Interactions of arginine and polyamines on growth and metabolism in Atlantic salmon

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-Synne

Abstract

Arginine and methionine are indispensable amino acids (AA) for Atlantic salmon, meaning that salmon is unable to produce these AA endogenously and is fully dependent on dietary supply. In addition to be substrates for protein synthesis, these AA are involved in several metabolic pathways in the fish. Arginine is used for production of nitric oxide, creatine, urea and polyamines, while methionine is converted to the methyl donor S-adenosyl methionine (SAM), which is important for polyamine production by supplying the aminopropyl donor decarboxylated SAM. Thus, both arginine and methionine may influence polyamine production. Polyamines are essential for cell growth and differentiation and can modulate gene expression and energy metabolism, stabilize proteins and cell membranes. Polyamines can also induce apoptosis and formation of reactive oxygen species under too high concentrations. Dietary arginine supplementation have been demonstrated to reduce visceral mass while increasing lean mass in pigs and rodents, which has been linked to increased energy consumption caused by increased polyamine production. Hence we aimed to investigate whether arginine supplementation to Atlantic salmon could affect growth and deposition pattern, and to what extent any effects was linked to increased production and catabolism of polyamines. We also assessed if methionine affected polyamine production by modulating SAM availability.

Two feeding trials were conducted, in juvenile and adult Atlantic salmon, with graded supplementation of arginine beyond the established requirement for growth. Tissue samples were taken to analyse gene expression, amino acids, polyamines and other metabolic parameters. Liver cells were isolated from the adult salmon fed graded inclusions of arginine and cultured *in vitro* together with activator/inhibitor of polyamine metabolism, in order to assess the importance of polyamine metabolism for cell survival and lipid metabolism. Metabolomic analysis applying HPLC-QTOF MS were performed on liver and plasma samples from the adult salmon to assess how arginine affects metabolic pathways. At last, a feeding trial were performed using

juvenile salmon fed a methionine deficient diet or a diet with methionine just above requirement to assess the impact on polyamine metabolism and turnover.

In juvenile salmon fed increasing arginine an effect on growth was observed, but this effect was not linear. The growth was due to an effect on both protein and lipid deposition, appeared to be equally distributed between tissues and was likely due to a faster overall growth. In adult salmon arginine had no effect on growth or deposition of fat or protein. Juvenile salmon appeared to have increased turnover of polyamines in the liver and this was associated with increased expression of carnitine palmitoyl transferase-1 (CPT-1), that increased oxidation of long-chained fatty acids in the liver. Arginine did not affect polyamine concentrations in adult salmon, and we could not document a clear effect on energy metabolism or growth. Metabolomic investigation of plasma and liver however, showed that arginine affected metabolism, even if no growth differences were observed. This revealed novel interactions of arginine in the metabolism of Atlantic salmon, such as interactions with heme, vitamin D, prostaglandins and branched chain AA. These interactions warrants further exploration. Cell studies on the isolated liver cells further confirmed that arginine significantly affected the metabolome and indicated an involvement of arginine in glucose metabolism. The cell studies also showed a difference in the initial and long-term responses to arginine in liver cells, suggesting an adaptation to long-term arginine supplementation. Also, arginine appeared to affect metabolism and cell survival trough pathways independent of polyamines. Finally, the methionine trial demonstrated that methionine deficiency strongly affects tissue concentrations of polyamines and SAM, suggesting that methionine is crucial to maintain polyamine homeostasis and thus avoid cell damage in liver of Atlantic salmon.

List of publications

Paper I

Andersen, S.M., Holen, E., Aksnes, A., Rønnestad, I., Zerrahn, J.E. and Espe, M. (2013). "Dietary arginine affects energy metabolism through polyamine turnover in juvenile Atlantic salmon *(Salmo salar)*." British Journal of Nutrition **110**, 1968-1977.

Paper II

Andersen, S.M., Holen, E., Aksnes, A., Rønnestad, I., Zerrahn, J.E. and Espe, M. (2014). ”Adult Atlantic salmon *(Salmo salar L.)* adapts to long-term surplus dietary arginine supplementation.” Aquaculture Nutrition, in press

Paper III

Andersen, S.M., Taylor, R., Holen, E., Aksnes, A. and Espe, M. (2014). “Arginine concentration and exposure time affects polyamine and glucose metabolism in primary liver cells isolated from Atlantic salmon.” Amino Acids **46**, 1225-1233

Paper IV

Andersen, S.M., Assaad, H.I., Lin, G., Wang, J., Aksnes, A., Wu, G. and Espe, M. (2014). ”Metabolomic analysis of plasma and liver from surplus arginine fed Atlantic salmon.” Frontiers in Bioscience, in press

Paper V

Espe, M., Andersen, S.M., Holen, E., Rønnestad, I., Veiseth-Kent, E., Zerrahn, J.E. and Aksnes, A. (2014). ” Methionine deficiency does not increase polyamine turnover through depletion of liver S-adenosylmethionine (SAM) in juvenile Atlantic salmon.” British Journal of Nutrition, in press

The papers are from now on referred to by their roman numbers

Abbreviations

AA	Amino acids
ACC	Acetyl CoA carboxylase
ACO	Acetyl CoA oxidase
AMPK	5'-AMP-activated protein kinase
ANOVA	Analysis of variance
APAO	Acetylated polyamines oxidase
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BAX	BCL-2 associated x-protein
CPSIII	Carbamoyl phosphate synthase III
CPT-1	Carnitine palmitoyl transferase 1
DAA	Dispensable amino acid
dcSAM	Decarboxylated SAM
DENSPM	N^{1},N^{11}-Diethylnorspermine
DFMO	Alpha difluoromethylornithine
FAS	Fatty acid synthase
GLUT-4	Glucose transporter-4
GPX-3	Glutathione peroxidase 3
HO	Heme oxygenase
HPLC	High performance liquid chromatography
IDAA	Indispensable amino acid
MAT	Methionine adenosyltransferase
MS	Mass spectrometry
MTA	5'methylthioadenosine
mTOR	Mammalian target of rapamyacin
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
OAT	Ornithine aminotransferase
OCT	Ornithine carbamoyltransferase
ODC	Ornithine decarboxylase
P5C	Pyrroline-5-carboxylate
PC	Phosphatidylcholine
PCA	Principal component analysis
PGC-1α	PPAR-γ coactivator 1α
PPAR-α	Peroxisome proliferator-activated receptor-α
qPCR	Quantitative real-time polymerase chain reaction

Q-TOF	Quadrupole time-of-flight
ROS	Reactive oxygen species
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SAMdc	SAM decarboxylase
SMO	Spermine oxidase
SSAT	Spermidine/spermine-N1-acetyltransferase
TCA	Tricarboxylic acid cycle (citric acid cycle/Kreb's cycle)
TNF-α	Tumour necrosis factor-α
UCP	Uncoupling protein
WAT	White adipose tissue

Contents

1. Introduction

1.1 Amino acids in Atlantic salmon feed

Atlantic salmon (*Salmo salar L.)* is found naturally in the North Atlantic Ocean and in connected rivers. Fisheries is an important part of Norway's cultural and economical history, but today most of the commercially available salmon comes from aquaculture. Fish farming of salmon started in the 1970's and has since developed into a major industry in coastal areas. A traditional salmon diet is high in protein, and juvenile salmon require up to 60% protein in their diets (NRC 2011). Fishmeal have until recently been the main protein source in salmon feed, however, the feed industry does not longer solely rely on fishmeal as the protein source. As the aquaculture industry is increasing globally, while catches of wild fish used for fishmeal production remains fairly constant, the prices and demand go up. This has led to a trend towards higher inclusion of plant protein in fish feed (Torstensen et al. 2008; Espe 2012). Replacing fishmeal with plant proteins leads to reduced growth and protein utilization in several fish species (Gomes et al. 1995; Kaushik et al. 1995; Fournier et al. 2004), suggesting a change in requirement of some amino acids (AA, (Fournier et al. 2003). Non-protein nitrogen compounds in the diets have been shown to increase feed intake (Fournier et al. 2002; Espe et al. 2012), but a minimum of 5% fishmeal inclusion is still considered necessary for optimal growth (Espe et al. 2007). As plant proteins very in their AA profiles and also differ compared to fishmeal, different plant protein ingredients have to be mixed in order to obtain a satisfactory AA profile in the fish feed (El-Mowafi et al. 2010; Conceicao et al. 2012). AA are traditionally classified as dispensable (DAA) or indispensable (IDAA), depending on whether they can be synthesised endogenously or not (Table 1). In later years, the term functional AA have received more attention, relating to AA that are involved in key metabolic pathways and can modulate immune function, health, development, reproduction and growth (Wu 2010). Functional fish feed, by supplementing

functional AA such as arginine, show promise to improve disease resistance, optimize growth and enhance sustainability of aquaculture production (Li et al. 2009; Martinez-Rubio et al. 2012).

Table 1 - *Content of amino acids in various plant proteins compared to fishmeal. + indicates higher concentration than fishmeal, while – indicates lower concentration. Double signs indicate more than 50% difference. Blank squares indicate similar amino acid concentration as fishmeal. Modified from Conceicao et al. (2012). * - Dispensable amino acids for fish.*

Amino acid	Corn gluten	Wheat gluten	Soybean	Rapeseed	Lupin
Indispensable amino acids for fish					
Val	-	-	-		-
Trp	--	-	+	+	-
Thr	-	-	-		-
Phe + *Tyr	++	+	+	-	+
Met + *Cys	+		-	+	-
Lys	--	--	-	-	-
Leu	++				
His	-	-	+	+	-
Arg	-	-	+		++
Ile		-			
Dispensable amino acids for fish					
Ser	+	+	+	+	+
Glu	++	++	+	+	++
Asp	-	--	+	-	+
Pro	++	++	+	+	
Gly	--	-	-	-	-
Ala	+	--	-	-	-

1.2 Arginine metabolism

Arginine is a basic AA that is especially abundant in seafood, meat and dairy products (Wu and Morris 1998). Apart from being a building block for proteins, arginine is involved in several metabolic pathways. It is essential for cell growth and survival, involved in immune functions, reproduction, cancer and hair growth, among others (Wu et al. 2009). Arginine is categorized as an IDAA in fish, as an endogenous pathway of arginine production has not yet been described (Li et al. 2009). In mammals however, arginine is described as a conditionally IDAA, as glutamate, glutamine and proline can be converted into ornithine, which again can be synthesised

to arginine via citrulline in healthy individuals, consuming carbamoyl phosphate (Fig 1). In fish, carbamoyl phosphate is synthesised by carbamoyl phosphate synthase III (CPSIII), a mitochondrial located enzyme requiring glutamine, not ammonia, as a substrate (Anderson 1981; Ball et al. 2007). In rainbow trout *(Oncorhynchus mykiss)* no hepatic activity of CPSIII or ornithine carbamoyl-transferase (OCT) were observed, possibly explaining the low *de novo* synthesis of arginine (Korte et al. 1997). There have been indications of endogenous synthesis of arginine from glutamate in channel catfish *(Ictalurus punctatus)* (Buentello and Gatlin 2000) but these findings are not yet conclusive. Chickens and cats both lack the enzyme pyrroline-5-carboxylate (P5C) synthase and this is also believed to be the reason for a lack of arginine production via citrulline in fish (Wu et al. 2009). The rate of proline conversion into P5C is also considered insignificant (Li et al. 2009).

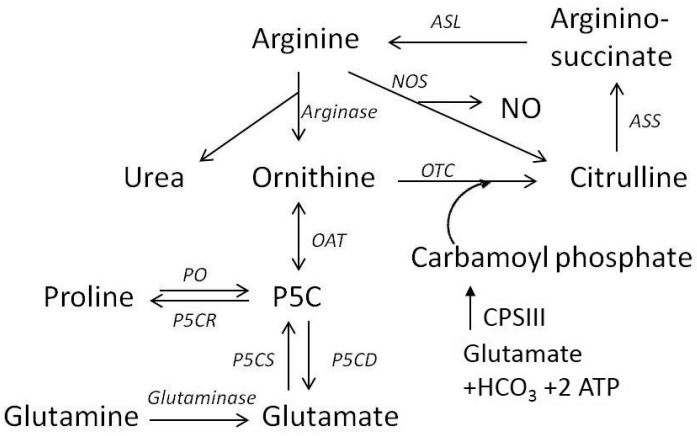

Figure 1 *– Pathways for endogenous arginine synthesis. Proline and glutamate can be converted to P5C by PO and P5CS respectively. P5C is then the substrate for ornithine production by OAT. Ornithine is then converted to citrulline by OCT, consuming carbamoyl phosphate. Citrulline can be syntesised back to arginine by a two-step process. In mammals, production of citrulline is mainly by the enterocytes, while arginine synthesis from citrulline occurs in the kidneys. When arginine concentrations are high, the pathway can be reversed, and proline and glutamate can be synthesized from P5C. Enzymes in italics. P5C – pyrroline-5-carboxylate, NO – nitric oxide, NOS – NO synthase, OCT – ornithine carbamoyltransferase, ASS – argininosuccinate synthase, ASL – argininosuccinate lyase, OAT – ornithine aminotransferase, P5CR – P5C reductase, P5CS – P5C synthase, P5CD – P5C dehydrogenase, PO – proline oxidase, CPSIII – carbamoyl phosphate synthase III*

In fish, arginine is involved in a range of metabolic processes (Fig. 2), including synthesis of nitric oxide (NO), creatine and polyamines in addition to be part of the urea cycle and substrate for protein synthesis. Arginine is used to synthesize NO and citrulline by various tetrahydrobiopterin-dependent NO synthase (NOS) enzymes in virtually all cell types (Mori 2007). NO is a potent vasodilator, increasing blood flow to organs and thus allowing for higher uptake of nutrients for oxidation (McKnight et al. 2010). NO also participates in facilitating neurological function, cell signalling, regulating osmolality and macrophage activation in fish (Li et al. 2009). Creatine is the storage molecule for energy in the muscle and is thus required in order to increase

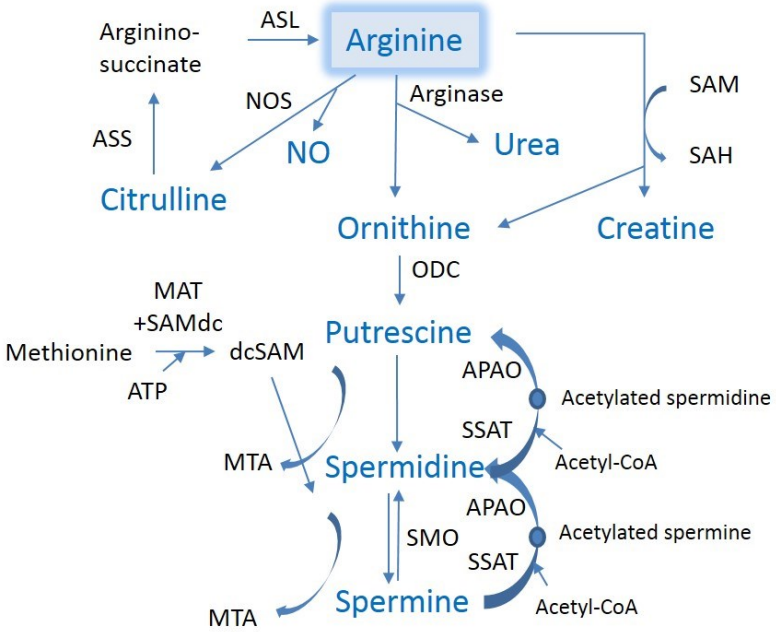

Figure 2 *- Arginine involvement in cellular metabolic pathways. Arginine can be used to produce nitric oxide (NO) and citrulline by NO synthase (NOS), to produce creatine as well as being part of the urea cycle, producing urea and ornithine by the enzyme arginase. Ornithine can further be used to produce putrescine by ornithine decarboxylase (ODC), the rate-limiting enzyme for polyamine production. Putrescine can then be converted to spermidine and spermine, an energy dependent process, consuming decarboxylated S-adenosylmethionine (dcSAM), which is made from ATP and methionine. Spermine and spermidine can then be acetylated by spermidine/spermine-acetyltransferase (SSAT), consuming acetyl-CoA, enabling them to be transported out of the cell or to be converted back to the shorter polyamine by acetylated polyamine oxidase (APAO). MTA – 5'methylthioadenosine, MAT – methionine adenosyl transferase, SAH–S-adenosylhomocysteine, ASS – argininosuccinate synthase, ASL – argininosuccinate lyase, SMO – spermine oxidase.*

muscle mass. Creatine synthesis occurs via a three-step process requiring arginine and glycine as well as consuming approximately 40% of all methyl groups provided from methionine derived S-adenosylmethionine (SAM) (Brosnan et al. 2011). SAM is made from methionine by methionine adenosyl transferase (MAT) in an ATP-dependent process and act as the methyl donor for a range of transmethylation reactions (Mato et al. 2002). Creatine is continuously broken down to creatinine which is excreted in urine, and the creatine pool thus needs to be continuously replaced, either through dietary or endogenously produced creatine (Brosnan et al. 2011). Arginine can also be converted to ornithine and urea by the enzyme arginase, which competes with NOS for the substrate arginine (Morris 2009). Arginine is thus important in order to eliminate nitrogen through the urea cycle. Two isoforms of arginase are known; arginase-1 is known to be almost exclusively expressed in the cytosol of mammalian liver cells while arginase-2 is localized in the mitochondrial matrix of several tissues (Mori 2007). The product ornithine can further be used to produce the polyamine putrescine by the enzyme ornithine decarboxylase (ODC), which again may be used to synthesize the polyamines spermidine and spermine (Pegg 2006), consuming decarboxylated SAM (dcSAM).

1.3 Arginine and Polyamines

Polyamines are small, positively charged organic molecules, which are present in all eukaryotic cells (Janne et al. 2006). As the names imply, spermidine and spermine were first discovered in human semen, as early as in 1678, and is responsible for the typical odor of semen (Leeuwenhoek 1687). Polyamines are essential for cell cycle progression and differentiation, and they can bind to negatively charged molecules such as RNA, DNA and membrane phospholipids, modulating gene expression and stabilizing DNA and chromatin structure (Wang et al. 2004). Production of spermidine and spermine is by spermidine and spermine synthase, respectively, requiring dcSAM as an aminopropyl donor. dcSAM is made from SAM by SAM decarboxylase (SAMdc) and is solely diverted to polyamine production (Pegg 2009).

The enzyme spermidine/spermine-N1-acetyltransferase (SSAT) can acetylate spermine and spermidine, enabling them to be transported out of the cell, or to be converted to shorter polyamines by the enzyme acetylated polyamines oxidase (APAO (Pegg 2008)). SSAT consumes acetyl-CoA, and increased turnover of polyamines as such might increase energy consumption of the cell, by increasing consumption of both ATP and acetyl-CoA (Kee et al. 2004; Jell et al. 2007). Spermine can also be directly oxidized to spermidine by spermine oxidase (SMO, (Wang et al. 2001). ODC and SSAT are the rate-limiting enzymes in polyamine metabolism, and several studies have been performed both *in vivo* (Jell et al. 2007; Pirinen et al. 2007) and *in vitro* (Soderstjerna et al. 2010; Vuohelainen et al. 2010) targeting these enzymes.

Knockout of arginase in mice are lethal at an early stage (Iyer et al. 2002), demonstrating the importance for polyamine production in cell growth and survival. SSAT knockout in mice on the other hand, is not lethal and only slight alterations in the polyamine pools were observed (Niiranen et al. 2006). Jell et al (2007) constructed transgene mice overexpressing SSAT, which led to a lean phenotype, with markedly less adipose tissue, while knockout mice of SSAT demonstrated an obese phenotype. The lean phenotype also exhibited markedly reduced pools of acetyl-CoA and malonyl CoA along with increased glucose and palmitate oxidation in white adipose tissue (WAT), demonstrating the importance of polyamine turnover in energy metabolism. Further, Pirinen et al (2007) showed that these mice had improved glucose tolerance and insulin sensitivity and increased number and size of mitochondria in WAT along with increased expression of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), 5'-AMP-activated protein kinase (AMPK) and genes involved in oxidative phosphorylation.

Induction of polyamine catabolism also has the potential to induce apoptosis and increase oxidative stress through formation of reactive oxygen species (ROS), as both APAO and SMO releases hydrogen peroxide and reactive aldehydes (Babbar et al. 2007; Larque et al. 2007). As APAO is located in the peroxisomes while SMO is located in the cytosol, SMO is expected to be the main producer of cytotoxic ROS

(Pledgie et al. 2005; Babbar et al. 2007). ROS can cause damage to DNA, lipids and proteins, as well as induce apoptosis or mutations that may develop into cancer. On the other hand, arginine have also shown to ameliorate oxidative stress, as arginine increased the antioxidant capacity in liver of piglets injected with diquat, simultaneously decreasing expression of inflammatory cytokines (Zheng et al. 2013). This demonstrates the importance of controlling cellular concentrations of polyamines, as too high concentrations can induce apoptosis and cancer, while a depletion can halt cell proliferation and growth (Babbar et al. 2007).

1.4 Arginine interactions with energy metabolism

Dietary arginine supplementation has shown to cause reduced adipose mass, while maintaining lean mass in diabetic or obese humans (Lucotti et al. 2006) rodents (Jobgen et al. 2009; Clemmensen et al. 2012), sheep (Satterfield et al. 2012) and growing pigs (Tan et al. 2009). Part of this effect has been related to arginine's ability to differentially regulate gene expression in muscle and adipose tissue (Jobgen et al. 2009; Tan et al. 2011), causing increased lipogenesis in muscle and lipolysis in adipose tissue. Transcription and translation of several genes have been demonstrated to be influenced by polyamines, via polyamine response elements (Pegg 2009), though arginine and NO also are able to directly affect gene expression (McKnight et al. 2010). Arginine induces muscle gain both by increasing protein synthesis, demonstrated by activation of mammalian target of rapamyacin (mTOR) in pigs, by inhibiting proteolysis as well as by inducing lipogenesis in muscle (Yao et al. 2008; Bauchart-Thevret et al. 2010). In obese rodent models the lipid reducing effect of arginine in adipose and visceral tissues are greater than the increase in lean mass, resulting in an overall weight loss (Jobgen et al. 2009), while in metabolically healthy growing pigs, the increase in lean mass is greater than the loss of visceral fat, resulting in overall weight gain (Tan et al. 2009). Overall, arginine have greater effect on body lipid deposition and metabolism in already metabolically challenged rodents (Jobgen et al. 2009; Clemmensen et al. 2012) or in knockout models (Pirinen et al.

2007), while studies in healthy humans have yielded limited results (Evans et al. 2004).

1.4.1 Arginine and lipid metabolism

The lipid reducing effect of arginine has been related to increased turnover of polyamines, an energy consuming process. Synthesis of dcSAM from methionine requires ATP, while acetylation of polyamines by SSAT consumes acetyl-CoA (Kee et al. 2004). Decreasing acetyl-CoA concentrations will lead to decreasing malonyl-CoA concentrations, releasing malonyl-CoA's inhibitory effect on carnitine palmitoyltransferase-1 (CPT-1), the rate-limiting enzyme transporting long-chained fatty acids into the mitochondria for β-oxidation (McGarry and Brown 1997). Increased turnover of polyamines thus have the potential to increase β-oxidation, improving metabolic health (Jell et al. 2007). Each polyamine cycle consumes 4 ATP equivalents and the following drop in ATP/AMP ratio will activate AMPK (Pirinen et al. 2007) which acts to increase ATP production through glucose and fatty acid oxidation while inhibiting lipogenesis (Winder and Hardie 1999).

Arginine can also increase transport of long chain fatty acid for β-oxidation through the NO pathway (fig. 3). NO can inhibit acetyl-CoA carboxylase (ACC) by stimulating phosporylation of AMPK or activating guanylyl cyclase, increasing cGMP production, both of which will inhibit ACC, and increase mitochondrial β-oxidation (Fu et al. 2005; Jobgen et al. 2006). NO can also directly increase the activity of CPT-1 (McKnight et al. 2010). As a potent vasodilator NO can increase energy uptake by increasing blood flow to peripheral organs, allowing for increased nutrient uptake. At last, NO can also increase mitochondrial biogenesis and oxidative phosporylation through cGMP mediated increased expression of PGC-1α (Nisoli and Carruba 2006). This increases cellular capacity for ATP generation. Even though NO has beneficial effects at physiological concentrations and may protect the cells from apoptosis, it is important to consider that too high concentrations can have pathological effects, leading to cell damage and apoptosis (Mori 2007).

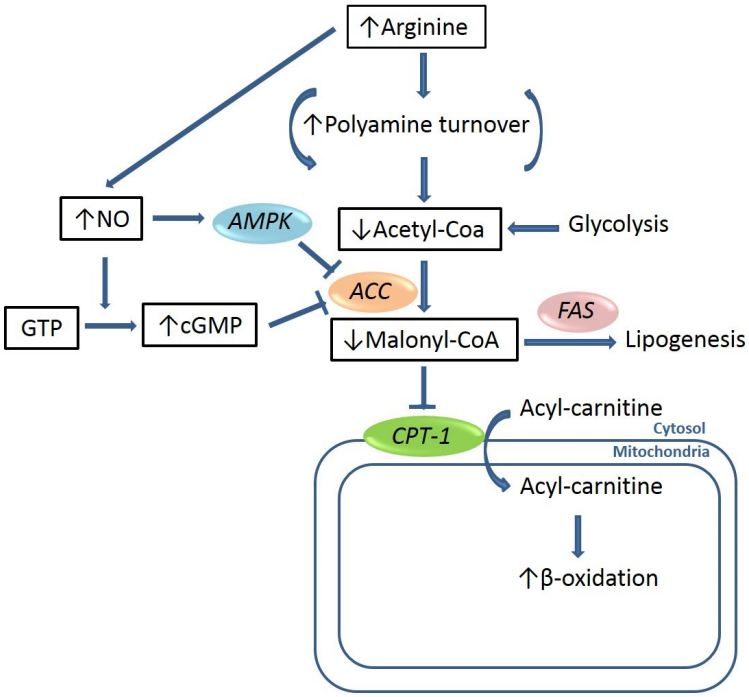

Figure 3 *- Proposed mechanisms for arginine to increase energy consumption, leading to a leaner phenotype. Arginine can increase polyamine turnover due to increased substrate availability and activated SSAT which will deplete cellular stores of acetyl-CoA and ATP. Less substrate will then be available for ACC to produce malonyl-CoA, and as malonyl-CoA concentrations decrease it will release its inhibitory effect on CPT-1, leading to increased transport of long chained fatty acids into the mitochondria for b-oxidation. Lipogenesis will also decrease as less malonyl-CoA is available for FAS, while glycolysis will increase to produce more acetyl-CoA. NO, produced from arginine, can also affect this pathway by activating AMPK or producing cGMP which both phosphorylates and inactivates ACC.*

The lipid reducing effect of arginine have also been related to increased growth of brown adipose tissue (BAT) in mammals, through activation of uncoupling proteins (UCPs), the only proteins able to mediate non-shivering thermogenesis (Tan et al. 2012). For a long time the consensus was that only placental mammals possessed the genes for UCPs, but later research have discovered the existence of UCP 1-3 also in fish (Jastroch et al. 2005). However, their function in fish appears to be more related to ROS production, rather than thermogenesis (Tseng et al. 2011). The current opinion is that fish do not possess BAT, and this could potentially limit the lipid reducing effect of arginine in fish.

1.4.2 Arginine and glucose metabolism

Several studies have shown an effect of arginine on glucose metabolism, increasing glucose oxidation and enhancing insulin sensitivity in Zucker diabetic fatty rats (Fu et al. 2005), diet-induced obese rodents (Jobgen et al. 2009; Clemmensen et al. 2012) and transgene mice (Jell et al. 2007). However, in a transgenic line overexpressing SSAT insulin production and sensitivity was decreased (Cerrada-Gimenez et al. 2012), illustrating the importance of keeping polyamine catabolism within physiological range, and suggesting that arginine may affect glucose metabolism through other pathways than polyamines. Arginine is indeed a potent stimulant for endrocrinological functions in fish and is in fact a better activator for insulin release than glucose in salmon (Mommsen et al. 2001). Injections of arginine in Pacific salmon *(Oncorhynchus kisutch)* efficiently decrease plasma glucose (Plisetskaya et al. 1991), suggesting higher uptake of glucose for energy consumption (Fig. 4). Arginine also stimulates release of other pancreatic hormones such as glucagon and growth hormone, and injection of arginine into feeding rainbow trout resulted in decreased levels of plasma fatty acids and liver glycogen (Mommsen et al. 2001).

NO can inhibit gluconeogenesis and glycogen synthesis in the liver, through activation of AMPK which stimulates glucose transporter-4 (GLUT-4) translocation and phosphorylates and inactivates ACC (Fig 4, (Jobgen et al. 2006). NO stimulated glucose uptake in skeletal muscle and adipose tissue through GLUT-4 translocation to the plasma membrane have also been suggested to be mediated through cGMP rather than insulin signaling (Tanaka et al. 2003; Fu et al. 2005). Genes for AMPK and GLUT-4 was upregulated in WAT of SSAT mice (Pirinen et al. 2007), increasing glucose oxidation, while GLUT-4 was downregulated in adipose tissue of arginine supplemented growing-finishing pigs (Tan et al. 2011). The latter was associated with decreased expression of genes for lipogenesis, limiting substrate availability of glucose, and increased lipolysis.

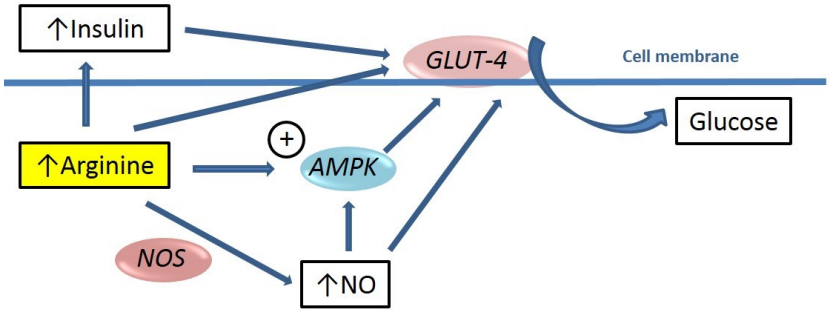

Figure 4 *- Possible ways for arginine and NO to increase glucose uptake in insulin sensitive tissue through upregulation and translocation of GLUT-4 to the cell membrane. Arginine activates insulin release, activates AMPK and increase NO production, all known to increase translocation of GLUT-4 to the plasma membrane.*

1.5 Arginine supplementation in fish

Few studies have examined the effect of surplus dietary arginine in fish (Table 2), while several others have studied the effects of combined supplementation of both arginine and glutamine/glutamate or lysine (not discussed herein). Also, a number of studies have aimed to determine requirement of arginine in a range of fish species. Overall, surplus arginine supplementation beyond requirement have proved promising in enhancing growth in several species including red drum *(Sciaenops ocellatus)* (Cheng et al. 2011), channel catfish (Pohlenz et al. 2013) and hybrid striped bass *(Morone chrysops x M. saxatilis)* (Cheng et al. 2012), which is mainly related to improved protein retention. However, in experiments with salmon, arginine has shown varying effects. Plisetskaya et al (1991) observed an initial growth boost in Pacific salmon after arginine supplementation, while this effect diminished over time. This was seen in relation to increased plasma insulin levels, decreasing appetite over time. In general, growth appears to be more affected by arginine in juvenile stages than in adult fish. Surplus arginine also appears more effective in enhancing growth when supplemented to a plant protein diet than to a fishmeal diet (Tulli et al. 2007). No trials have shown any effect of arginine on overall survival in fish, which is in

accordance with others stating arginine as safe when supplemented up to 30g/d in humans (Luiking and Deutz 2007).

Table 2 *– Effects of surplus arginine supplementation in fish trials. Arrows indicate up- or downregulation after arginine supplementation, = – not affected. PR – protein retention, FE- feed efficiency, NR – not reported, FCR – feed conversion ratio, HSI – hepatosomatic index.*

Fish specie	Arg range	Fish size	Time weeks	Growth	PR	FE	Others	Reference
Jian carp *(Cyprinus carpio)*	9.8 – 24.5 g/kg	6 g	9	↑	↑	↑	↑ intestinal absorption and digestibility	(Chen et al. 2012)
Red drum *(Sciaenops ocellatus)*	2.3% dry wt.	6.9 g	7	↑	NR	=	Improved intestinal structure	(Cheng et al. 2011)
Channel catfish *(Ictalurus punctatus)*	0.5-4% of diet	23 g	6	↑	↑		↑ arginine deposition, and plasma arg, orn, cit, gln and glu	(Pohlenz et al. 2014)
Chinook/Coho salmon *(O.tshawytscha/ kisutch)*	0-60 g/kg dry diet	2/8.2 g	8/4	↓/=	NR	NR	↑ plasma insulin. A short stimulatory effect on growth	(Plisetskaya et al. 1991)
Rainbow trout *(O. mykiss)*	0-50 g/kg dry diet	9.1 g	8	↑	NR	NR	↑ plasma insulin ↑FCR and HSI	(Plisetskaya et al. 1991)
Sea bass *(Dicentrarchus labrax)*	16 – 40 g/kg	8.5 g	10	↑/=†	↑/=†	↑/=†	Arginase activity higher in fishmeal fed fish.	(Tulli et al. 2007)
Turbot *(Psetta maxima)*	16 – 40 g/kg	7.4g	12	↑/=†	↑/=†	↑/=†	Plant proteins greatly reduced growth.	(Fournier et al. 2003)
Rainbow trout	16 – 40 g/kg	9.3g	12	-[1]/↓†	↓/=†	=/=†	↑ Urea excretion ↓HSI	(Fournier et al. 2003)

† - Plant protein based diets vs. fishmeal diets, [1]- Weight gain increased in group given 3% arginine, but not in the group given 4% arginine, compared to control of 1.6% arginine

1.5.1 Functional aspects of arginine supplementation in fish

In fish, arginine shows promise as a functional AA (Wu 2010). Apart from affecting growth and energy retention, arginine has been reported to affect reproduction (Lefevre et al. 2011), endocrine functions (Pohlenz et al. 2013) and immune resistance (Costas et al. 2011) in fish. Polyamines have been shown to play a role in reproduction in mammals, and an absence of polyamines is related to infertility and embryonal arrest (Lefevre et al. 2011). Little research is performed with arginine and reproduction in fish, but Peres et al (1997) reported that spermine supplementation to sea bass larvae resulted in increased survival rate and activation of pancreatic enzymes. Arginine has proven effective to aid the immune system under stress and disease (Wu et al. 2000). Today, arginine is given as supplement in several disease states, including sepsis (Davis and Anstey 2011), burns (Yan et al. 2007), wound healing (Witte and Barbul 2003) hypertension (Gokce 2004) and after intensive physical activity (Zajac et al. 2010), due to increased arginine utilization and breakdown after arginase release from injured tissues or due to the vasodilating effect of NO. Increased inclusion of arginine in channel catfish diets has shown to correlate with survival when exposed to the bacteria *Edwardsiella ictaluria,* normally causing sepsis or encephalitis in infected fish (Buentello and Gatlin 2001). The authors suggested this was due to prolonged NO production by macrophages after increased plasma arginine levels (Buentello and Gatlin 2001). Further *in vivo* and *in vitro* experiments in channel catfish confirmed this positive effect on the immune system, as arginine supplementation increased hematocrit, hemoglobin and erythrocyte count, improved macrophage killing and phagocytosis abilities as well as enhanced native T-cells and B-lymphocytes proliferation after mitogenic exposure (Buentello et al. 2007; Pohlenz et al. 2012). Stress is known to alter AA requirement as well as to impair disease resistance in Senegalese sole *(Solea senegalensis)* (Aragao et al. 2008), and Costas et al (2011) found that dietary arginine supplement to Senegalese sole increased the respiratory burst after mitogenic exposure. In a later paper they also found that plasma cortisol levels were reduced in stressed turbot *(Scophthalmus maximus)* after arginine supplementation (Costas et al. 2013). Reduced plasma

cortisol concentrations after arginine supplementation in response to stress have also been found in swine (Ma et al. 2010; Yao et al. 2011) and humans (Smriga et al. 2007). This demonstrates that arginine is indeed a functional AA, and highlights the possible benefits of adding arginine beyond the requirement of growth in fish feed, especially under challenging conditions.

1.5.2 Arginine interactions with lysine

Arginine is known to interact antagonistically with lysine in several species including Atlantic salmon (Kim et al. 1992; Berge et al. 1999; Wu et al. 2009; Zhou et al. 2011). Both AA use the same transporter proteins in the intestine and across cell membranes, and can therefore inhibit uptake of each other. High concentrations of lysine in Atlantic salmon feed have shown to reduce arginine and ornithine concentrations in plasma and muscle (Berge et al. 1998). Lysine is also known to inhibit arginase activity in Atlantic salmon (Berge et al. 1999). When Berge et al (2002) fed Atlantic salmon diets marginal in lysine, arginine supplementation improved growth. When on the other hand dietary arginine was marginal, lysine supplementation also improved growth, though not to the same extent (Berge et al. 2002). In juvenile cobia *(Rachycentron canadum)* the optimal ratio between lysine and arginine seems to be close to 1:1, and imbalanced ratios reduce feed intake and growth (Nguyen et al. 2014).

1.6 Methionine involvement in polyamine metabolism

As arginine, methionine is an IDAA in fish. Plant proteins such as soy and lupin are low in methionine (Table 1), and methionine and lysine is often the first limiting AA in plant proteins. In addition, plant proteins are absent or low in taurine and choline, products of methionine metabolism (Fig. 5). Methionine is involved in a range of metabolic processes, but this thesis focus only on its interactions with polyamine metabolism. SAM is produced from methionine by MAT which again is used to

synthesise dcSAM. The aminopropyl donor dcSAM is then solely directed to polyamine synthesis from arginine (Pegg et al. 1998). A previous study showed that methionine supplementation in the diet to Atlantic salmon correlated with liver SAM (Espe et al. 2008). As such, methionine has the potential to affect polyamine production by regulating availability of dcSAM. When Espe et al (2010) fed Atlantic salmon diets deficient in methionine, they observed decreased liver concentrations of both SAM and SAH as well as increased bile acid concentrations in plasma and faeces. Further, they showed that taurine supplementation to a plant-protein based diet

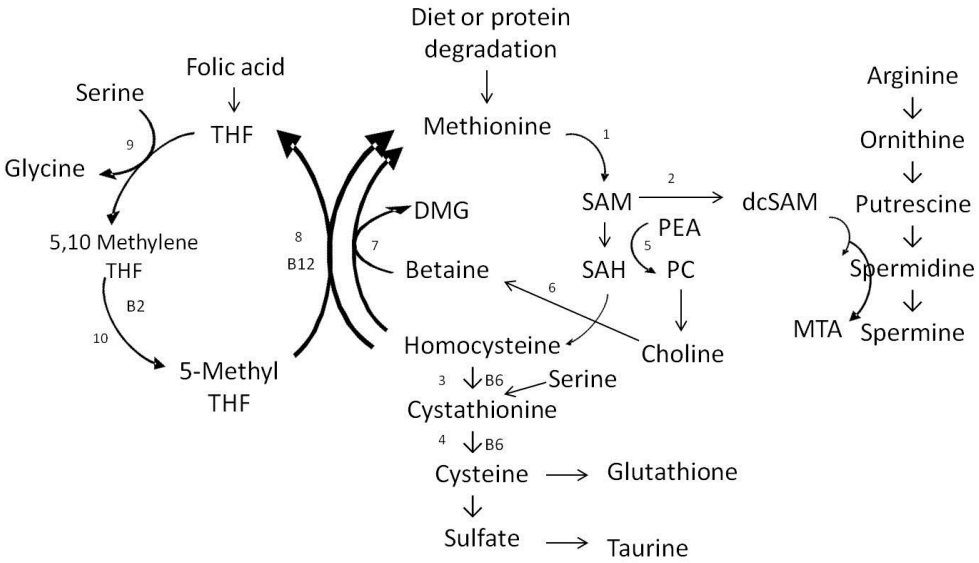

Figure 5 *- Methionine metabolic pathways and interaction with polyamine synthesis from arginine. SAM is produced from methionine by MAT, and can then be converted to dcSAM by SAMdc. dcSAM is solely directed towards polyamine production and is used to make spermidine and spermine. SAM is also used in a range of other cellular metabolic pathways, and it's demethylated product SAH can then be restored back to methionine via a multistep process. SAM – S-adenosyl methionine, SAH – S-adenosyl homocysteine, dcSAM – decarboxylated SAM, MTA - 5'methylthioadenosine, PEA – phosphatidylethanolamine, PC – phosphatidylcholine, DMG – dimethylglycine, THF – tetrahydrofolate. Enzymes: 1- methyl adenosyl transferase, 2 – SAM decarboxylase, 3 – cystathionine-β-synthase, 4 – cystathionine-γ-lyase, 5 – Phosphatidyl-ethanolamine N-methyltransferase, 6 – choline oxidase, 7 – betaine-homocysteine methyltransferase, 8 – methionine synthase, 9 – serine hydroxymethyltransferase, 10 – methylene tetrahydrofolate reductase*

increased liver concentrations of all arginine derived polyamines, simultaneously decreasing lipid accumulation (Espe et al. 2012). This could be related to a methionine sparing effect of taurine, leaving more SAM available for polyamine production, leading to a leaner phenotype, as described previously. Correa-Fiz et al (2012) observed similar effects in mice, where methionine supplemented drinking water decreased putrescine and SAM concentrations in liver, while spermidine, spermine and SAH concentrations increased highlighting the importance of methionine for polyamine metabolism in mammals.

2. Aims of the study

The aims of the work conducted in this thesis were to assess how arginine and methionine affect fat and protein metabolism in Atlantic salmon and how they interact with polyamine metabolism. This was done by:

- Investigating the effect of dietary arginine supplementation on growth and deposition patterns in juvenile and adult Atlantic salmon
- Assessing the effect of surplus dietary arginine on polyamine metabolism in juvenile and adult Atlantic salmon
- Using key inhibitors of the polyamine pathway in primary liver cells isolated from Atlantic salmon to assess the relationship between arginine, polyamine turnover and energy metabolism
- Learning and applying a metabolomic approach to assess metabolic effects after dietary supplementation with arginine
- Investigating whether methionine limitation affects polyamine metabolism through SAM depletion in juvenile Atlantic salmon

3. Methodical considerations

3.1 Fish feeding trials

All feeding trials (paper I, II and V) took place at indoor research facilities of EWOS Innovation AS in Dirdal, Norway under closely monitored conditions. The fish were fed and monitored by technical staff at the research station.

3.2 Fish diets

For the arginine trials (papers I and II), four plant-protein based diets were prepared with arginine at requirement (Berge et al. 1997), and then supplemented with increasing crystalline arginine concentrations. This resulted in increasing nitrogen concentrations, which could partly explain the increased weight gain observed. For the adult salmon (paper II) this problem was managed by adding glycine to diets Arg0 – Arg10, resulting in isonitrogenous compositions. This appeared reasonable at the moment, as glycine has been addressed as a DAA in fish. Later we discovered reports that glycine may act as a functional AA (Wu 2010) and is involved in several metabolic pathways in the cells (Wang et al. 2013). As glycine supplementation have been demonstrated to increase weight gain in shrimp *(Litopenaeus vannamei)* (Xie et al. 2014) it is not unlikely that a possible weight gain effect from arginine in the adult salmon (paper II) is masked by an effect from the added glycine. In the methionine trial (paper V), tapioca was added as a filler to the unsupplemented diet, as the difference in methionine was regarded too little to significantly affect the total nitrogen content. To avoid lysine interactions with arginine in our feeding trials, as described in section 1.5.2, lysine was kept at a minimum in the diets described in papers I and II, while crystalline lysine was added to the experimental diets in the methionine trial to have similar concentrations to the fishmeal control diet (paper V).

There was an unknown technical incidence during the extrusion of the feed for the juvenile salmon (paper I), which caused decreased fat absorption into the pellets. This resulted in a low fat content of all the four experimental diets, though fat content was the same in all four. Therefore, the obtained results might not have been the same if given a diet with a fat content at requirement. However, these results might indicate that arginine supplementation can increase weight gain when juveniles are given a low fat diet. A fish meal control diet was also included as a positive control for growth, but as this diet had twice the fat content as the experimental diets, the results were not comparable, and this diet was not included in the final manuscript (paper I).

3.3 Chemical analyses

The methods for energy, fat and protein determination in fish and feed (papers I, II and V) are accredited methods at NIFES, according to ISO standards and should thus give reliable and precise results. The methods for free AA, polyamines and SAM/SAH are frequently used in the lab and follow good laboratory practice method (papers I, II and V). Modifications were done on the polyamine method, in order to accommodate for small amounts of sample material. This method can also detect acetylated polyamines, however, their concentrations were below the detection limit, and the method therefore needs to be refined before these compounds can be reliably measured. Acetylated polyamines are thus not included in papers I, II or V. The whole fish total AA analyses were done by EVONIK in Germany following an accredited protocol. ATP, acetyl-CoA and NO were measured using commercial kits, relying on the accuracy of the kit. NO is hard to measure as it is reactive, rapidly diffuses and with a half-life of seconds (Hunter et al. 2013), and thus make it hard to get an accurate picture of NO production. The tissues also had to be homogenised before analysis, possibly affecting the analysed NO.

3.4 Gene expression

It is important to note that gene expression is merely a snapshot of the moment. It provides you a picture of the relative number of transcripts at that exact moment. As gene expression was analysed 8 (papers I and V) or 12 (paper II) weeks after commencing experimental diets, we do not have information about how expression has changed over these weeks. Nor do gene expression directly relate to protein abundance or activity, or how this affects the organism. In our data SSAT is a good example. In both juvenile and adult salmon dietary arginine did not affect SSAT expression (papers I and II). However, both western blot representing protein abundance and enzyme activity were affected, as enzyme activity increased with dietary arginine. RNA transcription is the first step towards final protein expression and activity, and many modifications, such as transcriptional regulation, protein degradation, cleavage, methylation and phosphorylation plays an important effect along the way. Therefore, even though very little effect was observed on gene expression in the adult salmon (paper II), arginine still may have an effect on these metabolic pathways, by affecting protein activity or degradation. In fact, this makes more sense, as inhibiting degradation rather than increasing production is a more energy conserving mechanism. As we observed in our cell studies (paper III), arginine supplementation had an early effect on gene expression, while this effect was absent after long-term supplementation. We assume that gene expression was upregulated as a first response, then after sustained exposure to surplus arginine, other mechanisms took over to maintain a high protein activity. Also, some of the genes investigated, like AMPK and ACC, are known to be regulated by activation/deactivation by phosphorylation/dephosphorylation (Zong et al. 2002), and as such the phosphorylation status of these enzymes are probably more important for overall activity than gene expression. Caspase-3 is activated by cleavage and RNA concentration does not reflect whether apoptosis is activated or not. Even though measured Ct values of our qPCR parallels were good, there was large individual variance between the fish, even within the same tanks, contributing to high SEM. This

could contribute to why limited effects were observed on gene expression in papers II, III and V.

3.5 Cell culture studies

For the cell study (paper III) primary liver cells were isolated from the adult salmon at the end of the arginine feeding trial described in paper II. The cells were then transported to our research facility at NIFES, and stored at 8°C overnight, before the number of live cells were counted the following morning and the cells were plated. As the cells were stored in the same medium, this may have caused any metabolic differences to even out. However, NMR clearly showed they were metabolically different, validating further comparison between treatment groups. This is discussed in paper III. Two compounds were used, difluoromethylornithine (DFMO) and N-1, N-11-Diethylnorspermine (DENSPM), inhibitor of ODC and activator of SSAT respectively. xCelligence, a system allowing for real-time monitoring of cell viability and impedance (Ke et al. 2011) was used in a pilot to optimize concentrations of the compounds and time before sampling. Initially, we also tested a medium containing 6x the amount of arginine compared to our control medium, but as this appeared to have negative effects of cell survival, this dosage was omitted from the final study. Our aim was to include a fourth treatment group where we added both DFMO and DENSPM, but due to a technical error this had to be omitted.

Cell culture is only a model of what happens *in vivo*. Using inhibitors and activators *in vitro* still gives a good indication of how cellular pathways are affected *in vivo*. However, as only one cell type are cultured, this approach miss the effects of inter-organ communication. Also, as the medium used did not contain any fatty acids this could explain why little effect was observed on genes involved in lipid metabolism. In fact, RNA was also isolated from the isolated liver cells before culturing, and when compared to expression in the same cells after 48 hours of culturing a drastic decrease in CPT-1 expression was observed from both dietary groups.

In our setup we also included cells for immunocytochemistry. We failed to test for live/dead cells after DFMO/ DENSPM application and to use our SSAT antibody to investigate whether distribution and/or intensity of the protein would be affected. The reason was that many of the cells had detached from the plate and was washed of after removing the antibodies, especially from the DFMO treated cells. That had the potential to give false results as it is likely to assume that the cells that detached had died, while the few left would be the ones strong enough to survive. Our staining could indicate higher number of dead cells in the DFMO treated cells, but due to a high loss of cells this could not be quantified and was omitted from the paper. The same happened when we applied the SSAT antibody, but we were able to localize SSAT to the cytosol of Atlantic salmon liver cells (Fig.6), as is also described in mammalian cells (Pegg 2008).

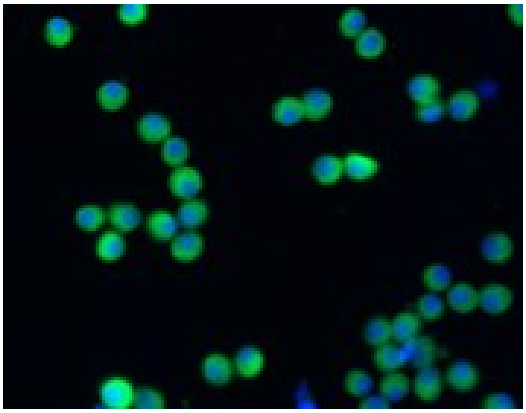

Figure 6 *– Green fluorescent staining of spermidine/spermine-acetyltransferase (SSAT) in isolated liver cells. Blue staining of the nucleus.*

3.6 Metabolomic analysis

Two types of metabolomic analysis were used to investigate the effect of arginine; nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC) connected to a quadrupole time-of-flight mass spectrometry (Q-TOF MS), followed by MS/MS for metabolite verification. In the latter, when using MS to detect the metabolites, separation is first required, here performed with HPLC, allowing for a wide range of analytes to be measured. NMR does not require such separation and thus allow for the samples to be recovered and used for further analysis. However, NMR is less sensitive than MS based methods. The HPLC Q-TOF MS method used

has excellent sensitivity, but with lower reproducibility and it is unable to detect metabolites with very low polarity. A study comparing the use of NMR and LC/MS on zebrafish *(Danio rerio)* livers found that NMR was more efficient at detecting small biochemical compounds such as AA and glucose than LC/MS (Ong et al. 2009), which is in agreement with our results, where only the NMR detected differences in these compounds.

The NMR conducted on the media from our cell study (paper III) was performed by Dr. Richard Taylor at EWOS Innovation, hence the rest of this discussion will focus on the HPLC Q-TOF MS method, applied on plasma and liver samples from the adult salmon (paper IV). The samples used for HPLC- Q-TOF MS (paper IV) were immediately flash frozen on liquid nitrogen after sampling, then stored on -80°C for more than a year, until samples were extracted and freeze-dried for metabolomics analysis in Norway before they were transported to Beijing, China for analysis. There is a possibility that some of the samples may have degraded either during storage or during transportation, as it took about 3 weeks from extraction until initiation of the analysis. Especially, concern is directed towards the fact that no effects were observed on any other AA than valine by the metabolomic analysis, even though our analysis of free AA in plasma from the same feeding trial (paper II) detected differences in several AA. This could indicate a degradation of some molecules or it could be related to the low number of replicates or a combination of these.

In our metabolomic study (paper IV) only 3 replicates were compared in each dietary group, limiting the power of statistical analysis. Originally four replicates were included, but as one sample had clearly been degraded, this sample was excluded and we were left with only 3 replicates. This is the lowest possible number of replicates allowing for statistical interpretation, and also why we did not observe more differences. Future studies should aim at having a higher number of replicates, by using more tanks, or at least by analysing several individual fish from each tank instead pooling the samples.

At last, it would have been interesting to have performed metabolomics on the juveniles in paper I, as these fish displayed more effects of arginine on weight difference than the adult salmon. Unfortunately, blood collection was limited from the juveniles, and the entire volume was used for analysis of free AA.

3.7 Statistical evaluations

In papers I, II and V one-way ANOVA was used to assess the effects of dietary arginine/methionine supplementation, after testing for homogeneity in variance using Levene's test. A Tukeys post-hoc test was used to assess for differences between treatments means. Regression analysis was also performed on some of the results. A student t-test was used when comparing a diet with and without surplus of arginine (papers I and II). Two-way ANOVA was used in the cell study (paper III), when assessing the effect of both arginine supplementation and treatment. The PCA analyses described in papers III and IV (performed by Dr. Taylor and Dr. Houssein, respectively) were applied to assess whether the groups could be differentiated metabolically. For the NMR (paper III) the number of replicates was high, increasing the power of this analysis. As pointed out before, the power of the statistical analysis from the HPLC-Q-TOF MS (paper IV) is not optimal as there were only three replicates in each group. As we decided only to compare the un-supplemented and the high arginine group, regression analysis could not be performed.

4. Discussion

4.1 Growth effects

4.1.1 Effects of dietary arginine on growth and deposition

Dietary arginine affected growth differently in the two life stages. In juvenile salmon (paper I) weight differences were observed, while no weight differences were observed in the adult salmon (paper II). For comparison, arginine is regarded as an IDAA in human preterm infants and neonates only (Wu et al. 2004; van Vught et al. 2013). It is interesting that in juveniles (paper I) weight gain slightly dropped when arginine was supplemented just above the expected requirement, and then increased when supplemented higher arginine doses (Fig.7). This is different to what was observed in juvenile carp (Chen et al. 2012) and catfish (Pohlenz et al. 2014) where arginine supplementation increased both weight and protein gain in a linear manner, though excess arginine have been reported to suppress growth in Nile tilapia *(Oreochromis niloticus)* (Santiago and Lovell 1988). The difference could be related to a slightly lower initial weight of fish fed diet B or to arginine interactions with lysine absorption (Berge et al. 1999), possibly leading to a deficiency of lysine when supplementing arginine. This is however not reflected in whole body lysine gain, which slightly increases with arginine supplementation. The growth effect in the juveniles also correlates with increased feed intake, and the fish fed diet B appeared to eat less than fish fed the other diets (Fig.7). Gene expression analysis of neuropeptides involved in central control of appetite however (pro-opiomelanocortin A and B, neuropeptide Y and Agouti-related peptide, performed by Prof. Ivar Rønnestad, data not shown), were not different between treatments. A similar correlation between growth and feed intake was not observed in the adult salmon (paper II, Fig. 7). As hepato- and visceralsomatic indexes were not affected in either of the trials, the weight difference in the juveniles appeared equally distributed

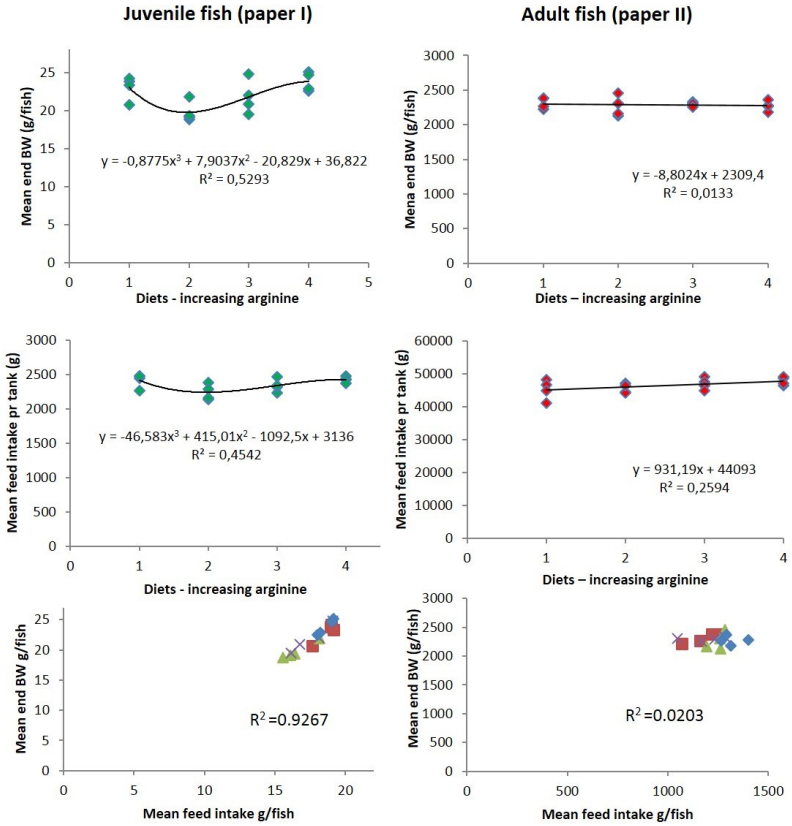

Figure 7 - Effects of supplementing surplus dietary arginine to a plant-protein based diets on growth, feed intake and the relationship between feed intake and growth in juvenile and adult salmon. In the juveniles (paper I) there is a correlation between how much they eat and how much they grow, while this trend is not clear in the adults (paper II). Diets 1 to 4 refer to the diets used, with diet 1 being the unsupplemented diet. ■- diet 1, ▲- diet 2, x – diet 3, ♦- diet 4. BW – body weight.

between tissues and fat/protein accretion, contrary to what have been observed in pigs (Tan et al. 2009) and mice (Clemmensen et al. 2012).

As no differences in growth or protein and fat content in muscle or liver in adult salmon (paper II), arginine supplementation did not appear to affect deposition of fat and protein in adult Atlantic salmon. The inability of arginine to enhance protein and lipid deposition in the muscle is different to what observed in mammals (Tan et al. 2009) and some fish, but similar to what observed in Pacific salmonids (Plisetskaya et al. 1991), supporting our findings. This suggests a difference in arginine metabolism

between mammals and fish as well as between fish species, highlighting the importance of determining pathways for arginine metabolism in salmon. In line with other results our trials suggests that arginine is better suited as a supplement in juveniles, possibly related to arginine's involvement in cellular differentiation and proliferation (Pegg 2009) and effect on early intestinal development (Rhoads and Wu 2009; Chen et al. 2012). As such, it would be interesting to evaluate the effect of high dietary arginine to spawning salmon on their offspring as growth restricted piglets have been shown to have low plasma arginine, and that growth can be regained by early supplementation of arginine (Wu et al. 2004; Lin et al. 2012).

4.1.2 Methionine deficiency – signs of muscle proteolysis and inflammation

A methionine restricted diet can increase fat oxidation in humans (Plaisance et al. 2011), decrease ROS formation (Caro et al. 2008) and increase life span in rats (Orentreich et al. 1993). In adult Atlantic salmon however, methionine restriction increase FAS activity (Espe et al. 2010), which is supported by the relative increase in liver weight observed in our study. The juvenile salmon fed the methionine deficient diet exhibited decreased growth which was associated with reduced protein gain (paper V). It is likely that methionine deficiency increased proteolysis in the muscle to maintain liver homeostasis. This is indicated by increased concentrations of free lysine in liver, plasma and muscle, increased free arginine in the muscle as well as decreased protein gain. Later studies have confirmed that methionine deficiency does induce muscle proteolysis in Atlantic salmon muscle (Espe et al, unpublished). Taurine and cystathionine concentrations were lower in the methionine deficient muscle and liver, respectively, suggesting a priority to remethylate methionine from homocysteine rather than use it for transulfuration or to utilise SAM for other methylation pathways (Espe et al. 2010). Decreased taurine and increased polyamine concentrations in the muscle could increase the susceptibility to apoptosis (Espe and Holen 2013) and induce ROS formation, opposite to that observed in rats (Caro et al. 2008).

Expression of tumor necrosis factor-α (TNF-α) was higher in the liver of the salmon fed the methionine deficient diet (paper V), possibly indicating increased inflammation in the liver. This is also associated with a decrease in GPX-3 expression. A suppressive effect of SAM, phosphatidylcholine (PC) and spermidine on TNF-α production has previously been reported in human leukocytes (Yu et al. 2006). As SAM decreased while spermidine concentration was unaltered in the methionine supplemented liver, this might be related to a protective effect of PC rather than from polyamines. TNF-α has been reported to increase SSAT expression (Babbar et al. 2006) and induce ROS formation through SMO activation (Babbar and Casero 2006). SSAT activity was unaltered in the liver, but activated SMO could be related to the change in GPX-3 expression.

4.2 Polyamine metabolism – a tightly regulated system

Polyamines were measured in liver, muscle and WAT after arginine supplementation (papers I and II) and in liver and muscle in the methionine trial (paper V). In the juveniles, putrescine concentration in the liver correlated with arginine in the diet (paper I), suggesting arginine is directed towards polyamine production. In the adults on the other hand (paper II), no effect of arginine supplementation on polyamine concentrations were observed in either of the tissues examined, corresponding to what have previously been observed when feeding Atlantic salmon diets with various ratios of arginine and lysine (Berge et al. 2002). In the juveniles, the increase in putrescine was accompanied by increased activity of SSAT in the liver (paper I). This suggests that we have increased catabolism of polyamines in liver, which is why spermidine and spermine concentrations are unaltered, supported by declining acetyl-CoA concentrations in the liver. Similar results were also observed in transgene mice overexpressing SSAT, where increased SSAT activity in WAT increased putrescine concentrations without affecting spermidine or spermine concentrations (Jell et al. 2007). Pirinen et al (2007) observed decreased spermine concentrations in WAT of their transgene mice overexpressing SSAT, which is similar to our observed decline

of spermine in the muscle. However, SSAT activity in the muscle was not analysed in the juvenile salmon, so we cannot determine whether polyamine turnover was currently affected by arginine in the muscle. ODC expression was upregulated in WAT in arginine supplemented juveniles, but this was not reflected in measured polyamines concentrations.

The activity and abundance of SSAT show a tendency to increase in the liver of arginine supplemented adult salmon (P=0.08 and 0.09, respectively, paper II), indicating a small difference in polyamine catabolism. However, this was not reflected in liver polyamine concentrations. SAM concentration was reduced in the liver from the adult salmon fed a high arginine diet but not in the juveniles, suggesting SAM as a limiting factor for polyamine production in the adult salmon, possibly contributing to why no effects were observed on polyamine concentrations. Also, expression of neither arginase-1 nor 2 were affected, though arginase expression is reported to correlate with dietary arginine in sea bass (Tulli et al. 2007) and could explain the increase in urea. ODC expression on the other hand, which is considered the first limiting enzyme in polyamine production (Pegg 2006) was upregulated in the liver (paper II), though this was not reflected in putrescine concentrations. In mice overexpressing one or both of ODC and SAMdc, cellular concentrations of polyamines were unaltered and pulse labelling of fibroblasts indicated increased acetylation and secretion of polyamines (Heljasvaara et al. 1997). This could also be the case in the liver of the adult salmon, as SSAT activity is slightly higher, and the liver might act as a producer of polyamines for other tissues. That indicates a tightly regulated system to keep polyamine concentrations stable, also demonstrated by the ability of spermine and spermidine to suppress SAMdc and ODC *in vitro* (Shantz et al. 1992). NO is also known to effectively inhibit SAMdc and ODC (Hillary and Pegg 2003), indicating that arginine can affect availability of dcSAM through several metabolic pathways.

Interestingly, dietary inclusion of methionine appears to have greater effect on polyamine concentrations in the liver than arginine supplementation. Juveniles fed the

methionine deficient diet (paper V) had increased concentrations of liver putrescine and SAM, while spermine concentration was lower compared to the fish fed methionine just above requirement. This is similar to what observed in mice supplemented methionine in the drinking water (Correa-Fiz et al. 2012). However, the increase of SAM in the methionine deficient group (paper V) contradicts previous work in adult salmon showing that liver SAM correlates with methionine intake (Espe et al. 2008). Opposite to methionine, arginine appeared to increase SSAT abundance and activity in the liver of arginine supplemented salmon (papers I and II), suggesting increased turnover of polyamines. In arginine supplemented adults (paper II), and to a less extent in juveniles (paper I), this was accompanied with a fall in SAM, suggesting a depletion of available SAM. The increased SAM in fish fed the methionine deficient diet could thus represent lower polyamine production in the liver, leaving more putrescine and less spermine. Notably, in mammals, dcSAM concentrations is normally only 1-2% of the SAM content (Pegg 2009), while this relation is not known in fish. So even though methionine deficiency did not limit SAM availability for polyamine production, it may have affected availability of dcSAM. Methionine's inability to affect SSAT expression and activity suggests that SAM concentrations do not affect SSAT activity in the liver. Hence the increase in putrescine may be due to increased production by ODC or SMO. Increased activity of SMO would explain the decrease in spermine, and could lead to oxidative stress due to release of hydrogen peroxide (Pledgie et al. 2005).

In muscle of the adult salmon, neither SSAT activity nor abundance were affected paper II). Together with our polyamine, gene expression and SAM/SAH data this suggests that dietary arginine supplementation does not affect polyamine metabolism in the muscle of adult salmon, contrary to what observed in rats (Fu et al. 2005) and pigs (Tan et al. 2009). Berge et al (1998; 2002) found ODC activity in the muscle of Atlantic salmon to be almost absent, and only about 1/100 the activity of the liver. This corresponds with our data showing lower polyamine concentrations in muscle compared to liver and WAT. Arginase activity have also been reported to be relatively low in fish muscle compared to other organs, and was not detected at all in white

muscle of Atlantic salmon (Portugal and Aksnes 1983). It is possible that the muscle is dependent on uptake of putrescine for polyamine production, limiting the effect of arginine on polyamine production in the muscle. SSAT activity in the muscle is still similar to liver activity (paper II), indicating importance of polyamine metabolism also in the muscle.

In muscle of the fish fed the methionine deficient diet, ornithine, citrulline, spermidine and spermine concentrations all increased (paper V). This could be related to the higher cellular concentrations of free arginine and lysine (due to proteolysis), increasing polyamine production. However, as ODC activity is almost absent in Atlantic salmon muscle (Berge et al. 2002), it is possible that most of the ornithine is catabolised via other pathways, while the increase in spermine and spermidine reflects an inability to control polyamine catabolism.

Taken together, our results indicate an importance of maintaining constant cellular polyamine concentrations, as depletion of polyamines are linked to induction of apoptosis (Oredsson et al. 2007). On the other hand, increased turnover and secretion of polyamines might have positive effects on growth and gene regulation in moderate doses, as long as kept within physiological concentrations (Babbar et al. 2007). While the arginine supplemented diets (papers I and II) were designed to assess the effects of surplus arginine, the methionine trial (paper V) is looking at a deficiency of methionine. The changes observed in polyamine metabolism during methionine deficiency could thus be a sign that the cells are no longer able to maintain polyamine homeostasis, while they can handle the surplus of arginine. This again indicate induction of apoptosis and cellular stress due to methionine deficiency. It would be interesting to see whether a combined supplementation of methionine and arginine would affect polyamine metabolism, as it would be expected to maintain SAM availability, concomitantly inducing SSAT activity, thus increasing polyamine turnover while maintaining polyamine concentrations within normal range. This may enhance cell growth and lipid metabolism, improve glucose tolerance and simultaneously protect against apoptosis and oxidative stress.

4.2.1 Targetting polyamine metabolism in Atlantic salmon liver cells *in vitro*

In the cell study (paper III) DFMO and DENSPM were added primary liver cells isolated from the adult salmon at the end of the arginine feeding trial described in paper II. Our theory was that DFMO would inhibit polyamine turnover while DENSPM would boost it, both depleting cellular polyamine pools, and that this would affect lipid metabolism. Due to limited material, polyamine concentrations could not be measured after treatments and the ability of the compounds to deplete cellular polyamines are therefore unknown. Dietary arginine supplementation before isolation appeared to have more effect on polyamine metabolism than DFMO or DENSPM. SSAT abundance was increased in the high arginine group, while DENSPM only had a small additional effect on protein abundance. Both incubation time and dosages were similar to studies performed by others (Odenlund et al. 2009; Soderstjerna et al. 2010) and our xCelligence pilot study did indicate these conditions as optimal incubation time and dosages. Still, the metabolic rate is expected to be slow due to lower incubation temperature used for fish cells compared to mammals. There was no effect on expression of genes involved in polyamine metabolism in liver cells isolated from the 12 week feeding trial (paper III), opposite to what observed in mice fibroblasts where DENSPM induced both SSAT mRNA and protein abundance (Uimari et al. 2009). This supports our theory of a metabolic adaptation to dietary arginine, but could also be due to a general change in gene expression after culture. Also, if some of the cells were detaching due to DFMO/DENSPM treatment, RNA from these cells would not be included and could mask some effects. In the short-term study in paper III, DFMO upregulated ODC expression. This is expected as DFMO inhibits ODC, and the cell then upregulates expression due to depletion of putrescine. DENSPM is known to also have an inhibitory effect on ODC (Uimari et al. 2009) and inhibited ODC expression in our short-term study. DFMO is reported to deplete cellular polyamine pools and prevent cell growth and adipogenesis in 3T3L1 adipocyte cells by inhibit expression of FAS and other genes involved in lipogenesis, all which was restored by spermidine supplementation (Vuohelainen et al. 2010). In

the short term cell study, FAS expression was only reduced by arginine supplementation, and to some extent DENSPM (p=0.08). However, DFMO did inhibit ACC expression, suggesting an early preventive effect on adipogenesis by depleting cellular polyamines. Whether this was due to a depletion of spermidine, as for Vuohelainen et al (2010) is not known. Pirinen et al (2007) observed that DFMO in the drinking water to mice increased ATP *in vivo*, while DENSPM depleted ATP *in vitro*, none of which was currently observed in salmon liver cells. The inability of DENSPM to reduce ATP through SSAT activation could be due to abundance of energy in the media, as nutrient deprived medium has been reported to be needed to observe a decrease in ATP from DENSPM (Pirinen et al. 2007). This also corresponds with our results that arginine is a stronger inducer of SSAT activity than the spermine analogue DENSPM.

In the short term cell study, DFMO inhibited caspase-3 in all cells and increased BAX expression in the control cells only (paper III). DFMO is expected to increase arginine concentrations, and as such this could be an effect of increased arginine, rather than an effect of polyamines. DENSPM have been reported to induce apoptosis through polyamine depletion and caspase-3 induction in cancer cell lines (Oredsson et al. 2007; Soderstjerna et al. 2010), while in a chondrocyte cell line DENSPM depleted polyamines without activating caspase-3 (Stanic et al. 2009). In liver cells isolated from fish supplemented arginine DENSPM increased polyamine catabolism by SSAT but had no effect on pro-apoptotic genes, suggesting that increased polyamine catabolism does not affect cell viability at the concentrations used in our work. Arginine supplementation also decreased BAX expression, demonstrating a pro-survival effect of arginine, though it is unsure whether this is mediated through polyamines. Neither BAX nor caspase-3 expression was affected after 12 weeks supplementation (papers II and III), implying that long term supplementation of arginine does not affect cell survival in healthy individuals. Still, as no information about the activation status of these proteins are available, we cannot determine whether there truly is an effect on apoptosis or not. Spermine and spermidine pools were constant in papers I and II and should thus not affect viability due to polyamine

depletion. Overall, our cell study data (paper III) indicates that arginine can affect cell survival and apoptosis through pathways independent of polyamines. Still, the data is limited and more research is needed to understand how arginine and polyamines affect cell survival and apoptosis in Atlantic salmon.

4.3 Does arginine modulate energy metabolism?

An important observation with arginine supplementation is that it appears most effective in reducing visceral mass and building lean mass in metabolically challenged animals, such as diet-induced obese (Jobgen et al. 2009; Clemmensen et al. 2012; Satterfield et al. 2012) or diabetic animals (Fu et al. 2005). In more metabolically healthy pigs (Go et al. 2012) and humans (Evans et al. 2004) no lipid reducing effect has been observed. As all our diets were designed to meet the requirements and thus give metabolically healthy fish, this could possibly explain the limited effect observed in the arginine feeding trials.

As discussed in papers II and III it appears that the salmon adapts to long-term supplementation to arginine, which has also been suggested in pigs (Mohan et al. 2012). In the adult salmon it appears that excess arginine is catabolised via the urea cycle, as indicated by increasing concentrations of urea in liver, plasma and muscle, while ornithine and putrescine concentrations were unaltered (paper II). These results are in line with what found in seabass, where plasma urea increased and weight gain stabilised once arginine requirement was met in the diet (Tibaldi et al. 1995). Hence, salmon adapts to long-term supplementation of arginine by excreting the surplus arginine as urea or using it for energy production. Studies with U-C^{14} arginine in rainbow trout and Atlantic salmon have reported increased oxidation to $^{14}CO_2$ once requirement of arginine were met, supporting our theory that the excess arginine is catabolised and used as energy (Kaushik et al. 1988; Lall et al. 1994). Notably, urea can also be produced during degradation of purines (Andersen et al. 2006), but as arginine was the only variable in our diets, this should not have affected our results.

Arginine can enter the TCA cycle as fumarate produced in the urea cycle, via the aspartate-argininosuccinate shunt (Fig. 8). The NMR in paper III indicated an effect on several TCA cycle intermediates and fumarate concentrations were lower in media from the high arginine cells suggesting higher activity through the TCA cycle. Arginine can also be used for energy production in the TCA cycle by transaminases working on arginine or ornithine to produce α-ketoglutarate. Notably, liver activity of the urea cycle enzymes (except for arginase) are reported to be low or absent in several fish species (Chadwick and Wright 1999; Gouillou-Coustans et al. 2002), which would limit arginine influx to the TCA cycle and suggest arginine as the sole nitrogen source for liver urea production. However, the localisation of urea cycle enzymes in other tissues have been suggested, and requires further attention.

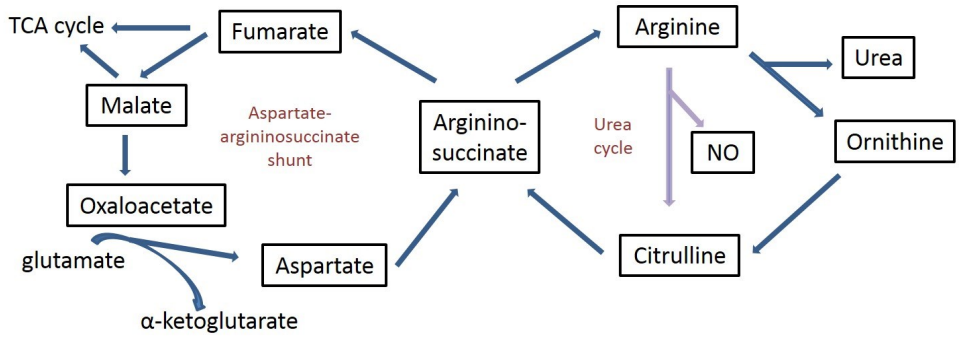

Figure 8 *– The relationship of the urea cycle to the TCA cycle. Excess arginine can be processed through the urea cycle producing urea and fumarate that can enter the TCA cycle through the aspartate-argininosuccinate shunt. All steps takes place in the cytosol, except the formation of citrulline which occurs in mitochondria.*

4.3.1 Arginine improve lipid metabolism in liver of juvenile salmon

In juvenile salmon (paper I) arginine supplementation appeared to increase fatty acid oxidation in the liver. Increased dietary arginine increased polyamine turnover in the liver as indicated by increased SSAT activity. This was associated with a tendency in decreased acetyl-CoA concentrations and increased expression of CPT-1 (Fig. 9), demonstrating a positive effect of arginine on long-chain fatty acid oxidation in liver. This did not affect hepatosomatic index, possibly indicating a healthy liver in all the

salmons but may have affected lipid content and classes of the liver as these were not analysed. In WAT on the other hand, we could not document any effect on polyamine turnover or CPT-1 expression. As viscera somatic indexes also were unaltered, arginine did not appear effective in decreasing WAT mass in juvenile Atlantic salmon, opposite to what observed in mice (Clemmensen et al. 2012) and pigs (Tan et al. 2009). Arginine deficiency have previously been reported to increase lipid retention in Atlantic salmon smolts, with no effects from excess arginine (Lall et al. 1994), supporting our findings. Muscle tissue was not further analysed in the juveniles (paper I), but as the effect on weight gain correlated with both protein and lipid gain, this suggests an effect on overall growth which is equally distributed between tissues. Overall, in metabolically healthy salmon, arginine supplementation does not affect lipid metabolism in WAT and muscle to an extent that reduce overall lipid accumulation.

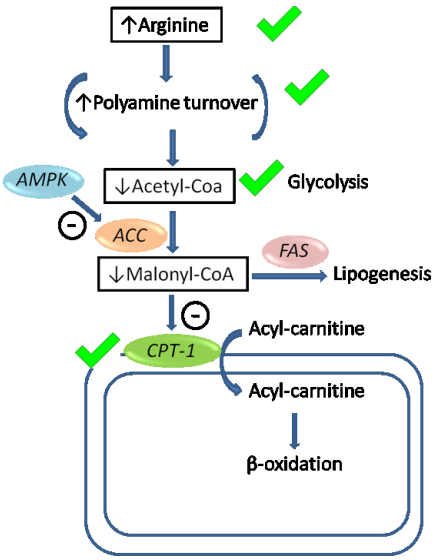

Figure 9 *– Effect of arginine supplementation in liver of juvenile salmon. Arginine increased polyamine turnover, decreased acetyl-CoA and increased CPT-1 expression, suggesting increased oxidation of long-chained fatty acids.*

In adult salmon (paper II) we aimed to better understand the effect of arginine on lipid metabolism by doing a larger screening of gene expression in liver, WAT and muscle. The positive effect on liver metabolism observed in the juvenile salmon (paper I) was not observed in the adults (paper II), as polyamine concentrations were unaltered and no effect on CPT-1 expression was observed. In the adults more genes involved in fat metabolism were analysed by qPCR, with no effect from dietary arginine in any of the tissues (paper II). As such, arginine did not have the same effect on fat metabolism in adult Atlantic salmon as have been reported in mammals (Jobgen et al. 2009; Tan et al. 2009). As suggested in both papers II and III this may be due to adaptation to long-

term arginine supplementation, which also has been reported by others (Mohan et al. 2012). Our short-term cell study (paper III) showed that supplementation of arginine in the media to liver cells downregulated the expression of FAS and ACO after 48 h culturing, suggesting that arginine supplementation initially inhibit lipogenesis and induce lipolysis. Whether this effect is maintained after continuous exposure by post-transcriptional mechanisms is not known, but it is unlikely as liver weight and fat content was not affected after long-term dietary supplementation (paper II).

Activated polyamine catabolism affect cholesterol synthesis in mice (Pirinen et al. 2010) via AMPK and PPAR-α. In salmon, arginine did not appear to affect cholesterol synthesis as plasma bile acid and expression of PPAR-α (paper II) and

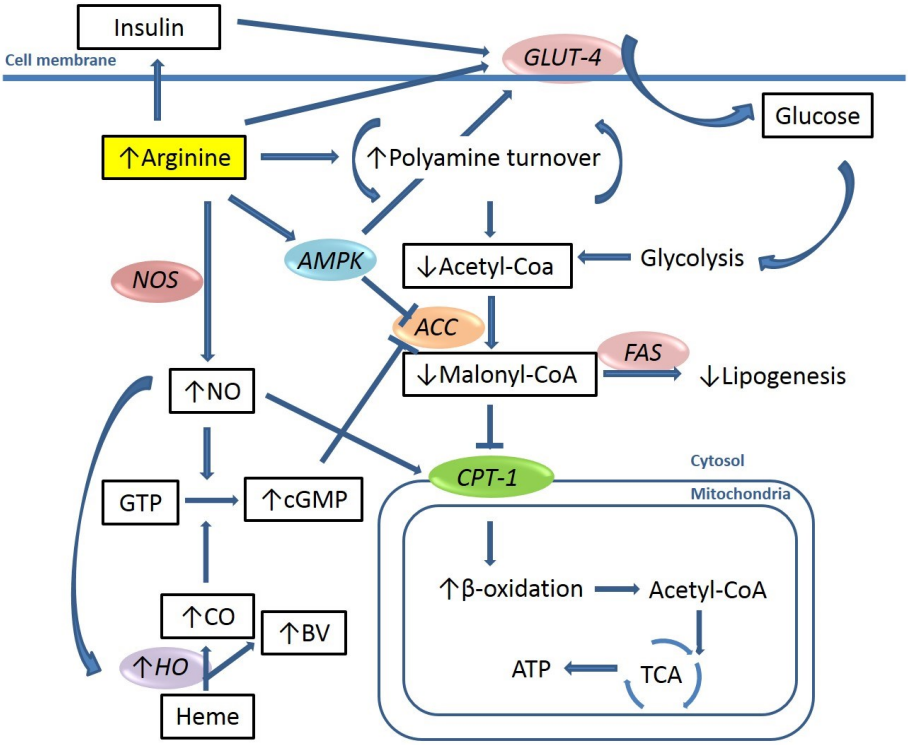

Figure 10 *– Possible pathways for arginine to increase fat and glucose oxidation in liver cells. Arginine can increase transport into the mitochondria via CPT-1 through increased polyamine turnover, NO and cGMP production. Glucose uptake can by enhanced through insulin, AMPK, NO and cGMP activation of enzymes in the TCA cycle.*

AMPK (from feeding trial, paper III) were unaltered.

Fu et al (2005) reported a massive upregulation of heme oxygenase (HO)-3 after dietary arginine supplementation in obese Zucker diabetic rats which was accompanied by increased lipolysis and glucose oxidation and reduced plasma glucose. HO, which is inducible by NO, catalyses the breakdown of heme to biliverdin and CO and stimulates production of cGMP by guanylyl cyclase (Garcia-Villafranca et al. 2003). Our metabolomic analysis of the liver demonstrated increased biliverdin concentrations after arginine supplementation (paper IV), indicating activation of HO by NO, possibly increasing fatty acid and glucose oxidation in the liver by inhibiting ACC and activating enzymes of the citric acid cycle (TCA, Fig 10). This corresponds with the NMR results (paper III) and supports the effects on TCA intermediates.

4.3.2 Arginine may enhance glucose utilisation

In paper III we observed that arginine affected glucose metabolism. Liver cells isolated from salmon fed a high arginine diet and cultured *in vitro* had lower glucose concentration in the media after 48 hours compared to liver cells from salmon fed arginine at requirement. The same effect on glucose has later been confirmed in a co-culture study with Atlantic salmon liver and head kidney cells in our group (Holen et al. 2014), but is opposite to what observed in arginine supplemented pigs (He et al. 2009). Fish don't readily utilize glucose and postprandial hyperglycemia may lead to reduced growth (Hemre et al. 2002; Polakof et al. 2012). Arginine is known to reduce plasma glucose and increase insulin sensitivity in salmon (Plisetskaya et al. 1991). By increasing cellular uptake and utilization of glucose, arginine have the potential to spare AA for protein synthesis and improve the metabolic status of the fish (Fig 10). Increased glucose oxidation after arginine supplementation (Fu et al. 2005) and SSAT activation (Koponen et al. 2012) have been linked to upregulation and activation of AMPK. In the short-term experiment in paper III, arginine supplementation to the medium of isolated liver cells decreased expression of the β1 subunit of AMPK and

expression was further decreased by DENSPM, contrary to what was expected, while no effect was observed in the long-term experiment. In the long-term experiment (paper III) intracellular ATP concentrations declined with arginine supplementation, which suggest activation of AMPK due to decreased ATP/AMP ratio (Winder and Hardie 1999). Before culture however (paper II), liver ATP concentration appeared to increase with increasing arginine supplementation ($p=0.07$). The latter suggests that AMPK was not activated in adult salmon *in vivo*, which may explain why no differences in fat metabolism or growth were observed. After culturing however, ATP appears to be depleted faster in the arginine treated cells, which could be due to increased polyamine catabolism as demonstrated by increased SSAT abundance in these cells. As arginine stimulate glucose uptake in Atlantic salmon (Plisetskaya et al. 1991; Mommsen et al. 2001) glycolysis is expected to increase acetyl-CoA production (Fig. 10). This would lead to increased ATP production through the TCA cycle, supported by increasing ATP concentration in the liver (paper II). By maintaining ATP and acetyl-CoA, AMPK and CPT-1 activity would not be activated, and no effect on fatty acid oxidation would be observed (Fig.10). Insulin have also been reported to increase lipogenesis in WAT of rainbow trout (Polakof et al. 2011), suggesting increased uptake and storage of glucose in WAT as a reason why no reduction in WAT mass was observed.

4.4 Arginine interactions with other amino acids

Arginine and lysine is known to affect uptake of each other in Atlantic salmon (Berge et al. 1999; Berge et al. 2002), especially arginine is known to have an inhibitory effect on lysine uptake. Interestingly, arginine supplementation appeared to increase concentration of free lysine in plasma and muscle. This indicates that lysine is still efficiently absorbed from the intestine or that a decrease in lysine uptake has led to increased breakdown of muscle protein to maintain lysine availability. However, in both paper I and II (table 3), whole body lysine gain was higher in the arginine supplemented fish, rather suggesting a lysine sparing effect of arginine. In the cell

study (paper III), we also observed increased lysine concentrations in the media when ODC was inhibited, even though arginine in the media was unaffected.

In adult salmon (paper II), arginine supplementation affected concentrations of several other free AA in plasma and muscle. The liver however, appeared to be unaffected. Muscle and plasma probably act as pools/transportation for AA, demonstrating an importance at keeping liver free AA concentrations constant. The changes observed in glycine and serine is probably related to the supplemented glycine as it correlates nicely with glycine concentration in the feed. However, serine is also consumed in the methionine pathway (Fig. 5), and glycine is also involved in creatine production (Brosnan et al. 2011). Increased consumption through these pathways could have contributed to the difference observed. The fact that 78% of glycine in the un-supplemented diet is used for protein accretion (Table 3) also highlights the importance of this AA as a "functional IDAA" in fish (Wu 2010).

Table 3 *- Mean amino acid gain in adult Atlantic salmon. From Arg0 and Arg15 diets described in paper II. Data calculated as tank means. Proline content was analyzed in whole fish only, not in the feed. AA – amino acid.*

	Arg 0				Arg 15					
AA	In feed(g/kg)	Eaten (g)	Gain (g)	%of eaten AA	In feed (g/kg)	Eaten (g)	Gain (g)	%of eaten AA	% change	Gain diff (g)
MET	8,4	205	112	55 %	8,4	197	114	58 %	3 %	2,2
CYS	6,1	149	41	28 %	5,9	139	42	30 %	2 %	0,2
M+C	14,5	354	154	43 %	14,3	336	157	47 %	3 %	2,9
LYS	18,9	462	294	64 %	19,1	449	304	68 %	4 %	10,6
THR	13,4	327	168	51 %	13,7	322	172	53 %	2 %	4,1
TRP	3,8	93	42	46 %	3,8	89	44	49 %	3 %	1,5
ARG	21,1	516	231	45 %	36,1	848	243	29 %	-16 %	12,2
ILE	16,2	396	166	42 %	16,2	381	165	43 %	1 %	-1,0
LEU	37,8	924	282	31 %	38,1	895	285	32 %	1 %	2,5
VAL	17,8	435	200	46 %	17,8	418	200	48 %	2 %	0,6
HIS	9	220	98	45 %	9,1	214	100	47 %	2 %	1,9
PHE	20,3	496	151	30 %	20,4	479	151	32 %	1 %	0,5
GLY	30	733	285	39 %	15,8	371	290	78 %	39 %	4,5
SER	18,8	459	163	35 %	19	446	163	36 %	1 %	0,2
PRO			176				193			17,3
ALA	20,8	508	241	47 %	21	493	247	50 %	3 %	5,9
ASP	31,8	777	366	47 %	32	752	367	49 %	2 %	1,1
GLU	81,6	1994	498	25 %	81,7	1919	506	26 %	1 %	8,0

Contrary to what expected, muscle and plasma citrulline decreased after arginine supplementation (paper II) and differ from what observed in channel catfish, where arginine increased plasma concentrations of citrulline as well as of glutamate and glutamine (Pohlenz et al. 2014). This indicate species differences in arginine metabolism. Proline gain increased with dietary arginine (Table 3 (paper II)), suggesting that ornithine is converted to P5C rather than to citrulline. In support of this, Tulli et al (2007) found that in seabass, hepatic OCT activity showed a negative correlation with dietary arginine while hepatic activity of CPSIII was absent. Arginase activity still increased (Tulli et al. 2007), suggesting ornithine formation subsequently used for energy or for polyamine or proline production. CPSIII was only fund in muscle tissue of adult rainbow trout (Korte et al. 1997) indicating CPSIII as a limiting factor for citrulline production (Ball et al. 2007). CPSIII and OCT expression are shown to be higher in early developmental stages in zebrafish while arginase expression is higher in adult stages (Caldovic et al. 2014). As fish may excrete nitrogen directly as ammonia over the gills, this has been linked to a higher need for detoxification of nitrogen through the urea cycle in early developmental stages. The urea cycle enzymes have also been linked to maintenance of acid-base balance as CPSIII consumes bicarbonate, and OCT is expected to regulate ornithine availability for polyamine production (Monzani and Moraes 2008).

Free methionine was not affected by arginine in neither of the tissues examined in juveniles or adults (papers I and II). When polyamine turnover increase, dcSAM consumption increase, which could potentially affect methionine concentrations within tissues. Our results indicate that methionine is not a limiting factor for polyamine turnover in these experiments. Methionine gain was also similar between the dietary groups, though the fish fed the highest dietary arginine inclusion were a little more efficient in storing dietary methionine (Table 3). Proline gain increased with arginine supplementation, suggesting that excess arginine is converted to proline, even though expression of the enzyme converting P5C to proline was downregulated in WAT of these salmon. Whether there is effective conversion between arginine and proline/glutamate in fish still needs to be determined (Buentello and Gatlin 2000).

Carnosine and β-alanine concentrations increased in the muscle of the adult salmon fed a high arginine diet, while free histidine concentrations decreased (paper II). Carnosine is synthesised from histidine and β-alanine, and is found in high concentrations in the muscle where it functions as a pH-buffer, antioxidant, protects against oxidative stress and improves the capacity for prolonged intense exercise (Abe 2000; Song et al. 2014). It is also known to facilitate NO production (Takahashi et al. 2009). This could indicate increased buffering capacity and swimming capacity of arginine supplemented salmon, supported by decreased lactate concentrations in media from the high arginine liver cells (paper III). Carnosine can be further synthesized into anserine which is catabolized to 1-methyl-histidine and β-alanine, concentrations of which correlated in the plasma of adult salmon (paper II, not shown), with highest plasma concentrations in salmon fed the Arg10 diet. This suggest a higher turnover of carnosine/ anserine production. Anserine and 1-methyl-histidine were not affected in the muscle. Studies in yeast have shown that the aminoaldehydes released during polyamine catabolism can be converted to β-alanine (White et al. 2001). This could explain the increase in muscle β-alanine and carnosine as β-alanine is known as the limiting factor in carnosine production (Dunnett and Harris 1999). However, this is highly speculative and needs to be further investigated.

4.5 Metabolic effects of arignine

Both NMR and HPLC Q-TOF MS data confirmed that surplus dietary arginine has a metabolic effect in Atlantic salmon, even though no growth differences were observed (papers III and IV). Fish from the feeding trial in paper II was used for both experiments. In the cell study (paper III) liver cells were extracted from fish fed the Arg5 and the Arg15 diet, while for the metabolomics work in paper IV liver and plasma were taken from fish fed the Arg0 and the Arg15 diet described in paper II. Also, for the cell study, cells were cultured *in vitro* before NMR were done on the medium, not the cells, and as such the results between paper III and IV are really not

comparable, but rather contributes to a greater understanding of arginine's effect in Atlantic salmon.

The NMR data showed that dietary concentration of arginine before isolation had greater effect on the metabolism than addition of DFMO or DENSPM after plating the isolated cells. This indicates that the effects observed by arginine were mediated through polyamine-independent pathways or that these changes were strongly present before isolation of the cells. DFMO did induce metabolic changes, and the PCA analysis after NMR showed that DFMO treated cells were different from DENSPM treated, but that the DENSPM treated cells overlapped with the control cells. This could be related to the observation that DENSPM only weakly induced SSAT abundance. It would have been interesting to compare the metabolomic effects in our long-term cell study with the short-term cell study. NMR performed on serum from arginine supplemented pigs showed a decrease in lipids, low density lipoproteins and very low density lipoproteins (He et al. 2009). No effects on lipid metabolism was observed in cell media based on our NMR data (paper III) or on plasma phospholipids or triglycerides in adult salmon (paper II) due to arginine supplementation. This corresponds with decreased fat mass pigs, and no difference in fat mass in salmon. However, similarly to He et al (2009), we observed effects on TCA cycle intermediates and several AA, including increasing concentrations of lysine with arginine supplementation.

Our metabolomic analysis (paper IV) shows that this is an effective tool to discover interactions between and to understand metabolic consequences of nutritional interventions. We also described novel interactions of arginine in Atlantic salmon, like the increase of biliverdin, a vitamin D3 metabolite and PGF-2α in the liver. How arginine affects these compounds and how these pathways can be modulated through the diet requires further investigations. Arginine also affected branched chain AA, as valine concentrations were reduced in plasma of arginine supplemented salmon, corresponding to what has been observed in pigs (He et al. 2009).

5. Conclusions

Surplus dietary arginine had a limited effect on growth in juvenile Atlantic salmon, while no effect was observed in the adult stage. Arginine supplementation did not affects deposition patterns of lipids and protein at either life stage.

Methionine deficiency reduced growth, likely due to increased muscle proteolysis to maintain constant liver sulphur metabolism.

Arginine appeared to increase transport of fatty acids into mitochondria in the liver of juveniles, based on the increased expression of CPT-1, thereby possibly improving their metabolic status. This effect appeared related to an increased polyamine turnover. Arginine supplementation did not affect fatty acid metabolism in adult Atlantic.

Arginine supplementation increased SSAT activity, thus maintaining tissue concentrations of polyamines, suggesting a tightly regulated system to maintain polyamine concentrations. This effect was strongest in the juveniles.

Adult Atlantic salmon adapted metabolically to long-term supplementation of arginine.

Short-term supplementation with arginine to isolated liver cells affected gene expression for lipid metabolism and apoptosis, and this appeared to be unrelated to polyamine metabolism.

Dietary arginine supplementation affects glucose metabolism in Atlantic salmon, by altering glucose transportation across liver cells.

Metabolomic analysis of plasma and liver samples showed that even though arginine supplementation in adult Atlantic salmon did not affect growth, it does affect several metabolic pathways. HPLC Q-TOF MS is a powerful tool to screen for metabolomic effects of dietary changes.

Methionine deficiency had more effect on polyamine concentrations than surplus of arginine. This suggests an inability to maintain cellular homeostasis of polyamines during methionine deficiency. Polyamine concentrations in liver and muscle appeared to be more dependent on SAM from methionine than of dietary arginine. Still, arginine caused SSAT abundance to increase, possibly indicating a higher turnover, thus maintaining steady polyamine concentrations.

6. Future perspectives

This thesis has demonstrated that dietary arginine and methionine can modulate metabolism and polyamine pathway in Atlantic salmon. It will be interesting to further investigate if and to what extent there is interconversion between arginine and citrulline, proline and glutamate in Atlantic salmon. And if so, to investigate how metabolism of these AA affect each other and whether citrulline can be supplemented to spare arginine, as the majority of arginine is known to be metabolised in the gastro-intestinal tract during first pass metabolism. As conversion to citrulline and arginine takes place in the intestine and kidneys of mammals, respectively, these tissues should be included in future studies aiming to better understand arginine metabolism in fish. *In vitro* studies can be applied to assess metabolism of arginine in different tissues, while *in vivo* studies or *in vitro* co-cultures can be used to investigate transport of arginine and its metabolites between tissues in Atlantic salmon, an area with limited knowledge today. Specific enzyme inhibitors can also be injected *in vivo* before analysing plasma metabolites. It will be interesting to determine if these metabolic pathways differ with age and disease states and to determine whether and how they can be modulated *in vivo*.

Arginine have shown potential to modulate the immune response and to improve intestinal growth, areas which will be interesting to focus on in the next step. Especially, arginine appears to have beneficial effects under stressful periods, as it reduces cortisol production, so supplementation of arginine may prove interesting as a "booster" before sea transfer, handling or during disease outbreaks. Cell culture studies with liver and head kidney cells in co-culture will be useful to assess effects on the immune system.

In paper III we showed that dietary arginine supplementation *in vivo* affected glucose metabolism *in vitro*. Future studies are needed to assess how, and to elucidate whether this can be applied to a salmon diet to improve energy utilization and metabolic health. Methionine have also been reported to affect glucose metabolism in fish, and

interactions between AA and carbohydrate metabolism is a developing field which could aid to improve the limited ability to use carbohydrates and to counteract the glucose-intolerant status of Atlantic salmon.

Future studies should also aim to investigate the effect of arginine and polyamines on cell proliferation and apoptosis. Cell culture studies are good tools to assess safe doses to avoid apoptosis and to optimise cell viability. As previous studies have demonstrated a positive effect on intestinal health and differentiation, this would an appropriate tissue to analyse effects of arginine and polyamines on cell growth and differentiation. This could be done with primary tissue cultures to reduce the numbers of animals required. Also, the metabolism by the intestinal flora is known to affect growth and health of the entire organism and this could be modulated by dietary AA.

Our metabolomics work showed interactions with vitamin D, heme and prostaglandins, among others. These interactions should be further investigated to elucidate how AA can be used to modulate metabolic health in fish. This could be achieved by using isolated liver cells or blood cells for investigation. A NOS inhibitor could be applied to investigate interactions with both vitamin D and heme.

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