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Metabolomic analysis of plasma and liver from surplus arginine fed Atlantic salmon

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1. ABSTRACT

The aim of this study was to determine the metabolic effect of surplus arginine (36.1 g/kg dry matter) compared to a control diet with required arginine (21.1 g/kg dry matter) in adult Atlantic salmon (Salmo salar L.). Although the feeding trial had no significant effect on growth, there were significant differences in the metabolite profile in both plasma and liver in experimental group as compared to the control group. There was increased concentrations of biliverdin, PGF-2 alpha, oxidized glutathione, selenocysteine, two monoacylglycerols and a tripeptide in the liver as well as decreased concentrations of valine and a vitamin D3 metabolite in plasma of arginine supplemented fish. These results indicate that while surplus arginine does not affect growth or body weight, it induces metabolic changes in Atlantic salmon.

2. INTRODUCTION

Arginine is an indispensable amino acid in fish, and is important in a range of metabolic processes including polyamine, nitric oxide (NO), creatine and urea synthesis (1). In mammals, arginine has been shown to be an effective supplement to enhance growth, while simultaneously reducing adiposity and sparing lean muscle mass (2-4). These effects have been linked to arginine's direct effect on gene regulation (5), through NO production (6) and via increased oxidation of glucose and fatty acids (7). Feeding trials with dietary arginine supplementation have demonstrated increased growth and protein deposition in some fish species (8, 9), while results in different salmon species have not shown a similar trend (10). The growth enhancing effects of arginine in fish varies, but appears more prominent in juveniles than adults (11-13). This could possibly be due to a higher requirement of arginine in juveniles, or an inability to produce arginine from glutamine and citrulline in the earlier life stages. Humans and rodents are able to synthesize arginine from glutamine and proline, while this pathway has not been established in fish (14). In addition, arginine supplementation in fish has shown to increase immune resistance and to protect against metabolic stress (9, 15, 16).

Table 1. Diet formulation and chemical analysis of dry matter of diets after preparation. All values are in g/kg unless otherwise stated

Diet	Control	Arginine
Diet formulation		
Fishmeal	50	50
Plant proteins*	510	510
L-arginine	0	15
Glycine	15	0
Fish oil	274	274
Premix/Binder†	145.9	145.9
Chemical analysis		
Dry matter		
Energy (MJ/kg)	22.8	23.3
Protein	420	420
Fat	250	260
Water	80	79
Ash	66	68
Amino acids		
Arg	21.1	36.1
Met	8.4	8.4
Cys	6.1	5.9
Lys	18.9	19.1
Thr	13.4	13.7
Trp	3.8	3.8
Ile	16.2	16.2
Leu	37.8	38.1
Val	17.8	17.8
His	9.0	9.1
Phe	20.3	20.4
Gly	30.0	15.8
Ser	18.8	19.0
Ala	20.8	21.0
Asp	31.8	32.0
Glu	81.6	81.7

*Plant protein blend: soy concentrate:wheat gluten:pea protein concentrate:corn gluten:sun flower meal (0.4.2:0.1.3:0.1.2: 0.1.6:0.1.7). †Premix contains minerals and vitamins to fulfill the requirement of Atlantic salmon (48)

To understand the underlying metabolic mechanisms of arginine it is important to investigate the metabolic changes occurring after arginine supplementation. Analysis of the plasma metabolite profile has previously been performed on pig plasma after arginine supplementation (17), identifying effects on free amino acids, lipid signaling molecules and tricarboxylic acid cycle intermediates. Similar metabolic studies with arginine have not yet been performed in Atlantic salmon, or any other fish species as far as the authors are aware. High performance liquid chromatography (HPLC) coupled to quadrupole time-of-flight (Q-TOF) mass spectrometry (MS) has shown effective when searching for unknown metabolites (18). This technique was applied in the present experiment to compare the metabolic profiles of plasma and liver samples from adult Atlantic salmon fed plant protein based diets with excess arginine supplementation or with required arginine concentration. An untargeted metabolomics approach was used to search for differences in the metabolome between fish fed the two diets. Both plasma and liver were analyzed as a multi-compartmental approach has been shown to enhance the understanding of nutrition on the whole organism (19).

3. MATERIALS AND METHODS

3.1. Feeding experiment

Formulation and chemical analyses of the diets as listed in Table 1. The fish were acclimatized on a commercial diet for two weeks in six tanks of 40 fish each before commencement of the trial. The Atlantic salmon (start weight 1.1 kg) were then fed a control (21.1 g arginine/kg dry matter) (20) or a high arginine diet (36.1 g arginine/kg dry matter) for 12 weeks. The experimental diets were randomly distributed to triplicate tanks of salmon and are from now on referred to as Control and Arginine diet. Glycine was added to the control diet to make the diets isonitrogenous and the diets were balanced in energy, fat, protein and all amino acids except arginine and glycine. Circular tanks with a capacity of 3.1 m^3 water were used in a temperature controlled environment with a 24-hour constant light regime. The tanks were supplied with flow through seawater (salinity 33 g L^{-1}) at a flow rate of 1.5 L kg^{-1} biomass min^{-1}, mean temperature 7.2 +/- 1.0°C. The fish were fed to apparent satiation three times a day, and feed intake for each tank was monitored. At the end of the trial, feeding was coordinated so that all

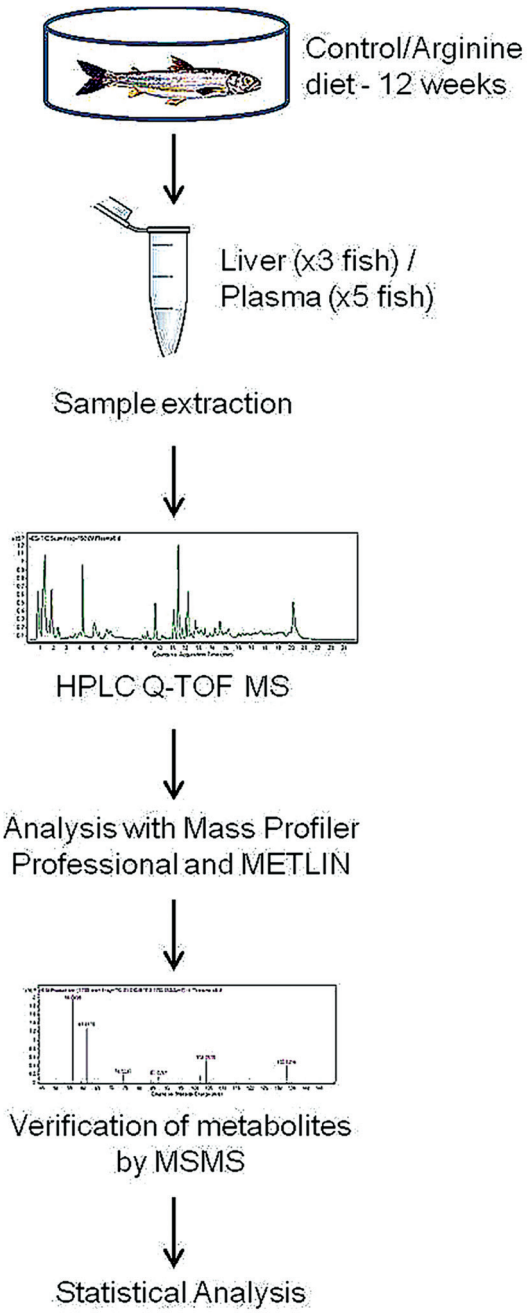

Figure 1. Workflow of metabolomic analysis. Fish were fed their respective diets for 12 weeks before pooled samples of liver and plasma were taken from 3 and 5 fish from each tank respectively. Metabolites were extracted and run on HPLC- Q-TOF MS for untargeted metabolic profiling. The Mass Profiler Professional software was used to find differences in the metabolomic profiles from the two diets. Metabolites were identified using the METLIN database and verified by MSMS. Statistical analysis was then performed.

samples were taken 5 hours post-prandially. Fish were anaesthetized with chlorobutanol (0.4 g L^{-1}) before blood was drawn from the caudal vein with a heparinized syringe, centrifuged, and pooled plasma from 5 fish from each tank was stored at -80°C until analyzed. Liver samples were taken from three fish from each tank, flash frozen in liquid nitrogen and stored at -80°C. The fish were 2.3 kg at the time of sampling and no growth differences were observed between the dietary treatments. The results from the growth study have been published elsewhere (21). The experiments complied with the guidelines of the Norwegian Regulation on Animal Experimentation and European Community Directive 86/609/EEC.

3.2. Sample preparation for metabolomic analysis

A summarized workflow of the metabolomic investigation is shown in Figure 1. Metabolites were extracted by mixing 200µl of plasma with 400µl ice-cold extraction mix (acetonitrile:methanol, 1:1, v:v). The samples were gassed with nitrogen and vortexed for 15 min at room temperature before they were centrifuged at 18 000 x g for 10 min at 4°C. 200µl of the supernatant was carefully collected and freeze-dried at -70°C. At the day of analysis the pellet was re-suspended in 160µl methanol and 40µl water, and the samples vigorously vortexed between each addition. The samples were centrifuged again at 15 000 g for 10 min at 4°C, and the supernatant was transferred to a conical insert and used for HPLC Q-TOF MS analysis. Liver samples were homogenized by vigorously mixing 0.2 g of pooled liver samples with 800µl of homogenization buffer (10 mM Tris, pH 7.6, with 1mM EDTA and 0.25 M sucrose). The samples were centrifuged at 8000g for 45 min at 4°C, and the supernatant stored at -80°C until extraction, using the same pretreatment method as described for plasma.

3.3. HPLC – Q-TOF MS analysis

5µl of the extracted samples were separated through HPLC (1290 Infinity, Agilent) followed by analysis with Q-TOF MS (model 6520, Agilent) operated in an electrospray ionization positive mode. The column used for HPLC was a Zorbax Eclipse Plus C18 column (1.8µm, 2.1x100mm, Agilent) and the column temperature was held at 40°C. The flow rate was 0.3 ml/min with solvent A (water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid). Metabolites were eluted over a linear gradient consisting of 5-50% B over 0-5 min, 50% B from 5-9 min, 50-95% B over 9-16 min, holding 95% B

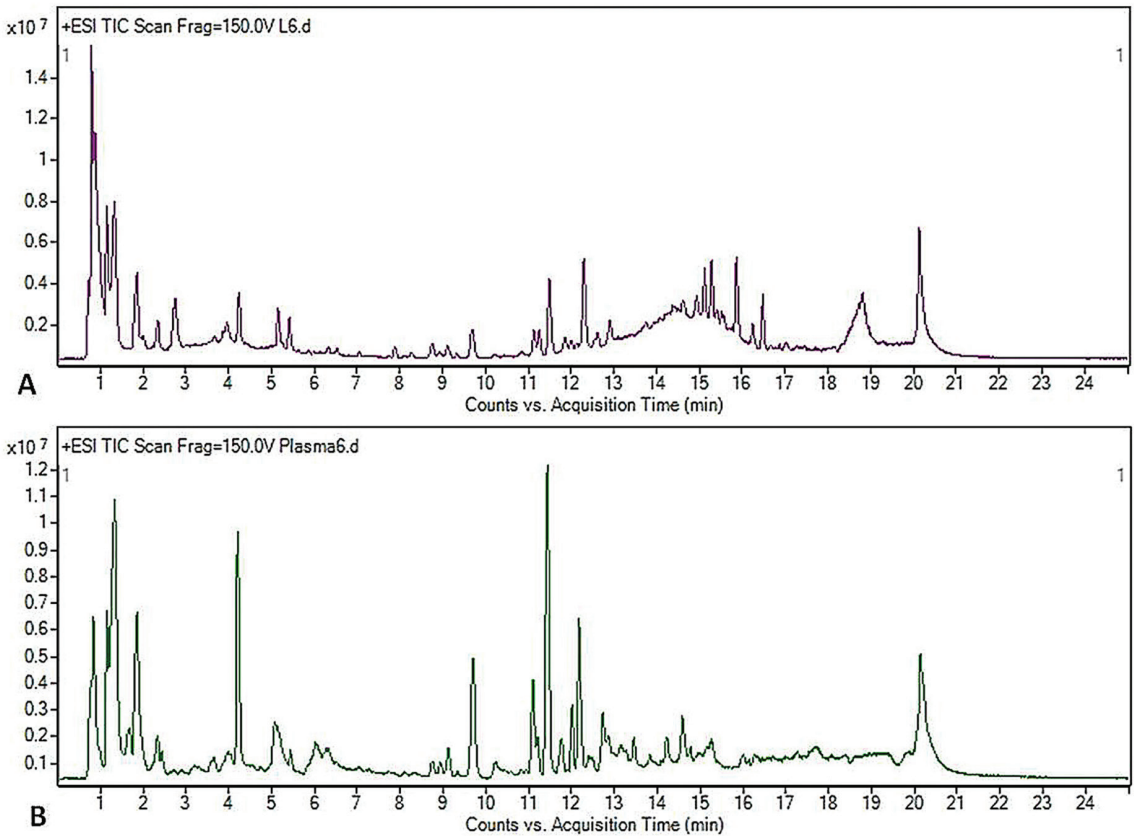

Figure 2. Total ion chromatography scans of A) liver and B) plasma samples after separation through HPLC Q-TOF MS.

until 19 min, and then 5% B from 19.01-25 min to re-equilibrate the column. The separated compounds went directly from the HPLC to the Q-TOF MS, which was set at a desolvation flow rate of 12L/min, a nebulizer pressure of 60 psig and a drying gas temperature of 350°C. Voltages for the capillary and fragmentor were 3500V and 150V, respectively. The Q-TOF MS data was collected in the range of 60 – 1000 *m/z* at the rate of 2 spectra/s. The corresponding scans of liver and plasma tissues after analysis with HPLC-Q-TOF MS are presented in Figure 2. Reference molecules of 121.050873 and 922.009798 *m/z* were continuously injected into the electrospray ionization source to perform internal calibration.

For quality control a mix of both plasma and liver samples were used. Blank samples of water or methanol were also used and run between each sample to clear the column.

3.4. Data processing

All data was processed in the Agilent MassHunter Qualitative Analysis and Mass Profiler Professional software. The Molecular Feature Extractor algorithm was applied before import into the Mass Profiler Professional software for further analysis. A student t-test was applied to search for compounds with a fold change of more than 2 and/or a P-value less than 0.0.5. Metabolites were classified using the METLIN, Massbank Database, Chemspider, Human metabolome database and the Kyoto Encyclopedia of Gene and Genome. The metabolites were confirmed by both retention time and mass spectra under the same chromatographic conditions as described.

3.5. Statistics

A student t-test was applied to check for significant differences between metabolites. A P-value less than 0.05 was considered significant.

Principal component analysis (PCA) was used to identify relationships within classes of metabolites and to visualize how they influenced differentiation between the two diets. All statistical analyses were run using the R. 3.02 software for Windows (22).

4. RESULTS

4.1. Metabolic profiling after HPLC-Q-TOF MS

Overall, there was great variation within the groups, especially for fish fed the arginine supplemented diet. As there were only three replicates within each group, few of the detected metabolites were present at statistically different concentrations between the experimental groups. Still, a few metabolites were found to be significantly affected by the diet after application of the student-test, and are displayed in Table 2. In plasma, two valine compounds and a vitamin D3 metabolite decreased after arginine supplementation. In the liver, fish fed the arginine supplemented diet had higher concentrations of biliverdin, PGF2alpha methyl ether, selenocysteine, two different monoacylglycerols, oxidized glutathione and a tripeptide compared to fish fed the control diet. The concentration of all the metabolites that differed significantly between the diets were standardized and then plotted individually for each tank. All the metabolites affected in the liver (Figure 3a) had higher concentrations, while all the affected metabolites in plasma had lower concentrations (Figure 3b) due to arginine supplementation.

4.2. Principal component analysis

Principal component analysis (PCA) is a data reduction technique (23). Here, we used the Standardized PCA approach (also called reduced PCA), instead of ordinary PCA, as it is more appropriate when the variables (metabolites concentration) are measured on scales with widely differing ranges which is the case for our data. The method starts by first standardizing the variables, by centering each variable and dividing it by its standard deviation. We aim at summarizing the metabolic measurements within each tank by a few, usually two or three, measurements (per tank) called PC scores, without losing useful information. The first two PC scores for each tank are shown in Figure 4c for the liver and 4d for the plasma. For instance, the 2^{nd} PC (y-axis) in Figure 4d clearly separates the tanks supplied with arginine (tanks A1, A2 and A3) from those supplied with the control treatment (C1, C2, and C3). Figure 4a and 4b are useful to further investigate which metabolites contributed to this separation. The x-axis in these correlation plots represents the correlation coefficient between each metabolite and the first PC, while the y-axis represents the correlation between each metabolite and the second PC in the liver and plasma, respectively. The standardized concentration of metabolites found to contribute to the difference between the diets, as having a 2-fold difference due to arginine supplementation are shown in green. When the correlation between the i^{th} PC, i=1 or 2 and a metabolite is highly positive (resp. negative), a tank with a high positive i^{th} PC score will generally have a high concentration of that metabolite. For example, the 2^{nd} PC (y-axis), in Figure 4b is strongly positively correlated with the metabolites Xa18, X2dVD, N5aav, and Xaanv (Table 3), all shown in green. Thus, tanks C1, C2 and C3, all having a high coordinate value on PC2 (y-axis in Figure 4d), will also have a high concentration in these metabolites, as demonstrated in Figure 3b. In other words, the metabolites in the plasma shown in green in Figure 4b are the main contributors to the separation seen in Figure 4d. The same can be said for the seven metabolites (Table 4) in the liver shown in green in Figure 4a.

5. DISCUSSION

Even though there were no growth differences, PCA analysis showed that arginine supplementation affects the metabolic profile of both liver and plasma, which may affect the health of Atlantic salmons (21). PCA analysis indicated that arginine supplementation affected the metabolic profile of both liver and plasma, which may influence health and welfare of the animals, even though no growth differences were observed (21). Interestingly, all affected metabolites increased in the liver and decreased in the plasma (Figure 2). This could be incidental, but could indicate increased general uptake of nutrients from the plasma. Due to the low numbers of replicates, only a few metabolites were present at significantly different concentrations. Thus, future design using a metabolomic approach in fish nutrition should aim to have a higher number of replicates for better results. This can be achieved by using more tanks for each diet or by analyzing several individual fish from each tank, increasing the number of measurements. The authors acknowledge this flaw, however the results still show there is an underlining metabolic effect of arginine and demonstrates the power of applying metabolomic analysis to investigate the effects of

Table 2. Metabolites affected in liver and plasma by arginine in the diet. RT-retention time, m/z – mass-to-charge ratio, MAG – monoacylglycerol

Metabolites	Formula	*m/z*	Mass error[2]	RT	P value	Regulation	Related pathway
Liver							
Glu Thr Leu	C15H27N3O7	362.1925	−2.21	2.674	0.03	↑	Tripeptide
Biliverdin IX	C33H34N4O6	583.2557	−1.14	7.152	0.04	↑	Heme breakdown
PGF 2alpha alcohol methyl ether	C21H38O4	377.2684	0.44	14.742	0.04	↑	Prostaglandin
Selenocysteine	C3H7NO2Se	201.933	0.34	0.787	0.02	↑	Selenoproteins
MAG (18:2)	C21H38O4	337.2736	−0.54	15.428	0.03	↑	Lipid metabolism
MAG (18:1)	C21H40O4	339.2893	−0.67	16.236	0.02	↑	Lipid metabolism
Oxidized glutathione	C20H32N6O12S2	613.1601	−1.41	1.159	0.04	↑	Oxidation
Plasma							
1alpha-25dihydroxy Vitamin D3	C28H42O4	460.3553	1.58	16.539	0.02	↓	Vit D3 metabolite
Valine	C5H11NO2	118.0859	3.24	0.871	0.04	↓	Branched chain amino acid
Valine+Na	C5H11NO2	140.0676	−3.35	0.805	0.04	↓	Branched chain amino acid

Table 3. Correlation coefficient between selected metabolites in the Liver (shown in green in Figure 4A) and the first two principal components

Metabolite	Name in Figure 4a	PC1	PC2
Glu Thr Leu	GTL	0.779	−0.017
Biliverdin	BIIX	0.844	−0.148
PGF 2alpha alcohol methyl ether	PGme	0.731	−0.654
Selenocysteine	Slnc	0.856	−0.413
MAG (18:2)	X91org	0.802	−0.398
MAG (18:1)	X1org	0.706	−0.601
Oxidized glutathione	Oxdg	0.917	0.186

Table 4. Correlation coefficient between selected metabolites in the Plasma (shown in green in Figure 4B) and the first two principal components

Metabolite	Name in Figure 4b	PC1	PC2
1alpha-25 dihydroxy vitamin D3	X2dVD	0.512	0.737
Valine	N5aav	0.203	0.799
Valine+Na	Xaanv	0.181	0.925

amino acid supplementation on fish nutrition and metabolism.

Another factor possibly affecting the results, is the use of glycine in the control diet, as glycine has been proposed as a functional amino acid (24) and may affect gene expression, cell signaling and antioxidant responses among others (25, 26). Glycine supplementation has recently been reported to increase weight gain in shrimp (27). If glycine

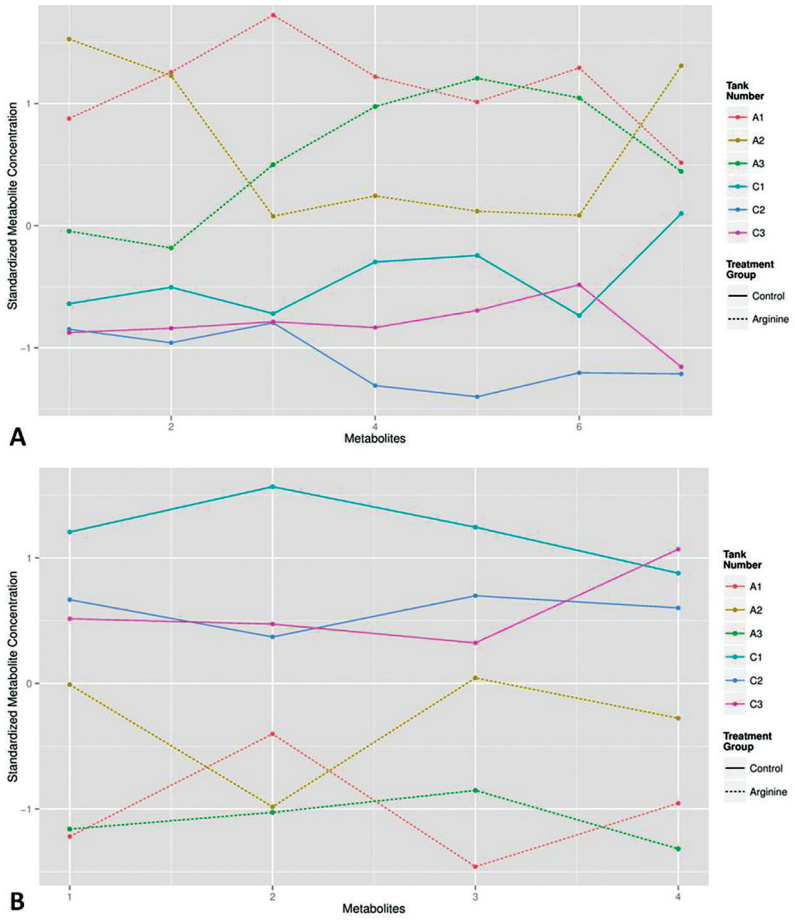

Figure 3. Standardized concentration in A) liver and B) plasma of metabolites significantly affected by arginine supplementation in the diet

did increase weight gain in the control group, this could explain why we did not observe a difference in weight gain between the experimental groups. However, total body gain of glycine did not differ between the two dietary treatments (results not shown). Alanine, having no documented interactions with arginine metabolism might have been a better substitute, and has successfully been used as an isonitrogenous control in other arginine trials (5, 28). Previous fish trials have also used a carbohydrate filler such as wheat grain or starch as the volume of added arginine is relatively small and does not significantly affect the total nitrogen content (29, 30).

Arginine supplementation led to a decrease of plasma valine, suggesting increased catabolism of branched chain amino acids. Valine is directed solely towards carbohydrate metabolism, and as such this might indicate increased need for glucose production, which is consistent with other reports of arginine stimulating glucose oxidation (31, 32). Increased plasma valine concentrations on the other hand, have been associated with insufficient insulin action and are often observed in obese and diabetic patients (33). This is in correlation with reports of arginine stimulating insulin secretion in rainbow trout (31) and decreasing extracellular glucose concentration in arginine supplemented liver cells from Atlantic salmon (34). The increased concentration of two monoacylglycerols in the liver further suggests an effect on energy metabolism, possibly indicating increased fatty acid oxidation. Indeed, there have been several reports of increased fatty acid oxidation after arginine supplementation in mammals (3, 7, 32). However, there was no difference in protein or fat distribution between the analyzed tissues in the current study (21).

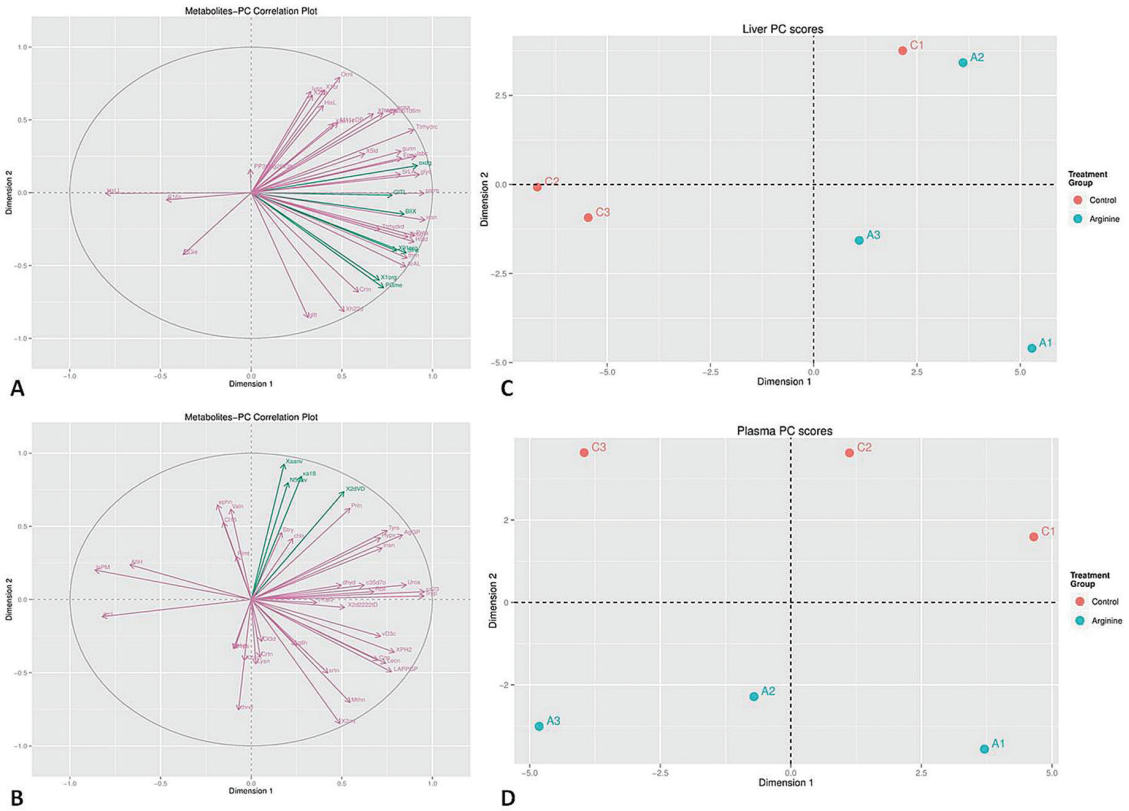

Figure 4. PC correlation plots of metabolites contributing to differences between the dietary treatment in A) liver and B) plasma. The x-axis represents the correlation between each metabolite and the first PC. The y-axis represents the correlation between each metabolite and the second PC. Metabolites significantly affected by the diet are marked in green. The PCA score plots of C) liver and D) plasma show separation due to dietary treatment

Several prostaglandins were found to have a more than 2-fold difference between the dietary groups, but due to the low numbers of replicates only PGF 2alpha alcohol methyl ether was significantly upregulated in liver from fish fed the arginine supplemented diet. Arginine has previously been shown to affect concentrations of another eicosanoid, leukotriene B5, in liver cells of Atlantic salmon (35), but the underlining pathways in which arginine affect eicosanoid metabolism are not determined. The effect of arginine on eicosanoid metabolism might be due to an immunostimulatory effect (36) or a sign of inflammation in the liver, possibly due to increased oxidative stress as indicated by the increase in oxidized glutathione. The effect of arginine on eicosanoids in Atlantic salmon needs to be further elucidated.

Biliverdin is the breakdown product of heme, which can be directly excreted in fish. It is a natural anti-oxidant, and in humans high levels of biliverdin have been linked to a decreased risk of cardiovascular disease and cancer (37). Heme is catabolized to biliverdin by heme-oxygenase (HO), expression of which is rapidly induced by oxidative stress and NO, indicating increased heme oxidation via NO induced HO activation in the arginine supplemented fish. Fu *et al* (38) observed a more than 7-fold increase of HO-3 expression in Zucker diabetic fatty rats after arginine supplementation, further suggesting this as a likely explanation for the increased biliverdin observed. Breakdown of heme by HO into biliverdin produces carbon monoxide (CO), which will activate guanylyl cyclase to produce cGMP. This will promote mitochondrial oxidation of both glucose and fatty acids (39), possibly improving the metabolic status of the fish. HO has itself been shown to have anti-inflammatory, anti-oxidant and anti-apoptosis effects, possibly indicating a pro-survival effect of arginine. However, the increase

in biliverdin could also be linked to oxidative stress in the liver after arginine supplementation, due to production of reactive oxygen species (ROS) (40, 41). The increase in oxidized glutathione in the liver further supports this theory. This is in correspondence with our previous observation of increased expression of glutathione peroxidase 3 in liver of Atlantic salmon after dietary arginine supplementation (21). High biliverdin concentrations have also been linked to green liver syndrome in red sea bream, where taurine supplementation reduced biliverdin concentrations as well as alleviating the symptoms (42). However, Atlantic salmon has the ability to store taurine in the liver, and therefore they do not develop this syndrome (43). It is interesting that heme itself is synthesized from glycine, and that biliverdin is increased in the Arginine group, the group without glycine supplementation. This indicates that heme production is not affected by the diets, rather it must be an effect of arginine, either increasing heme breakdown, or inhibiting further processing or elimination of biliverdin from the liver.

A metabolite of vitamin D3 was lower in plasma of the arginine supplemented salmon. Fish do not synthesize vitamin D themselves; rather vitamin D is accumulated through the food chain, making salmon and other oily fish one of the few dietary sources naturally high in vitamin D for human consumption. Atlantic salmon is therefore fully dependent on vitamin D from the diet (44). Vitamin D3 deficiency has been reported to impair arginine induced insulin secretion in rats (45). Vitamin D has been linked to the NO pathway, as humans with vitamin D deficiency exhibit lower plasma NO concentrations and slightly higher plasma arginine concentrations (46). Supplementation of vitamin D3 has also been shown to induce NO production in cultured endothelial cells (47). Vitamin D is involved in calcium homeostasis, a cofactor used by NO synthase when converting arginine into NO. If vitamin D3 is involved in arginine induced NO synthesis or insulin secretion in our arginine supplemented fish, this could explain the decreased plasma concentrations of the vitamin D3 metabolite observed after arginine supplementation. However, the exact pathways in which arginine and vitamin D3 metabolism interacts needs to be further elucidated.

Previous studies with arginine supplementation in fish have shown that arginine affects the intestinal microbial metabolism (13, 17) while no current changes in metabolites appeared to be related to microbial metabolism. Future studies should aim to investigate the effect on intestinal metabolism by arginine in Atlantic salmon.

Metabolomics is a useful approach to get a whole-organism overview of affected pathways. However, our result clearly highlights the importance of having a high number of replicates as well as to optimize diets and sample preparation before commencing analysis. In conclusion, our results show that dietary arginine supplementation affects several metabolic pathways in adult Atlantic salmon, which opens up for future studies on these interactions. To our knowledge, this is also the first study linking arginine to vitamin D and heme metabolism in fish.

6. ACKNOWLEDGEMENT

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Abbreviations: HPLC, high performance liquid chromatography, Q-TOF, quadrupole time-of-flight, MS, mass spectrometry, PCA, principal component analysis, HO - heme oxygenase, NO, nitric oxide, MAG - monoacylglycerol

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