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ORIGINAL ARTICLE

Arginine supplementation and exposure time affects polyamine and glucose metabolism in primary liver cells isolated from Atlantic salmon

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Abstract Arginine has been demonstrated to enhance glucose and lipid oxidation in mammals through activation of polyamine turnover. We aimed to investigate how arginine affects energy utilization through polyamine metabolism and whether this effect is time dependent. Primary liver cells were isolated from Atlantic salmon (2.2 kg body weight) fed diets containing 25.5 (low arginine, LA) or 36.1 (high arginine, HA) g arginine/kg dry matter for 12 weeks, to investigate the effect of long-term arginine supplementation. The cells were cultured for 24 h in L-15 medium to which either alpha-difluoromethylornithine (DFMO) or N^1,N^{11}-diethylnorspermine (DENSPM) was added. Analysis of the medium by nuclear magnetic resonance revealed significant differences between the two dietary groups as well as between cells exposed to DFMO and DENSPM, with decreased glucose, fumarate and lactate concentrations in media of the HA cells. Liver cells from fish fed the HA diet had higher spermidine/spermine-*N*1-acetyltransferase protein abundance and lower adenosine triphosphate concentration as compared to the LA-fed fish, while gene expression was not affected by either diet or treatment. Primary liver cells isolated from salmon fed a commercial diet and cultured in L-15 media with or without arginine supplementation (1.82 or 3.63 mM) for 48 h, representing short-term effect of arginine supplementation, showed differential expression of genes for apoptosis and polyamine synthesis due to arginine supplementation or inhibition by DFMO. Overall, arginine concentration and exposure time affected energy metabolism and gene regulation more than inhibition or activation of key enzymes of polyamine metabolism, suggesting a polyamine-independent influence of arginine on cellular energy metabolism and survival.

Keywords Arginine · Polyamine turnover · Atlantic salmon · DFMO · DENSPM · Glucose

Abbreviations

DFMO	Alpha-difluoromethylornithine
DENSPM	N^1,N^{11}-Diethylnorspermine
ODC	Ornithine decarboxylase
SSAT	Spermidine/spermine-*N*1-acetyltransferase
SAMdc	*S*-Adenosyl methionine decarboxylase
NO	Nitric oxide
CPT-1	Carnitine palmitoyl-transferase-1
AMPK	5′-Activated protein kinase
ATP	Adenosine triphosphate
NMR	Nuclear magnetic resonance
WB	Western blot
qPCR	Quantitative real-time polymerase chain reaction

Introduction

Opposite to mammals, arginine is defined as an indispensable amino acids in fish nutrition (NRC 2011). Arginine supplementation has been shown to affect energy metabolism in several mammalian species, increasing lipid oxidation in the viscera and simultaneously enhancing

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deposition of both lipids and proteins in the muscle (Clemmensen et al. 2011; Jobgen et al. 2009b; McKnight et al. 2010; Pirinen et al. 2007; Tan et al. 2009, 2012). Increased expression of lipolytic genes has been observed in both fish and mammals following arginine supplementation (Andersen et al. 2013; Tan et al. 2011; Jobgen et al. 2009a). This has been linked to arginine stimulation of polyamine metabolism, with the rate-limiting enzymes ornithine decarboxylase (ODC) and spermidine–spermine acetyltransferase (SSAT), as well as to the arginine–nitric oxide (NO) pathway. Feeding trials with surplus arginine in fish have been shown to stimulate growth and protein deposition in several species (Cheng et al. 2012; Pohlenz et al. 2013), while results in salmon have not shown a similar trend (Plisetskaya et al. 1991). The growth-enhancing effects of arginine vary, but generally appear more prominent in juvenile than adult fish (Andersen et al. 2013; Han et al. 2013). This could possibly be due to a higher requirement in juveniles, or an inability to produce arginine from glutamine and citrulline at the earlier life stages. Also, previous studies have indicated adaptation to arginine over time (Mohan et al. 2012). Arginine supplemented through the diet is known to correlate with ornithine concentrations in plasma, muscle and liver in Atlantic salmon (Andersen et al. 2013; Berge et al. 2002) and with arginase activity in the liver of black sea bream (Zhou et al. 2011), but the metabolic effects of arginine supplementation on lipid and glucose metabolism in Atlantic salmon is still unknown.

In this paper we examined whether supplementing arginine in the diet of adult Atlantic salmon could affect the polyamine turnover. The interactions with polyamine metabolism was investigated by applying alpha-difluoromethylornithine (DFMO) and N^1,N^{11}-diethylnorspermine (DENSPM), inhibitor of ODC and activator of SSAT, respectively, to primary liver cells isolated from Atlantic salmon fed surplus arginine. To compare the long- and short-term effect of arginine on gene expression, liver cells were also isolated from salmon fed a standard commercial diet and plated in medium with or without arginine supplementation for 48 h.

Methods

Feeding trial and isolation of primary liver cells

Primary liver cells were isolated from adult Atlantic salmon (*Salmo salar*) fed diets containing a low arginine (LA) or a high arginine (HA) inclusion for a period of 12 weeks. The LA diet was supplemented with 5 g arginine/kg, and the HA diet with 15 g/kg, resulting in diets containing 25.5 and 36.1 g arginine/kg dry feed, respectively. For comparison, the requirement for arginine in adult Atlantic salmon have been estimated as 21.1 g/kg dry feed (Berge et al. 1997). The diets contained a high inclusion of plant protein ingredients and a marine lipid source, and were balanced in fat, protein and energy content and in all indispensable amino acids except arginine. At the time of isolation, the fish were 2.2 ± 0.1 and 2.2 ± 02 kg in the LA and HA groups, respectively, and no significant differences from arginine supplementation were observed on growth parameters or deposition (Andersen et al. 2014, in press). Liver cells were isolated from five fish fed each diet, 6 h post-prandial, after anaesthetization with metacain (MS222). Isolation was performed in vivo by a two-step perfusion process as described by Krovel et al. (2008). Cells were washed three times in PBS, before the pellet was suspended in L-15 medium and carried from the breeding facility to the research facilities on ice and kept incubated at 8 °C overnight before being plated onto six-well plates. The experiments complied with the guidelines of the Norwegian Regulation on Animal Experimentation and European Community Directive 86/609/EEC.

Cell culturing

The cells were counted and plated on six-well plates at a concentration of 0.8 million cells/cm^2. All cells were incubated for 24 h at 8 °C to allow to settle before the medium was replaced and either 1 mM DFMO or 100 μM DENSPM was added to duplicate wells from each fish. As controls, duplicate wells containing L-15 medium only were used. After 24 h incubation in the respective treatments, the medium was harvested and stored at −80 °C until analysis. For gene expression, cells were trypsinated, dissolved in media and spun down at 2,200 rpm for 5 min. The pellet was then dissolved in RNA later and the samples stored at −80 °C. Extraction of cell proteins for Western blot (WB) was performed in 300 μl CelLytic (Sigma, Missouri, USA), according to the manufacturer's instructions and the following supernatant stored at −80 °C until use. The liver cells from the feeding trial represent long-term administration of arginine (12 weeks). To study the short-term effect (48 h) of arginine on gene expression, liver cells were isolated as described from three salmons of approximately 600 g, obtained from the University of Bergen, fed a standard commercial diet. These cells were suspended and plated in either standard L-15 medium (measured concentration of 1.82 mM arginine) (control) or L-15 medium supplemented with L-arginine (A8094, Sigma Aldrich) to concentrations of 3.63 mM (×2Arg) on the day of isolation. After 24 h incubation at 8 °C, the cells were treated with 1 mM DFMO or 100 μM DENSPM for 24 h before being harvested for gene expression analysis. All L-15 medium used was supplemented with 10 % fetal

bovine serum (BioWhittaker, cat#14-801F), pen/strep (50 U/ml, BioWhittaker, cat#17-602E) and 2 % 2 mM Glutamax™ 100× (Gibco, cat#35056).

Analysis of culture media using ^{1}H-NMR

As the liver cells isolated from salmon fed the LA or HA diet had been transported and stored in L-15 medium overnight before plating, there was concern regarding whether their different phenotypes would have been canceled out and the arginine effect lost. Therefore, metabolic analysis was performed on the medium from these cells at the end of treatment with DFMO or DENSPM. Previous studies have used a similar approach of cultured cells (Zulak et al. 2008) or media (Dowlatabadi et al. 2009) profiling by nuclear magnetic resonance (NMR) metabolomics. Cell culture media were mixed 1:4 with D_2O containing 1 mM 3-trimethylsilyl propanoic acid (TSP) and centrifuged at 13,000 rpm for 105 min. The supernatant was centrifuged through a 0.45 μm filter at 13,000 rpm for 10 min. 600 μl of the supernatant was put in a 5 mm-diameter glass tube for NMR analysis (Bruker biospin). Cell culture media were analyzed with a Bruker 500 MHz magnet equipped with a 5 mm ^{1}H BBI liquid probe and using a noesygppr pulse sequence to suppress the water peak. 64 scans were collected for each sample with a line broadening of 0.5 Hz. Spectra were phased and baseline corrected using Chenomx software, and all samples gave acceptable spectra without artifacts. Culture media samples were compared by first superimposing spectra to visually compare peaks which varied among samples as candidates for quantification; then specific molecules were quantified using peak integration software Chenomx NMR suite version 7.5 (Chenomx Inc., Edmonton, Alberta, Canada). Peaks were selected for measurement based on known metabolic function or extreme difference across the samples. In addition to the profiled components, the spectra were also analyzed as full spectra divided into bins of 0.01 ppm width from regions 0.6 to 9.4 ppm for a total of 880 bins per sample. The bins were normalized to the TSP standard.

RNA extraction and qPCR

RNA was extracted from the samples using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA concentration was determined at 260 nm with the NanoDrop ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A two-step quantitative real-time polymerase chain reaction (qPCR) was run as described (Torstensen et al. 2011). Briefly, 500 ng RNA was reverse transcribed into cDNA with duplicates of each sample, using a TaqMan® Gold RT-PCR Kit (Applied Biosystems, CA, USA), with the following protocol: 25 °C for 10 min, 60 °C for 48 min and 95 °C for 5 min, and stored at −20 °C. Gene expression was quantified with qPCR on the Lightcycler 480 (Roche Applied sciences, Basel, Switzerland), on the following program: 95 °C for 5 min, 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s, followed by a melt curve analysis and cooling to 4 °C. Relative quantities were calculated against the reference genes β-actin, elongation factor 1Ab and acidic ribosomal protein using the $2^{-\Delta\Delta C_t}$ method.

Western blot

The freezed samples from the long-term trial were mixed 1:1 in Laemmli sample buffer with β-mercaptoethanol and separated on an SDS-gel (12 %), Western blotted on a PVDF membrane and detected by Chemiluminesence Image Capture as described (Espe and Holen 2013). The gels were stained for SSAT (abcam: Anti-SAT1, ab54047) and abundance was calculated relative to protein content measured in each sample. The relative values of the LA controls were set to 100 and the other values calculated relative to this.

ATP

500 μl of cell lysate was deproteinized in perchloric acid using a deproteinizing sample preparation kit, according to the manufacturer's instructions. Adenosine triphosphate (ATP) was then measured in the deproteinized samples using an ATP fluorimetric kit. Both kits were from Bio-Vision, Milpitas, CA, USA.

Statistical analysis

Multivariate analysis of the NMR data was performed using Simca-P version 12.0.1 software (Umetrics, Umea, Sweden). Spectra were pareto scaled to minimize the influence of noise in the baseline regions. Analysis included clustering the data using principal component plots and class discrimination. Principal component analysis (PCA) was used as a way to summarize all observations and variables into the projected figures. The data is graphically shown as loading plots of the variables. To determine the variables most associated with PCA distribution, the Simca-P orthogonal partial least-squared discriminant analysis (OPLS-DA) was used, with R^2 as the percent of modeled variation and Q^2 as the percent of variation which can be

predicted by cross-validation analysis. When comparing the effects of diet and treatment, a two-way ANOVA was applied, with diet and treatment as factors, using the Statistica Program (Stat Soft Inc., version 11). Data are represented as mean ± SEM. Differences were regarded as significant when $p < 0.05$.

Results

Dietary arginine affects metabolic profiling more than DFMO and DENSPM administration

The PCA score plots showed that even 72 h after isolation and storing in L-15 medium, there was still a substantial difference between the cells isolated from the HA- and LA-fed salmon (Fig. 1a) with R^2 of 0.85 and Q^2 of 0.45 demonstrating this. OPLS-DA was used to determine differences among the treatments and identify compounds related to these differences. When comparing the controls with the DFMO and DENSPM treatments, R^2 was 0.45 and Q^2 0.14 with extensive overlap of the DENSPM group and the controls; so discrimination could not be made between treatments. When comparing DFMO versus DENSPM treatment, however, R^2 was 0.98 and Q^2 0.92, which was an excellent prediction for the metabolomics datasets (Fig. 1b), proving significant metabolic differences between the treatments. The corresponding loading plot of DFMO and DENSPM treatment was used to identify potential compounds contributing to the differences which were then measured as single-profiled components as discussed below. Values furthest from the center contributed most to predicting sample differences and were located in superimposed spectra for determining if they could be profiled and identified. Based on the whole spectra analysis, compounds were chosen for profiling. All spectra were superimposed to select metabolites for quantitative measurement using Chenomx software, and 17 compounds were selected for profiling (Table 1). Most of these are amino acids which differ and are resolved in the spectra. In addition, lactate, choline, pyruvate, glucose, galactose, phenylacetate and fumarate contributed to the difference. The Simca-P software was used to make a ranking of variables which contribute to high versus low grouping, also termed the variable importance in making the projection of OPLS. This suggests the relative importance as glucose > ornithine > lactate > fumarate for separating the HA- and LA-fed groups.

SSAT abundance and ATP increase after dietary arginine supplementation

WB of SSAT showed that SSAT abundance in the long-term feeding experiment was significantly higher in liver cells isolated from salmon fed the HA diet compared to salmon fed the LA diet, both with and without treatment with DENSPM (Fig. 2). DENSPM treatment induced SSAT abundance significantly in the HA cells, but not in the LA cells. Overall, ATP concentrations were higher in hepatocytes from salmon fed the LA diet (Fig. 3; $p = 0.009$). Still, no significant differences due to treatment by DFMO or DENSPM or between the two dietary groups within each treatment were observed in the long-term arginine feeding trial.

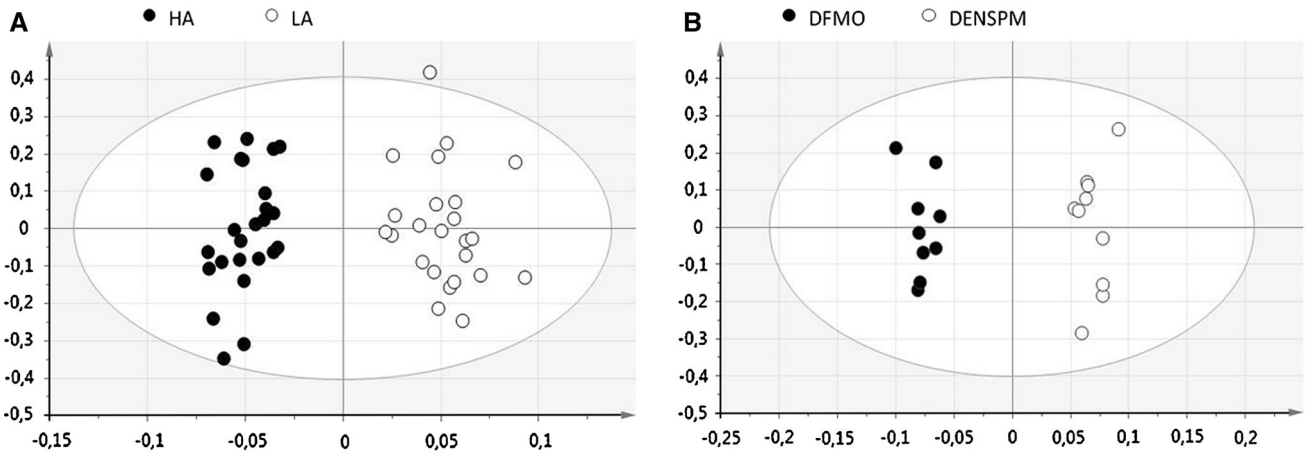

Fig. 1 NMR results. OPLS-DA plots of liver cells from **a** HA (*black symbol*) versus LA (*white symbol*)-fed salmon and **b** after treatment with DFMO (*black symbol*) or DENSPM (*white symbol*). The *X* axis is the score of component 1 and the *Y* axis the score of component 2. The ellipse in **a** and **b** represents the 95 % confidence region of the Hotelling's test

Table 1 NMR quantification of metabolites contributing to difference between groups in media after 24 h incubation with or without DFMO/DENSPM treatment

Treatment	Controls		DFMO		DENSPM		*p* values		
Diet	LA	HA	LA	HA	LA	HA	*D*	*T*	*D* × *T*
Alanine	18.9 ± 2.6	20.0 ± 5.8	19.6 ± 2.5	20.2 ± 3.3	24.3 ± 3.6	21.3 ± 3.1	0.51	0.67	0.76
Arginine	3.3 ± 0.6	4.0 ± 1.2	4.0 ± 1.0	4.6 ± 0.9	4.9 ± 1.2	4.7 ± 1.0	0.68	0.54	0.91
Choline	0.28 ± 0.02	0.24 ± 0.02	0.26 ± 0.04	0.22 ± 0.04	0.28 ± 0.06	0.26 ± 0.02	0.86	0.30	0.91
Fumarate	0.32 ± 0.18	0.18 ± 0.08	0.35 ± 0.24	0.18 ± 0.08	0.28 ± 0.15	0.20 ± 0.07	0.04	0.93	0.81
Galactose	19.5 ± 1.2	19.1 ± 0.8	20.2 ± 0.6	20.48 ± 0.9	20.6 ± 1.5	21.2 ± 1.2	0.85	0.33	0.90
Glucose	7.0 ± 0.7	5.7 ± 0.6	8.1 ± 0.8	5.9 ± 0.7	7.2 ± 0.7	6.0 ± 0.4	0.01	0.60	0.66
Glutamine	8.0 ± 0.4	8.2 ± 0.5	8.4 ± 0.4	8.5 ± 0.4	8.5 ± 0.5	8.3 ± 0.4	0.83	0.73	0.87
Glycine	14.6 ± 0.9	14.5 ± 0.4	14.9 ± 0.3	15.0 ± 0.8	15.2 ± 1.2	15.3 ± 1.0	0.92	0.68	0.96
Histidine	3.6 ± 0.4	3.6 ± 0.3	4.0 ± 0.2	3.9 ± 0.4	3.9 ± 0.6	3.8 ± 0.4	0.82	0.58	0.99
Lactate	25.9 ± 1.1	23.5 ± 1.3	27.8 ± 0.5	24.7 ± 1.4	25.9 ± 1.8	24.5 ± 1.3	0.04	0.51	0.82
Lysine	2.7 ± 0.2	4.1 ± 0.7	3.9 ± 0.3	4.2 ± 0.4	3.0 ± 0.3	3.3 ± 0.2	0.056	0.001	0.71
Ornithine	12.8 ± 0.9	11.8 ± 1.0	13.7 ± 1.2	10.7 ± 1.4	13.1 ± 1.0	12.0 ± 1.1	0.12	0.90	0.93
Phenylacetate	1.5 ± 0.2	1.86 ± 0.2	1.8 ± 0.2	2.0 ± 0.2	1.6 ± 0.2	1.9 ± 0.3	0.14	0.68	0.94
Pyruvate	0.42 ± 0.02	0.38 ± 0.05	0.38 ± 0.05	0.40 ± 0.03	0.56 ± 0.07	0.42 ± 0.05	0.18	0.07	0.22
Tyrosine	1.3 ± 0.4	1.8 ± 0.3	1.4 ± 0.3	1.9 ± 0.3	1.5 ± 0.4	2.0 ± 0.4	0.30	0.77	0.91

Values are expressed in μM as mean ± SE with $N = 5$. Two-way ANOVA with diet (*D*) and treatment (*T*) as categorical factors was used to calculate *p* values

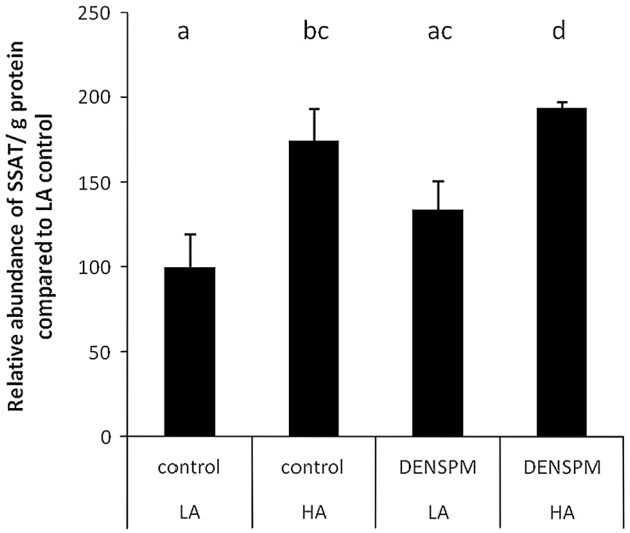

Fig. 2 WB of SSAT abundance in liver cells from the LA- and HA-fed salmon after cell culturing and incubation with or without DENSPM. Data shown as mean ± SEM, $N = 4$. Protein abundance in LA control is set to 100 and other values calculated relative to this. A two-way ANOVA test was applied with a post hoc Tukey's test. *Lowercase letters* indicate significant difference

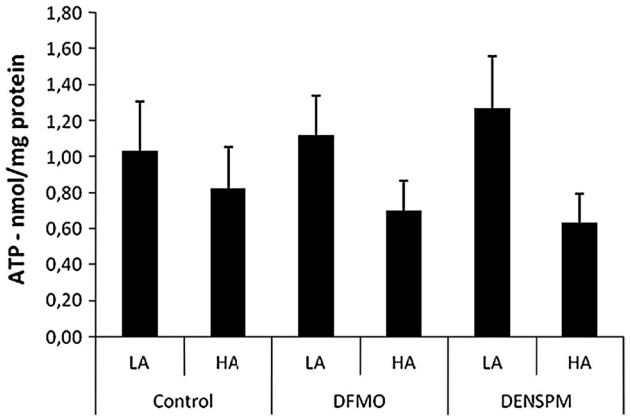

Fig. 3 ATP measured in isolated hepatocytes from salmon fed low arginine (LA) or high arginine (HA) diet, treated with DFMO or DENSPM for 24 h. Data shown as mean ± SEM, $N = 4$. Significance was calculated using two-way ANOVA. ATP decreased after arginine supplementation ($p = 0.05$), while no effect on ATP was observed after treatment with DFMO or DENSPM ($p = 0.95$)

Time-dependent response on gene expression

No effect on gene expression was observed in liver cells from long-term feeding experiment, due to arginine supplementation in the feed or to their respective treatments (Table 2). In the short-term experiment, a downregulation of ODC and BAX was observed in those cells cultured in x2Arg medium, regardless of DFMO/DENSPM treatment (Table 3) compared to culturing in L-15 medium. The difference in expression of ODC was further enhanced by treatment with DFMO or DENSPM, which was enhanced by DFMO and inhibited by DENSPM, resulting in significant difference in expression levels between the two treatments. DFMO also decreased the expression of caspase-3 at both arginine concentrations and showed

Table 2 Relative gene expression of SSAT, ODC, CPT-1, SAMdc, BAX, caspase-3, PRKAB1 and PRKAG1 in LA/HA hepatocytes compared to LA control, 24 h after incubation with or without DFMO or DENSPM

Accession number	Gene name	Control		DFMO		DENSPM		*p* value		
		LA	HA	LA	HA	LA	HA	*D*	*T*	*D* × *T*
NM_002970.2	Spermidine/spermine acetyltransferase	1.00 ± 0.14	1.15 ± 0.13	0.89 ± 0.09	0.95 ± 0.12	0.91 ± 0.13	1.08 ± 0.20	0.34	0.49	0.93
BT045263.1	Ornithine decarboxylase	1.00 ± 0.20	1.00 ± 0.10	1.21 ± 0.22	1.32 ± 0.20	0.97 ± 0.16	1.14 ± 0.17	0.65	0.41	0.75
NM_001165344.1	*S*-Adenosyl methionine decarboxylase	1.00 ± 0.25	0.75 ± 0.08	1.07 ± 0.19	0.76 ± 0.06	0.99 ± 0.16	0.78 ± 0.05	0.19	0.88	0.81
AM230810	Carnitine palmitoyl-transferase-1	1.00 ± 0.05	0.93 ± 0.06	0.97 ± 0.02	0.93 ± 0.04	0.90 ± 0.09	0.86 ± 0.07	0.30	0.33	0.93
DQ364432	Acetyl-CoA oxidase	1.00 ± 0.10	0.95 ± 0.09	1.03 ± 0.10	0.91 ± 0.05	1.14 ± 0.02	0.98 ± 0.04	0.11	0.53	0.86
DW573070	Acetyl-CoA carboxylase	1.00 ± 0.15	0.81 ± 0.18	0.95 ± 0.17	0.96 ± 0.19	0.89 ± 0.22	0.77 ± 0.16	0.48	0.79	0.83
BT060359	Fatty acid synthetase	1.00 ± 0.07	1.01 ± 0.15	0.92 ± 0.11	1.02 ± 0.03	1.08 ± 0.11	0.82 ± 0.10	0.84	0.53	0.26
NM_001141086	Bcl-2 associated x-protein	1.00 ± 0.10	1.26 ± 0.11	1.05 ± 0.16	1.26 ± 0.07	1.04 ± 0.11	0.98 ± 0.16	0.14	0.34	0.34
DQ008070	Caspase-3	1.00 ± 0.11	0.90 ± 0.16	0.93 ± 0.15	0.83 ± 0.17	0.98 ± 0.09	1.35 ± 0.36	0.74	0.34	0.38
NM_001141762.1	AMPKγ1	1.00 ± 0.14	0.87 ± 0.12	0.93 ± 0.01	0.93 ± 0.07	1.03 ± 0.05	0.97 ± 0.14	0.40	0.75	0.76
NM_001141359.1	AMPK β1	1.00 ± 0.27	1.01 ± 0.12	1.02 ± 0.28	0.96 ± 0.09	1.07 ± 0.29	1.05 ± 0.17	0.73	0.93	0.99

Mean ± SEM, $N = 5$. A two-way ANOVA test was applied with diet (*D*) and treatment (*T*) as categorical values

Table 3 Relative gene expression of polyamine and apoptosis related genes from the isolated liver cells cultured in L-15 or ×2Arg medium with or without DFMO or DENSPM treatment

Accession number	Gene name	Control		DFMO		DENSPM		*p* value		
		L-15	×2Arg	L-15	×2Arg	L-15	×2Arg	*D*	*T*	*D* × *T*
NM_002970.2	Spermidine/spermine acetyltransferase	1.00 ± 0.16	1.00 ± 0.28	0.72 ± 0.13	0.54 ± 0.11	0.91 ± 0.16	0.89 ± 0.13	0.61	0.13	0.86
BT045263.1	Ornithine decarboxylase	1.00 ± 0.09	0.45 ± 0.06	1.46 ± 0.25	0.59 ± 0.10	0.81 ± 0.18	0.23 ± 0.02	0.01	0.01	0.45
NM_001165344.1	*S*-Adenosyl methionine decarboxylase	1.00 ± 0.35	0.99 ± 0.28	0.61 ± 0.19	0.95 ± 0.24	1.04 ± 0.33	0.74 ± 0.16	0.98	0.82	0.49
AM230810	Carnitine palmitoyl-transferase-1	1.00 ± 0.19	1.03 ± 0.07	0.83 ± 0.14	0.79 ± 0.05	0.84 ± 0.04	0.93 ± 0.14	0.93	0.24	0.88
DQ364432	Acetyl-CoA oxidase	1.00 ± 0.20	0.83 ± 0.11	1.06 ± 0.11	0.71 ± 0.03	0.81 ± 0.07	0.59 ± 0.02	0.01	0.08	0.97
DW573070	Acetyl-CoA carboxylase	1.00 ± 0.13	1.28 ± 0.25	0.59 ± 0.11	0.77 ± 0.16	0.87 ± 0.15	1.03 ± 0.11	0.14	0.04	0.97
BT060359	Fatty acid synthetase	1.00 ± 0.12	0.74 ± 0.13	0.95 ± 0.07	0.69 ± 0.08	0.85 ± 0.05	0.49 ± 0.08	0.01	0.08	0.85
DQ008070	Caspase-3	1.00 ± 0.42	0.93 ± 0.12	0.38 ± 0.12	0.47 ± 0.03	0.94 ± 0.17	1.03 ± 0.17	0.91	0.02	0.66
NM_001141086	Bcl-2 associated x-protein	1.00 ± 0.07	0.80 ± 0.12	1.31 ± 0.07	0.85 ± 0.12	0.81 ± 0.11	0.65 ± 0.04	0.01	0.01	0.27
NM_001141762.1	AMPKγ1	1.00 ± 0.11	0.70 ± 0.10	0.70 ± 0.06	0.49 ± 0.05	0.90 ± 0.12	0.62 ± 0.11	0.37	0.43	0.40
NM_001141359.1	AMPK β1	1.00 ± 0.09	0.90 ± 0.03	1.06 ± 0.08	0.88 ± 0.06	0.89 ± 0.06	0.77 ± 0.04	0.01	0.06	0.85

Expression is calculated relative to the unsupplemented control. Mean ± SEM, $N = 3$. A two-way ANOVA was applied with diet (*D*) and treatment (*T*) as categorical values

indications of downregulating SSAT expression, though not significantly. No differences were observed on CPT-1 or SAMdc expression in response to arginine or treatments. Two of the genes for 5′-AMP-activated protein kinase (AMPK), PRKAB1 and PRKAG1 encoding the β1 and γ1 subunits respectively, were not affected by either diet or treatment in the long-term experiment, while PRKAG1 was lower expressed in the liver cells cultured in the x2Arg medium compared to the L-15 medium, and was downregulated after DFMO treatment in the short-term exposure trial.

Discussion

NMR analysis of the media from the HA- and LA-fed liver cells after 48 h of culturing proved that these were two metabolically different groups and that they had maintained their different phenotypes even after transportation and storage in L-15 medium overnight (Fig. 1a). The loading plot indicated significant variance due to several intermediates in the urea and citric acid cycle, demonstrating differential energy metabolism after arginine supplementation. Most significantly, the medium from the HA liver cells had lower concentrations of glucose, fumarate and lactate and higher lysine concentrations compared to the medium from the LA cells (Table 1). Arginine and SSAT induction have been reported to improve glucose metabolism by decreasing fasting levels of blood glucose and increase glucose tolerance (Koponen et al. 2012; Jobgen et al. 2009b) in rodent models. Arginine also has the potential to stimulate glucose uptake via NO and AMPK stimulation (Jobgen et al. 2006). However, neither of the AMPK genes investigated was affected in the long-term experiment. On the contrary, decreased expression of the regulatory subunit γ1 was observed in the short-term trial after arginine supplementation. As opposed to mammals, salmon do not have any requirement for dietary glucose (NRC 2011) and are often referred to as insulin resistant. A carbohydrate-rich meal will lead to persistent postprandial hyperglycemia which is associated with decreased growth (Hemre et al. 2002; Polakof et al. 2012). Mommsen et al. (2001) have shown that arginine is a potent stimulator of both insulin and glucagon release in rainbow trout, and chronic injections of insulin to rainbow trout was recently reported to increase gene expression of glucose transporters in the liver while decreasing glucose production (Polakof et al. 2010), possibly explaining the decrease in extracellular glucose from the HA-fed salmon. By increasing glucose oxidation, arginine may improve the metabolic profile of the fish, enhancing limited glucose utilization, sparing amino acids and promoting growth. Both NO and polyamines are known to affect mitochondrial biogenesis and oxidation (Jobgen et al. 2006; Tan et al. 2012) and might increase mitochondrial oxidation in the HA cells, explaining the decrease in glucose and lactate. Further research is required to clarify whether these observations are due to altered oxidation, synthesis or excretion of glucose in the HA cells.

The higher lysine concentrations after arginine supplementation is consistent with other reports (Andersen et al. 2013; He et al. 2009), but the underlining mechanisms have not been established. DFMO treatment increased lysine concentrations in the LA cells only, to concentrations similar to the HA cells. Inhibition of ODC is likely to increase arginine concentrations, suggesting that the observed increase in lysine is directly linked to high arginine concentrations, suggesting a lysine sparing effect of arginine. DENSPM treatment had no significant effect on any of the metabolites, which is consistent with the overlap of control and DENSPM treatments in the PCA plot. To our knowledge, DENSPM has not previously been applied to salmon, or any other fish species, with the possibility that it may not induce fish SSAT as effectively as mammalian SSAT.

Regulation of SSAT does not occur at the level of expression (Pegg 2008). Abundance of SSAT, however, was higher in the HA than the LA cells even after long-term feeding, isolation and cell culturing, suggesting higher polyamine turnover in the HA cells. SSAT abundance was further increased after activation with DENSPM, without affecting mRNA expression, contrary to that described by others (Uimari et al. 2009). In contrast to what was expected, ODC was downregulated after arginine supplementation in the short-term experiment, possibly due to increased degradation of ODC transcript by polyamines (Pegg 2009). Increased polyamine turnover by SSAT is expected to deplete cellular ATP pools, as was observed in HA cells. In transgenic mice overexpressing SSAT, administration of DFMO has been reported to restore their ATP pools (Pirinen et al. 2007). DFMO could not restore ATP in the HA cells, suggesting that this difference was present before culturing the isolated liver cells. Increased polyamine turnover has the potential to increase β-oxidation, as depletion of ATP, acetyl-CoA and then malonyl-CoA will release malonyl-CoA's inhibitory effect on CPT-1 (Jell et al. 2007). However, no effect on CPT-1 expression or any genes involved in lipid metabolism was observed in the long-term experiment. As the only available fat in the medium was from bovine serum, a rapid downregulation of genes related to β-oxidation is expected, partly explaining why no differences were observed. In the short-term experiment, however, arginine supplementation decreased the expression of fatty acid synthetase and acyl-CoA oxidase, indicating decrease of both fatty acid synthesis and oxidation. Also, AMPKγ1 is downregulated in

response to arginine, further indicating decreased lipolysis. It is possible that the arginine stimulates the cells to increase glucose oxidation, as indicated in the long-term experiment, possibly by activation of the arginine–NO pathway, thereby inhibiting lipolysis, though further studies are required to determine these mechanisms. DFMO downregulated acetyl-CoA carboxylase, indicating that arginine rather than polyamines have an inhibitory effect on fatty acid synthesis in salmon liver cells.

Previous studies have indicated an adaptation to arginine over time (Mohan et al. 2012; Wilson et al. 2007), possibly explaining why no effect on gene expression or weight differences was observed in the long-term experiment. Indeed, the current study confirmed a time-dependent response, as several genes were only affected in response to arginine in the short-term experiment. There was too little material to allow the analysis of polyamines or amino acids in vitro and, as NMR was only performed on media from the long-term experiment, time-dependent comparison on these parameters could not be done. The growth study, however, showed effects on free amino acids in plasma and muscle, while the only effect in liver was increased urea (Andersen et al. 2014, in press).

BAX expression decreased in the cells cultured in x2Arg medium from the short-term experiment only, demonstrating a time-dependent pro-survival effect of arginine administration. DENSPM is reported to inhibit cell growth and induce apoptosis in several cell lines (Oredsson et al. 2007; Stanic et al. 2009) and induce caspase-3 activity in a neuroblastoma cell line (Soderstjerna et al. 2010), which has been linked to DENSPM's ability to reduce the polyamine pool (Oredsson et al. 2007). DENSPM had no effect on apoptosis-related genes in the current study, suggesting that activation of polyamine catabolism in salmon liver cells did not trigger apoptosis. DFMO has been reported to attenuate proliferation of smooth vascular muscle cells without affecting viability and apoptosis (Odenlund et al. 2009), which is consistent with the absent effect on apoptosis-related genes observed in the cells from the long-term supplementation study. In the short-term experiment, however, DFMO caused a distinctive downregulation of caspase-3, independent of arginine concentration, while BAX was upregulated in the cells cultured in L-15 medium only. As DFMO is expected to increase cellular arginine concentrations, the positive effect on caspase-3 could be related to increased arginine availability. Overall, our results imply that arginine, rather than polyamine, concentrations are important for glucose metabolism and cell survival of liver cells from Atlantic salmon, and with regard to gene expression long-term arginine supplementation can lead to adaptation, though further research is required to elucidate this.

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