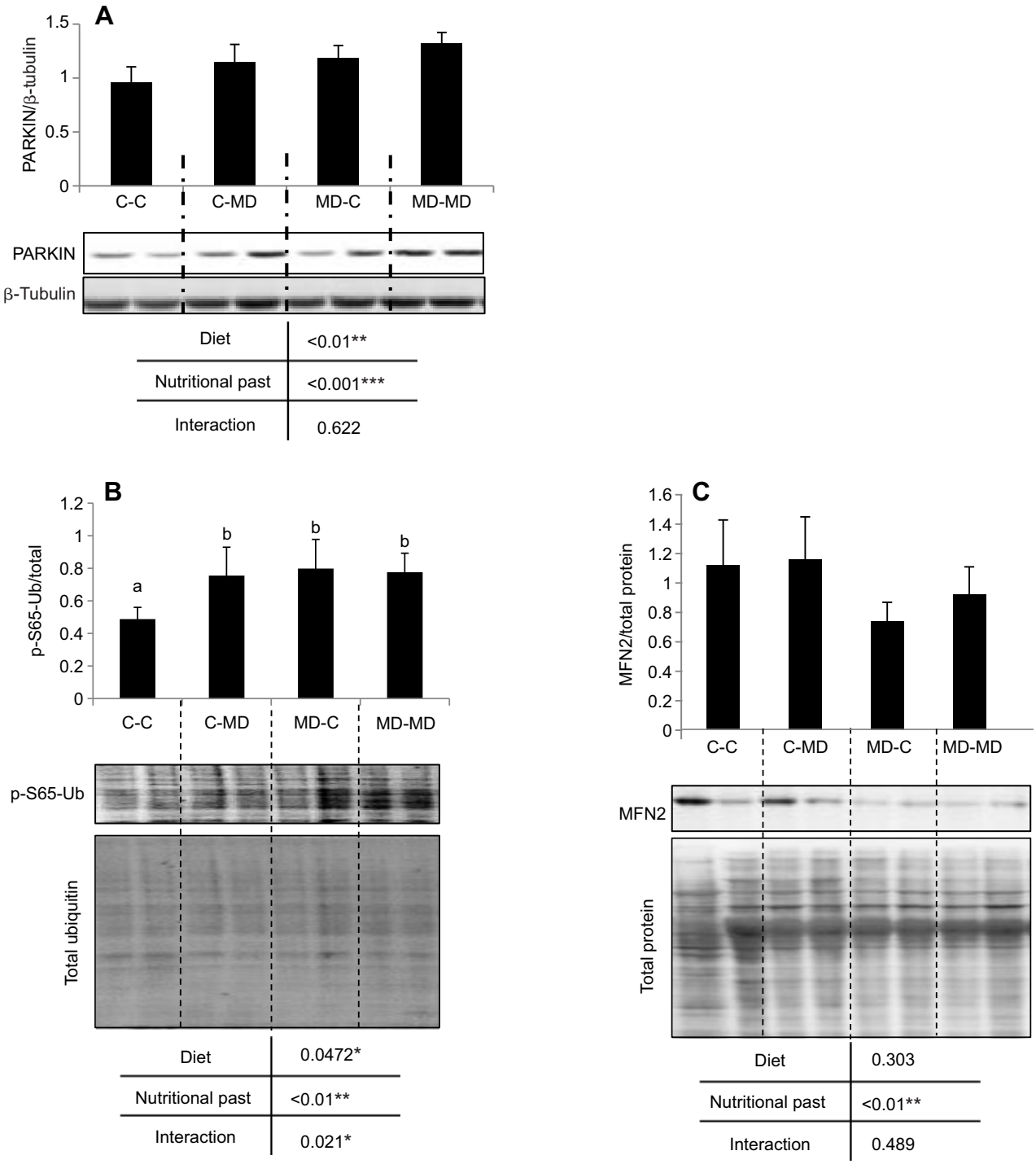

Fig. 5. Methionine deficiency has a direct and programmed effect on mitophagy. Levels of (A) PARKIN, (B) p-S65-Ub and (C) MFN2 in mitochondria isolated from liver of trout in the C-C, C-MD, MD-C or MD-MD group and sampled 16 h after the last meal. Western blot analysis was carried out on six individual samples per treatment; a representative blot is given and the graphs show the ratio of the targeted protein to β-tubulin, the total amount of ubiquitin or the total amount of protein used as a loading control. Values are means+s.d. (n=6) and were analysed using two-way ANOVA or PERMANOVA, followed by Tukey's *post hoc* test for multiple comparisons. Where the interaction between diet and nutritional past is significant (asterisks), lowercase letters in the graphs represent statistically significant differences (P<0.05, Tukey's HSD).

represents a potential critical factor in both direct and programmed nutritional control of cell homeostasis and metabolism. In this regard, we recently reported that feeding trout with a methionine-deficient diet for 6 weeks led to general mitochondrial defects associated with a sharp increase in mitochondrial degradation through mitophagy in the liver (Séité et al., 2018). However, the underlying mechanisms are still unclear, and given the role of methionine (as a methyl-group donor) in epigenetic processes, we considered it worth investigating whether an early methionine restriction could have programming consequences for mitochondria.

Here, we report that fish from fry fed a MD diet at first feeding exhibited significantly higher mass than fish from fry fed a control

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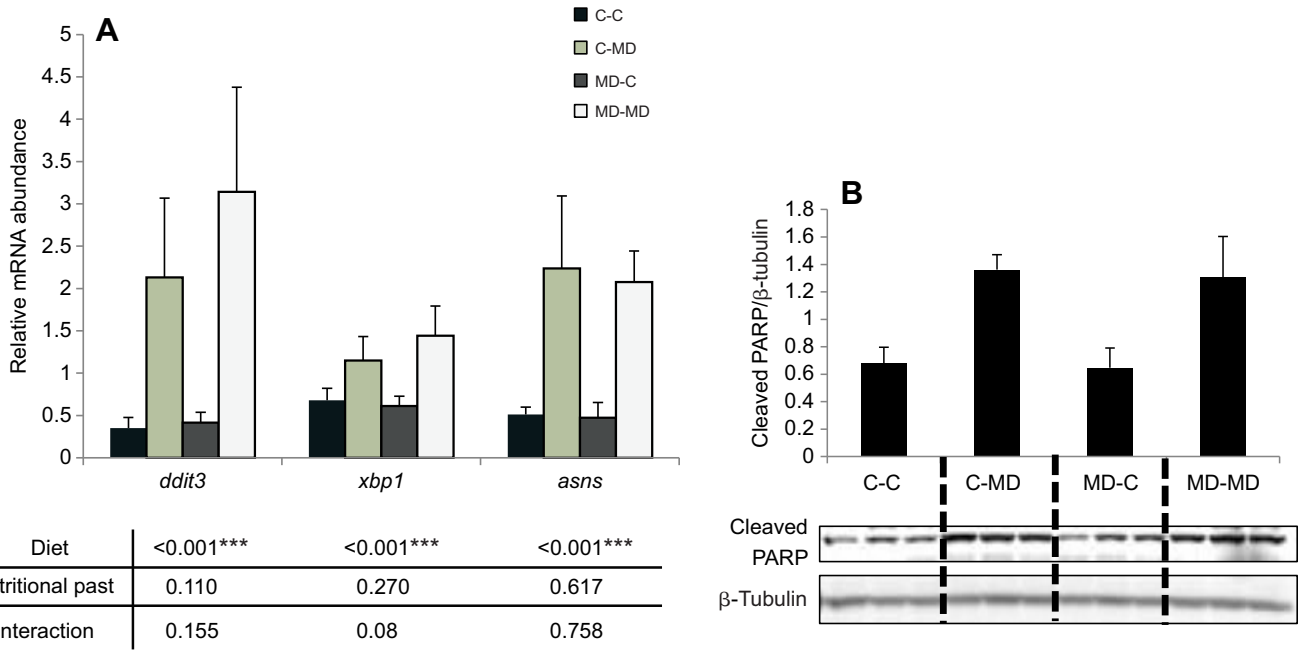

Diet	<0.001***	<0.001***	<0.001***
Nutritional past	0.110	0.270	0.617
Interaction	0.155	0.08	0.758

Fig. 6. Induction of endoplasmic reticulum (ER) stress- and apoptosis-related factors in the liver of fish fed the MD diet. (A) mRNA levels of *DNA-damage inducible transcript 3* (*ddit3*), *X-box binding protein 1* (*xbp1*) and *asparagine synthetase* (*asns*) were measured using RT-qPCR assays in liver of trout sampled 16 h after the last meal. Expression values were normalized to expression of *eef1α1ef1* mRNA. (B) Western blot analysis of cleaved PARP protein in the liver of trout sampled 16 h after the last meal. Western blot analysis was carried out on six individual samples per treatment; a representative blot is given and the graphs show the ratio of the targeted protein to β-tubulin used as a loading control. Values are means+s.d. (*n*=6) and were analysed using two-way ANOVA or PERMANOVA, followed by Tukey's *post hoc* test for multiple comparisons. Where the interaction between diet and nutritional past is significant (asterisks), lowercase letters in the graphs represent statistically significant differences ($P<0.05$, Tukey's HSD).

diet when they were subjected to a 2 week feeding challenge 18 weeks later. These results could be due either to an increase in growth performance or to important metabolic disorders (e.g. abnormal lipid stores) resulting in the fish being overweight. Several studies have previously shown that dietary methionine deficiency led to a direct body mass reduction in fish (Belghit et al., 2014; Gao et al., 2019; Mambrini et al., 1999; Séité et al., 2018), but to our knowledge no data are available on the programming impact of methionine deficiency on body mass. However, several previous findings in mammals demonstrated that maternal methyl-group donor nutrition might affect susceptibility to obesity and metabolic syndrome of offspring in adulthood (McGee et al., 2018). Interestingly, we observed that whatever the first feeding of fry, the 2 week challenge of juveniles with the MD diet increased the HSI, and this effect was even stronger in fish from fry fed the MD diet at the first meal. Such an effect of a dietary methionine deficiency on liver mass has already been reported and attributed to severe metabolic perturbation (Caballero et al., 2010; Craig and Moon, 2013). In this regard, hepatic protein, TG and glycogen levels were strongly affected in fish fed the MD diet at the last feeding challenge, and (at least for glycogen) even more in fish from fry fed the MD diet at the first meal. Furthermore, remaining fish that were fed the respective treatment diets for another week showed significantly lower body mass at week 23 for the final challenge group, whatever the first feeding of fry: 88.19 g C-C=93.26 g MD-C<75.45 g C-MD=78.4 g MD-MD. Overall, these data indicate that dietary methionine deficiency impacts the hepatic metabolism of trout both directly and in the long term, and provide relevant material for studying the mechanisms behind the direct effect of this diet on mitochondrial function, as well as assessing the programming consequences of early methionine restriction on these key metabolic organelles. In the future, it will be worth investigating whether other organs (e.g. muscles or viscera) are also affected. This could provide a better understanding for the observed effect of dietary methionine treatments on fish body mass.

With regards to the direct effect, the obtained results showed that feeding trout a diet deficient in methionine for 2 weeks reduced the levels of mitochondrial protein TIM23 in the liver, and concurrently induced two critical markers of mitophagy: PARKIN and p-S65-Ub. Such a direct effect of dietary methionine deficiency on both mitochondrial function (revealed by the decrease in the TIM23 quantity involved in mitochondrial protein transport) and mitophagy has recently been reported in rainbow trout, but the underlying mechanisms were not investigated (Séité et al., 2018). Here, we report that fish fed the MD diet in the last 2 week feeding challenge also exhibited an increase in ER stress (as revealed by the mRNA levels of *ddit3*, *xbp1* and *asns*), and we propose that this dietary methionine deficiency-induced ER stress probably plays an important role in the observed induction of mitophagy. Mitochondria and ER form structural and functional networks that are essential in the maintenance of cellular homeostasis and in the determination of cell fate under various physiological conditions (Marchi et al., 2014; Szymański et al., 2017). ER stress can be transmitted to mitochondria through alterations in the transfer of metabolites such as Ca^{2+} or through stress-sensitive signalling pathways that directly control mitochondrial integrity and mitophagy (Bouman et al., 2011; Guo et al., 2018; Kim et al., 2006; Marycz et al., 2018; Rainbolt et al., 2014; Senft and Ronai, 2015). In this regard, it is now well recognized that mitophagy is induced by the UPR during ER stress, in order to eliminate stress-damaged mitochondria and to protect cells from apoptosis (Kim et al., 2006; Maiuri et al., 2007), as noted above. For example, the transcription factor ATF4 of the PERK/ATF4 pathway is known to control the expression of parkin (Bouman et al., 2011). Surprisingly, we also noticed an increase in cleaved PARP levels in

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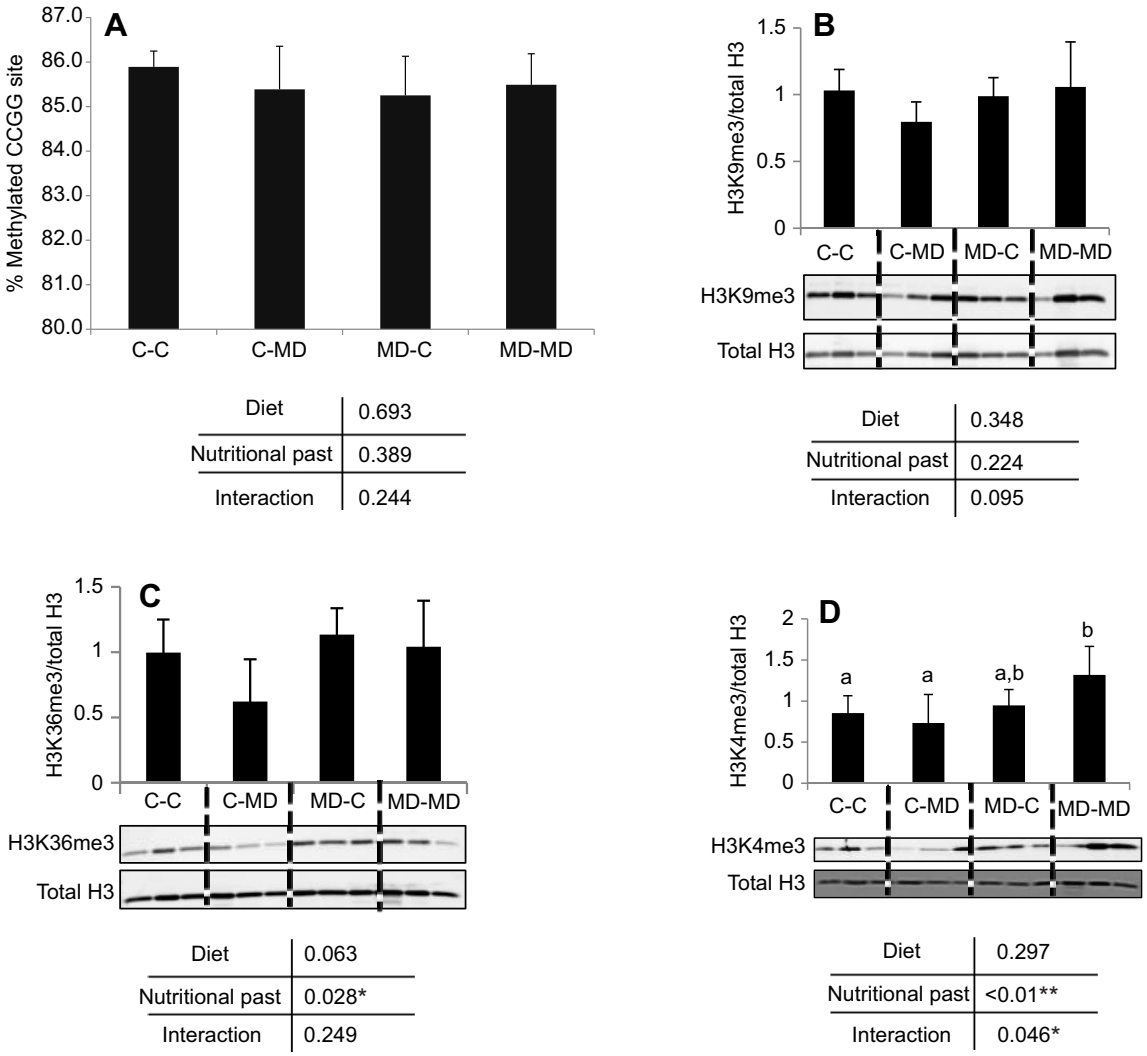

Fig. 7. Methionine deficiency at the first feeding affects the hepatic epigenetic landscape of fish at later juvenile stages. (A) Global DNA methylation in liver of fish sampled 16 h after the last meal. (B–D) Western blot analysis of global histone modifications at H3k9me3, H3K36me3 and H3K4me3 in liver of trout sampled 16 h after the last meal. Western blot analysis was carried out on six individual samples per treatment; a representative blot is given and the graphs show the ratio of the targeted histone marker to the total amount of H3 used as a loading control. Values are means+s.d. (n=6) and were analysed using two-way ANOVA or PERMANOVA, followed by Tukey's *post hoc* test for multiple comparisons. Where the interaction between diet and nutritional past is significant (asterisks), lowercase letters represent statistically significant differences (P<0.05, Tukey's HSD).

fish fed the MD diet during the last 2 week feeding trial, indicating that under the methionine deficiency conditions, the observed induction of mitophagy did not prevent the cells from initiating apoptosis. Collectively, the data obtained in the present study confirmed our previous findings on the direct effect of dietary methionine deficiency on both mitochondrial function and mitophagy, and suggest that it probably results from complex cross-talk between the ER, mitochondria and autophagy, which ultimately determines the cellular response (survival or death) to this nutritional challenge.

However, our results also evidenced that early methionine deficiency resulted in a long-term programming of mitophagy in rainbow trout, without impacting mitochondrial function, ER or apoptosis. These results suggest that other mechanisms are at play in the observed programmed effects of methionine deficiency. Methionine emerged as a key factor in modulating the cellular availability of SAM needed for all biological methylation (including DNA and histone methylation), and represents a potential critical actor in nutritional programming. This finding is supported by several studies which demonstrated that early imbalanced dietary methionine led to modulation of DNA and histone methylation later in life through the control of the one-carbon metabolism (Rees, 2002; Waterland, 2006; Waterland and Jirtle, 2004). Recently, we also reported that for both broodstock and early fry, methionine nutrition affected the methylation of several CpG sites and the mRNA levels of *bnip3a* (bcl-2/E1B-19K interacting protein 3) and *bnip3lb1* (also known as *nix)* genes involved in mitochondrial mediated apoptosis and/or mitophagy (Veron et al., 2018). In the present study, we did not observe any modulation of global DNA methylation in the livers of fish from fry fed the MD diet compared with their control counterparts. However, the levels of H3K4me3 as well as those of H3K36me3 were significantly affected by early methionine deficiency, supporting major epigenetic changes in MD diet-fed fish. Interestingly, mitochondrial stress adaptation in worms has recently been associated with heritable changes of H3K4me3 over the promoters of several mitochondrial stress response genes (Ma et al., 2019). This new finding thus raises the possibility that the H3K4me3 enrichment observed in methionine-

deficient fish may play a critical role in the induced programmed mitophagy.

Conclusions

In conclusion, we confirmed a previously reported direct effect of dietary methionine deficiency on both mitochondria and mitophagy in trout liver, and propose that ER stress could play an important role in this effect, highlighting the involvement of complex cross-talk between ER, mitochondria and autophagy in the maintenance of cellular homeostasis. Moreover, we show for the first time that an early nutritional stimulus with a methionine-deficient diet over a 2 week period resulted in the long-term programming of mitophagy, without impacting mitochondrial function, ER or apoptosis. Although the underlying mechanisms are not yet clear, the results demonstrate significant changes in both H3K4me3 and H3K36me3 levels in fish from fry fed the MD diet, indicating a possible involvement of epigenetic processes in the observed effects. Further studies are needed to evaluate this long-term persistent effect of methionine deficiency on mitophagy and to elucidate the mechanisms, whether epigenetic or not.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.S., K.M., S.P., I.S.; Methodology: S.S., V.V.; Validation: L.M., S.P., I.S.; Formal analysis: S.S., C.H., V.V.; Investigation: S.S.; Data curation: S.S., C.H.; Writing - original draft: S.S.; Writing - review & editing: K.M., C.H., L.M., S.P., I.S.; Supervision: K.M., I.S.; Project administration: I.S.; Funding acquisition: K.M., I.S.

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