Effects of dietary methionine on feed utilization, plasma amino acid profiles and gene expression in rainbow trout (Oncorhynchus mykiss)

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PhD Thesis

Written by Marine Rolland
Defended 26 June 2014

DTU Aqua
National Institute of Aquatic Resources

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Ph.D. Thesis by

Marine Rolland

March 2014

Technical University of Denmark

National Institute of Aquatic Resources

Section for Aquaculture

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Colophon

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Cover picture: Marine Rolland

Preface

The work presented in the thesis was undertaken during my enrolment as an industrial PhD student at BioMar A/S, Denmark and the National Institute of Aquatic Resources, Section for Aquaculture, Technical University of Denmark (DTU). The PhD was co-financed by BioMar A/S and granted by the Danish Ministry of Higher Education and Science.

The thesis, in its entirety, took place over three busy years. During these years I was given the chance to learn at my own pace thanks to the patience and support of my supervisors and colleagues. This thesis represents a mosaic of collaborations; every person that I went to at some point surely took part in the final piece as it stands today. I can surely not express on other's behalf how participating in the work felt like, but from where I have been standing, it was a great adventure. I "loveded" it.

The thesis could not have been achieved without the great help from my main supervisor at the DTU, Dr. Peter V. Skov. Thank you for your constant and precious support; not a single day has gone by, even Christmas, where your door was not open. I would also like to thank my second supervisor from DTU, Dr. Anne Johanne Dalsgaard, largely involve in this project, and always providing conscientious advice. I appreciated greatly the support from Dr. Bodil K. Larsen, who spent countless hours in the lab to teach me the methodology and patience needed when working on the HPLC. Thanks to Ulla, Brian and Dorthe for being so helpful during my time in the lab and fast with any assistance I required.

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Heartfelt thanks to all my dear friends for never letting me down even when I became the most absent friend in the world. A special thought to Dr. Jordan P. Feekings, a precious friend and co-worker. Many thanks to my sisters, Eline, Lucile and Solene, for giving me strength back when they went missing. Last but not least, thanks to Juliette for putting up with me, even during the numerous difficult moments, never failing to support me and providing me with valuable time to reenergize.

Hirtshals, March 2014

Marine Rolland

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Paper III: Rolland M, Dalsgaard J, Holm J, Gómez-Requeni P, Skov PV (2014) Dietary methionine level affects growth performance and hepatic gene expression of GH-IGF system and protein turnover regulators in rainbow trout (Oncorhynchus mykiss) fed plant protein-based diets. Comparative Biochemistry and Physiology, Part B

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List of abbreviations and biochemical terms

Acetyl-CoA:	Acetyl coenzyme A, key intermediate of metabolic pathways
Akt:	Tyrosine protein kinase is involved in the transduction of insulin or IGF signal
ALT1:	Alanine transferase, a transaminase which catalyses the transfer of an amino group from alanine to α-ketoglutarate, resulting in the synthesis of pyruvate
ANF:	Antinutritional factor
ATP:	Adenosine triphosphate carries energy at a cellular level from a catabolic to anabolic reactions
BCAAs:	Branched-chain amino acid, isoleucine, leucine and valine
CAA:	Crystalline amino acid
Capn:	Calpain, proteolytic enzyme of the calpain cascade, existing in two genes 1 and 2 (Capn 1 and Capn 2, respectively)
CAST:	Calpastatin, inhibitor of the calpain protease, existing in long and short sequences (CAST-L and CAST-S, respectively)
cDNA:	complementary Deoxyribonucleic acid, produce of the reverse transcription step
CPT1a:	Carnitine palmitoyltransferase 1 isoforms a, enzyme of the β-oxidation
CT:	Cycle threshold, number of cycle necessary to obtain a threshold level of fluorescence in the RT-PCR analysis
DE:	Digestible energy
D-L isomers:	Dextrorotary and levorotary are the chiral isomers resulting from the presence of an asymmetric carbon in molecule
DM:	Dry matter
DNA:	Deoxyribonucleic acid
DNase:	Deoxyribonuclease, enzyme responsible for DNA degradation
DP:	Digestible protein
E:	Efficiency of a RT-PCR, $E= 10^{-1/slope}$
EAA:	Essential amino acid
EER:	Energy efficiency ratio, unit of bodyweight gain per unit of dietary energy intake

EF1α: Elongation factor 1 alpha, used as a reference gene in the RT-PCR data analysis

elF4E: Elongation factor involved in the translation of mRNAs into proteins

$FADH_2$: The reduced version of flavin adenine dinucleotide (FAD). Used as electron carrier during oxidative phosphorylation

FAS: Fatty acid synthase, multi-enzyme protein catalysing fatty acid synthesis from acetyl-CoA

FBPase: Fructose-1,6-biphosphatase, enzyme of the gluconeogenesis

FCR: Feed conversion ratio, unit of feed intake per unit of weight gain

FM: Fish meal

FRET: Fluorescent resonance energy transfer

G6Pase: Glucose-6-phosphatase, enzymes catabolizing the final step of gluconeogenesis

G6PD: Glucose-6-phosphatase dehydrogenase, enzyme of the pentose phosphate pathway which supplies reducing power (NADPH) to the cells

GCN2: General control non-depressible protein 2, cellular pathway involve in the sensing amino acid deficiency

GDH: Glutamate dehydrogenase, enzyme of the amino acid catabolism that converts glutamate into α-ketoglutarate, resulting the production of an ammonia molecule

GH: Growth hormone

GHR: Growth hormone receptor

GLS: Glutamine synthetase, isoform 1 and 2, GLS01 and GSL02, respectively. Catalyse the synthesis of glutamine from glutamate and ammonia

GOT2: Aspartate aminotransferase, transfer amine group from amino acids to from glutamate

HSI: Hepato-somatic index. Liver weight relatively to the bodyweight

IGF: Insulin-like growth factor I or II (IGF-I and IGF-II, respectively)

IGFBP: Insulin like growth hormone binding protein

met-tRNA: Methionine transfer ribonucleic acid

MMT: Million metric tons

mRNA: Messenger ribonucleic acid

NADPH:	The reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$) which provides the reducing equivalents for biosynthetic reactions
NEAA:	Non-essential amino acid
PEPCK:	Phosphoenolpyruvate kinase, catalyse the first step *de novo* glucose synthesis from pyruvate
PER:	Protein efficiency ratio, unit of bodyweight gain per unit of dietary protein intake
PPC:	Pea protein concentrate
Prot20D:	Proteasome 20 delta, proteolytic complex of the ubiquitin pathway
RT-PCR:	Reverse transcription – polymerase chain reaction
S6K1:	Ribosomal protein S6 kinase-1, of the family of protein kinase, is involved in the signalling transduction
SGR:	Specific growth rate, as percentage weight gain per day
TCA:	Tricarboxylic acid cycle, citric acid cycle or Krebs cycle
TOR:	Target of rapamycin, cellular signalling pathway
α-ketoA:	Alpha-keto acid resulting from the deamination of an amino acid
β-oxidation:	Beta-oxidation is the process by which fatty acids are gradually degraded into acetyl-CoA molecules
4EBP1:	Binding protein of elongation factor, inhibitor of the protein synthesis

Dansk resumé

Den globale produktion af karnivore fiskearter, såsom laksefisk, er afhængig af ekstruderet foder for at kunne forsyne fiskene med optimale protein- og energimængder for at opnå maksimal vækstrate. Foder til opdrætsfisk har udviklet sig dramatisk som konsekvens af en global mangel på marine råvarer og en samtidig øget efterspørgsel på næringstæt foder. For at understøtte en øget og fortsat konkurrencedygtig produktion, aftager afhængigheden af dyre og knappe marine råvarer. Foder til opdrætsfisk indeholder en stigende mængder alternative proteinkilder af forskellig oprindelse. Plantebaserede råvarer er egnede kandidater til at erstatte fiskemel, grundet høj tilgængelighed, lave omkostninger og interessante ernæringsmæssige egenskaber. Til gengæld afviger aminosyreprofilerne af vegetabilsk protein i forhold til fiskemel og deres anvendelse i foder til opdrætsfisk resulterer i aminosyreunderskud i forhold til ernæringsmæssige behov. Supplementering med krystallinske aminosyrer er almindelig praksis for at afbalancere foderet og opretholde vækstrater. Fuldstændig erstatning af fiskemel med planteprotein suppleret med krystallinske aminosyrer resulterer ofte i dårligere vækst og foderudnyttelse. Forskelle i optagelsen af aminosyrer under fordøjelsen resulterer i en tidsmæssig forskydning i tilgængelighed for syntesen af protein og angives ofte som en forklaring på ringere vækstparametre. Aminosyrer er alsidige molekyler, der anvendes som byggesten for proteinsyntese men også som substrat i metabolske mellemtrin. Der er ligeledes en stigende bevismængde for at aminosyrer ikke blot er signalstoffer, som er i stand til at regulere metabolske processer, men også regulerer vækstprocesser.

Denne afhandling omfatter fire artikler, der undersøger effekten af indholdet og formen af methionin i foder på plasma aminosyreprofiler og på gen ekspression relateret til leveremetabolisme og vækstrelaterede mekanismer i regnbueørred (*Oncorhynchus mykiss*).

Resultaterne fra den første undersøgelse bliver præsenteres i artikel I og viser, at selve proteinkilden (fra fiske- eller plantemel) ikke påvirker plasma profilen af essentielle aminosyrer, men snarere afspejler diætens niveau. Omvendt resulterede tilskud med histidin, lysin og threonin i krystallinsk form til en plantebaseret diæt i en fremskyndelse af maksimale plasmakoncentrationer sammenlignet med aminosyrer tilført diæten i proteinbundet form. Desuden resulterede tilskud med krystallinsk methionin i en tydelig akkumulering i plasmaet sammenlignet med de fisk, der blev fodret diæter med samme methioninmængder bundet til protein. Kvælstofudnyttelsen for en diæt med manglende aminosyrer var ringere i forhold til diæter baseret på fiskemel. I plantebaserede diæter suppleret med krystallinske aminosyrer forbedredes kvælstofudnyttelsen, og nåede næsten niveauer svarende til kontroldiæter.

Forholdet mellem niveau og form af methionin i diæter (fri, coated eller proteinbundet) og plasma aminosyre profiler blev yderligere undersøgt i artikel II ved hjælp af statistike modeller fra et stort datasæt baseret på 504 individer og 20 variable. Ved brug af generaliserede additive modeller, vises det, at (1) methionin niveau og form i foderet forklarer 74 % af variansen i plasmakoncentrationer af methionin under fordøjelsen, og at

(2) foderets form af methionin og samtidige ændringer methioninkoncentrationer i plasma påvirker koncentrationerne af adskillige andre væsentlige aminosyrer (arginin, histidin, isoleucin, lysin, fenylalanin, threonin og valin) betydeligt. Endelig viste lineære modeller en positiv sammenhæng (R^2>0.9) mellem plasmakoncentrationerne af tregrenede aminosyrer (isoleucine, leucin og valin) ved fordøjelse af foder med forskellig methionin niveauer.

Resultaterne fra artikel III viste, at inklusionsniveau og kilde (krystallinsk eller proteinbundet) af methionin i diæten påvirkede ekspressionen af gener i levervæv der er relateret til I) den somatotropiske akse og II) proteinomsætning. Til dette forsøg blev syv fodertyper sammensat med forskelligt methionin indhold (varierende mellem 0,6 og 1,29 % tørstof) enten forsynet til diæterne i krystallinsk eller proteinbundet form. Ekspressionsniveauer af væksthormon receptor 1 og insulinlignende væksthormon I viste et lineært sammenhæng med foderets methioninindhold (P<0.01), hvilket afspejledes i fiskenes vækst. Derudover blev ekspressionen af fire komponenter af den somatotropiske akse væsentligt påvirket (P <0.05) af diæternes methioninindhold. Den tilsyneladende kapacitet for proteinnedbrydning i leveren faldt med stigende foder methioninniveauer på en mere eller mindre lineær facon. Til sammenligning syntes methionin kilden ikke at have væsentlig indflydelse på spredningsmønsteret af proteinnedbrydende enzymer. Resultaterne peger på, at methionins tilgængelighed, som konsekvens af diætens indhold og form, regulerer ekspressionen af gener involveret i GH/IGF responset og protein omsætning, hvilket yderligere påvirker vækstparametre.

Artikel IV præsenterer resultaterne af effekten af diæternes methioninniveau og form på leverens metabolske mellemtrin. Diæterne blev fodret til regnbueørreder i 37 dage efterfulgt af prøvetagning for I) hepatisk genekspression, II) hepatosomatisk indeks, III) postprandial ammoniumudskillelse, og IV) plasma methionin analyser. Ekspressionen af adskillige gener, der koder for enzymer, som medvirker til lipid metabolisme, glukoneogenese og aminosyre katabolisme viste et lineært respons (P <0.005) til gradvis forøgelse af foderets krystallinske methionin og en samtidig reduktion i kvælstofudskillelse og relativ levervægt. Derudover påvirkede kostens form af methionin de postpradiale plasma methionin koncentrationer og ekspressionen af specifikke levergener signifikant (P <0.05). Ammoniumudskillese var konsekvent højere hos fisk fodret med diæter forsynet med krystallinsk methionin end hos de fodret med proteinbundet methionin (P <0.05). Denne undersøgelse er den første til at vise, at ekspressionen af adskillige gener relateret til mellemtrin i energiomsætningen reagerer på stigende niveauer af methionin i diæten og at methioninkilden påvirker leverens energiomsætning på transskriptionsniveau.

Kostens indhold af aminosyrer, i hvert fald methionin, synes at være afgørende for regulering af adskillige gener, der er forbundet med vækst og mellemtrin i energiomsætning. Derudover synes genekspressionen at påvirkes betydeligt ved brug af supplering med krystallinske aminosyrer, der kan være en mulig faktor forskellen i udnyttelsen af plantebaserede diæter sammenlignet med foder baseret på fiskemel.

Abstract

Aquafeed formulation has evolved dramatically in response to shortages in marine raw materials, driven in part by the sustainable management of the wild stocks and an increased demand for nutrient-dense diets. Aquaculture of carnivorous species such as salmonids relies on extruded feeds with optimal protein and energy ratio to maximize the growth performances. To support the increasing demands, aquafeeds contain increasing contributions of protein products from alternative origin. Plant raw materials can be suitable substitutions for fish meal, benefiting from a high availability, low cost and similar nutritive properties. The major limitation in using pant derived protein, at least when using high quality protein concentrate, is the amino acid profiles of plant protein, which differs from that of fish meal. Their inclusion in aquafeed results in a product deficient in essential amino acids (EAA) compared to dietary requirements. Supplementation with amino acids in crystalline from (CAA) is a common practice to balance the dietary amino acid profile to achieve high growth performances. However, complete substitution of fish meal using plant proteins and CAAs often results in poorer growth performances. The reason for this is often suggested to be related to difference in amino acid uptake kinetics during digestion, resulting in a temporal mismatch in amino acid availability, resulting in poorer at protein synthesis site. In addition to their role as building blocks in protein synthesis, amino acids also serve as substrates for synthesis of metabolic intermediates, and increasing evidence shows that amino acids also function as signaling factors in the regulation of intermediary metabolism and growth related pathways.

The present thesis comprises four supporting papers, based on two laboratories studies, investigating the effect of dietary amino acid level and form on i) growth performances, ii) plasma amino acid profiles and iii) on the expression of genes involved in hepatic metabolic and growth-related pathways in rainbow trout (*Oncorhynchus mykiss*).

The results from the first study are presented in **Paper I** and show that the protein source itself (fish meal or plant based) does not affect the plasma EAA profiles, but rather that plasma EAA levels reflect the dietary level. Supplementation with histidine, lysine and threonine in crystalline form to a plant based diet was, on the other hand, found to result in their plasma concentrations peaking earlier during the digestive process when comparing to other AAs supplied as protein-bound. In addition, to these early peaks in the plasma concentration, supplementation with crystalline methionine resulted in what can be best described as an apparent “accumulation” in the plasma, compared to fish fed similar dietary level but in protein bound form. The study further showed that the nitrogen excretion resulting from feeding an AA deficient diet was higher than for the fish meal control diet. Supplementation of the plant meal diet with crystalline amino acids tended to improved nitrogen utilization, almost equaling the results obtained for the fish meal control diet.

The relationship between dietary methionine level and form (free, coated and bound), and plasma amino acid profiles was further investigated in **Paper II** by applying statistical modeling to a large dataset (504

individuals and 20 variables). Using generalized additive models, it was shown that i) dietary methionine level and form explained 74 % of the variance in methionine plasma concentrations observed during digestion, and ii) that the dietary form of methionine and concomitant changes in methionine plasma concentrations significantly affected the plasma concentrations of several other essential AAs (arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine and valine). Linear models revealed a positive relationship (R^2>0.9) between plasma concentrations of the three branched chain amino acids (BCAAs; isoleucine, leucine and valine) during digestion of meals differing in dietary methionine levels.

Results from **Paper III** showed that dietary level of methionine in a plant based diet affected the expression of hepatic genes related to i) the somatotropic axis and ii) protein turnover. For this purpose five diets were fed to juvenile rainbow trout under control condition. The diets were formulated to differ only in methionine content (ranging from 0.6 to 1.29 % dry matter), supplied as crystalline. The transcript levels of the growth hormone receptor I (GHR-I) and insulin-like growth hormone I (IGF-I) increased linearly with dietary methionine content (P<0.01), which was reflected in the overall growth performances. In addition, the expressions of four components of the somatotropic axis investigated were significantly (P<0.05) affected by dietary methionine. The apparent capacity for hepatic protein degradation decreased with increasing dietary methionine level in a more or less linear manner. The results suggest that methionine availability modulates the expression of genes involve in the GH/IGF response and protein turnover, further affecting growth performances.

Paper IV presents the results of the effects of dietary methionine level on hepatic intermediary metabolism using the same diets as in **Paper III**. The diets were fed to rainbow trout for 6 weeks, followed by sampling for i) hepatic gene expressions, ii) hepatosomatic index (HSI), iii) postprandial ammonia excretion, and iv) plasma methionine levels. The expression of several genes coding for enzymes involved in lipid metabolism (FAS, G6PD, CPT1a), gluconeogenesis (FBPase) and amino acid catabolism (ALT1, GHD and GLS01) responded in a linear manner (P<0.05) to gradual increase of dietary crystalline methionine; and were associated with a decrease in nitrogen excretion and relative liver mass. Nitrogen excretion was found to decrease linearly with increasing levels of dietary methionine (P <0.05). This study is the first to demonstrate that expression of several genes related to intermediary metabolism respond in a dose-response manner to increasing levels of dietary methionine.

Dietary methionine, possibly in concert with other amino acids, appears to be a key regulatory factor in the expression of several genes involved in growth and intermediary metabolism. Furthermore, the gene expression seems to be conjointly modulated by the raw material matrix, resulting in differences in amino acid utilization but also affecting postprandial amino acid profiles.

Objectives of the thesis

Carnivorous fish species require high levels of proteins and species-specific amino acid profiled in their diets. Aquafeeds are formulated with proteins originating from a variety of sources; marine or land-based, animal or vegetal, in order to provide high-performing, price-competitive and sustainable products. The raw materials commonly used vary in their amino acid profile, necessitating supplementation with crystalline amino acid to fulfil requirements.

The main objective of the thesis was to assess the effect of current practices in the aquafeed formulation on amino acid utilization and role as signalling factor in rainbow trout (*Oncorhynchus mykiss*).

Main hypothesis: The dietary level and source of methionine affects growth performances and feed utilization in rainbow trout, mediated by changes in amino acid bioavailability, and their role as substrate molecules and signalling factors.

The main hypothesis was divided in four sub-hypothesis and addressed in the different work packages of the PhD.

Hypothesis 1: Diet recipe (raw material and amino acid content) influences postprandial plasma amino acid patterns and protein utilization.

Hypothesis 2: Changes in the supplementation of one essential amino acid and protein source (plant or fish meal) affect postprandial plasma amino acid profiles.

Hypothesis 3: Dietary methionine level and protein source (plant or fish meal) affect growth performances at a molecular level through modulating the transcription of genes of the somatotropic axis and in protein turnover.

Hypothesis 4: Dietary methionine level and protein source (plant or fish meal) affect intermediary metabolism at the transcriptional level, resulting in different amino acid utilization.

Hypothesis 1 was addressed in a first experiment where rainbow trout were force fed three different diets : i) a pea protein concentrate diet (PPC); ii) a pea protein concentrate diet supplemented with four essential crystalline amino acids (PPC+); and iii) a fish meal (FM) based diet as control. In two consecutives parts, the fish were force fed 0.75 % of their body mass to measure oxygen consumption and ammonia excretion, using respirometric chambers; and sample blood and determine postprandial amino acid concentration in the plasma. The results are presented in **Paper I.**

A second experiment was designed to address the other hypotheses. For this purpose juvenile rainbow trout were fed for six weeks with diets (12 in total) differing mainly in their methionine level and form. Different levels of methionine were supplemented to a reference plant-based diet, either as crystalline (free) or agar-

coated. Additionally, diets with increasing level of protein-bound methionine were achieved by increasing the fish meal content while decreasing the share of protein originating from plant products.

To answer **hypothesis 2** statistical models were performed on a large dataset (20 variables and 504 individuals) to quantify the effect of dietary methionine level and form on plasma amino acids profiles. Postprandial plasma samples were obtained at succeeding time intervals and analysed for essential amino acid concentrations. The results are presented in **Paper II**. Finally, to address **hypothesis 3** and **4**, the growth performances obtained for selected dietary treatments were recorded and related to the hepatic expression of genes coding for proteins involved in the somatotropic axis and protein turnover (**Paper III)**. Additionally, hepatic transcription levels on gene related to intermediary metabolism were measured and associated with metabolic indicators (nitrogen excretion and relative liver size). **Paper IV** recounts the results obtained on the metabolic response to dietary methionine level. The results on the effect of protein source are presented as figures and tables in the Introduction and Background.

Introduction and background

Aquaculture is the fastest growing food-producing sector in the world, supplying 49 % of fish for human consumption in 2010 (FAO, 2012), and is expected to overcome fisheries production in tonnage. Carps, cyprinids and barbels are the major species produced (22.2 million metric tons; MMT) mainly in China and the Indian sub-continent, followed by tilapia (3.2 MMT) and salmonids (2.5 MMT) comprising mainly Atlantic salmon, *Salmo salar* (1.44 MMT) and rainbow trout, *Oncorhynchus mykiss* (0.7 MMT). More than half of the global farmed salmonids are produced in Europe (1.4 MMT), and make up 80 % of the total European aquaculture production volume (Failler, 2006). Simultaneous with the overall increase of the global production volumes has been intensification of rearing practices, i.e. increasing rearing densities, production rates and the use of compounded feeds. Compounded feeds are nutrient dense, made using blends of various raw materials and additives, formulated according to the nutritional requirements of the species, and finally processed (e.g. pelleted, extruded or crumbled). From 1995 to 2010, the share of fish and shrimp produced using compounded feed has increased from 44 % to 68 % of the total tonnage, which represents a rise from 4 to 21.5 MMT (Tacon and Metian, 2008) because of the concomitant increased aquaculture production volume. The use of nutrient dense diets for finfish culture has been increasing at a rate 2.5 times higher than the growth in the volume of aquaculture production (Tacon and Metian, 2008).

Historically and nutritionally fish meal (FM) has been the preferred protein source in aquafeeds for carnivorous species. However, FM production relies on wild forage fish stocks, which are now under a sustainable management to preserve this resource on the long term, and to ensure a constant availability of marine raw materials each year (FAO, 2012). The increasing demands for aquafeeds and the stagnant availability of FM have forced feed manufacturers to integrate other protein sources in the diets. Decreasing reliance of marine raw materials by supplementing the diets with land based proteins is perhaps the major challenge for the continued growth of the aquaculture sector. However, the access to protein sources is strongly competitive, and other sectors, such as food production for human or feed for other farmed species, face similar issues. Increasing diversity of dietary protein sources add flexibility to the recipe formulation, and help the feed manufacturer to keep price fluctuations to a minimum.

Plant raw materials are appropriate substitutes for fish meal, benefiting from high availability, low prices and suitable nutritional value. However, proteins of different origin vary in their amino acid composition (Médale and Kaushik, 2009). Optimizing nutrient content of a diet to fulfil both protein and energy requirements under the constraint of an ideal amino acid profile becomes a difficult exercise, and often requires supplementation with crystalline amino acids (CAAs) to balance the dietary profile.

Fish appear to have a high requirement for dietary protein, about twice of that of other vertebrates. However, the efficiency with which feed is converted into growth is about three-fold higher in fish than in other

vertebrates (Bowen, 1987). This is possible through three strategies: i) as fish are poikilothermic, they do not expend energy towards maintaining elevated body temperatures (Médale and Kaushik, 2009); ii) as fish are neutrally buoyant in water, energy requirements to maintain posture and locomotion are greatly reduced; and lastly iii) fish excrete nitrogen mainly through a minimal-cost process, i.e. passive diffusion of ammonia across the gills driven by a gradient between the blood and the surrounding water. In comparison, mammals and birds excrete urea and uric acid, respectively, after energy-expensive syntheses.

Salmonids have a requirement for a supply of dietary protein with a particular amino acid composition, i.e. not for protein *per se*. In that regard, fish fed a diet with a tight ratio of energy and protein should be carefully supplied with the right amount of essential amino acids (EAA) to ensure optimal feed utilization. Optimal levels of protein and dietary energy content will still lead to poor growth if the amino acid profile is not balanced to requirements (Green and Hardy, 2008). Fish utilize amino acids as an energy source, and amino acids will be oxidized to fuel metabolism in the case of insufficient energy supply by the diet or an amino acid unbalance.

Despite this, the growth performance of fish fed plant based diets supplemented with CAAs is typically not equal to that of fish fed FM diets. Fully replacing fish meal with plant protein, despite optimizing nutrient and energy contents of the diets, often result in poorer dietary protein utilization for growth (Bendiksen et al., 2011; Burr et al., 2012; Jalili et al., 2012). The timing at which amino acids are present at the protein synthesis site has been proposed to be pivotal for the utilization of amino acids. Amino acids derived from crystalline supplementation typically reach the systemic circulation earlier than protein-bound amino acids as the latter requires enzymatic digestion before being available for uptake across the gut wall (Shuhmacher et al., 1997). For protein synthesis to take place, the required proportions of amino acids should be available simultaneously, which may not be the case if uptake kinetics of CAAs differs from protein bound.

Amino acids are versatile molecules used as substrates for synthesis of various components, and recently there has been growing interest in their role as regulating factors in physiological functions. Firstly, they are used as building blocks for protein synthesis (e.g. muscular fibres, enzymes), but can also be used as a metabolic energy substrate (ideally minimal), or as precursors for many active compounds (e.g. non-essential amino acids, hormones, purine bases). The traditional approach in fish nutrition has been to consider mainly this aspect of amino acids function, whereby they exert their effect on growth. However, our understanding of the roles of amino acids in nutrition is growing, with increasing evidence showing that amino acids act as regulators of the expression of genes involved in metabolism and pathways involved in growth.

The present thesis brings forward understandings on the effects of common practices of the aquafeed production on the role of amino acids as metabolic substrates and regulation factors. At first a brief introduction to the current practices in formulation of salmonids aquafeed will be given, followed by a discussion on how diets affect the plasma free pool of amino acids. The following part introduces the roles of

amino acids as substrates for molecule synthesis or signal factors, and the implication of dietary changes on the regulation of the somatotropic axis, protein turnover, and intermediary metabolism. The most important results are recapitulated in part 2, followed by the general conclusion and future perspectives in chapter 3.

1. Current practices in commercial feed formulation for salmonids

During the last century, formulation of salmonids feed changed quite dramatically, from a wet-mixture of locally available ingredients (salmon eggs, slaughter house wastes, fresh or frozen fish) to extruded diets with low FM content (Grassl, 1956; Hardy and Barrows, 2002; Rana et al., 2009). In the production of today's aquafeeds, protein raw materials represent the highest expense, accounting for about 50 % of the total cost. The aquaculture sector is at present the biggest consumer of FM, estimated to be 50 % of the global production in 2012 (Pike, 2005). For several reasons, this reliability on marine raw materials is too high, and a large range of land based protein sources (plant or animal) present promising potential for FM substitution. The alternative plant products most widely used in compounded feed are soybean meal or protein concentrate, pea, corn and wheat gluten, canola or rapeseed cake.

From a wet feed sustaining growth, aquafeed has evolved into nutrient dense diets designed to optimize feed utilization with respect to growth, and reduce environmental impacts. The evaluation of the nutritional value of a feed has also progressed from measuring growth to utilizing molecular techniques. Measurements of growth performances and feed utilization are still the main indicators used to assess diet formulations. However, utilization of advanced technic pushes forwards the understandings on the dietary effect on specific metabolic pathways. This has resulted in the recent implementation of two concepts in commercial feed formulations: i) optimisation of the digestible protein: digestible energy (DP:DE) ratio; and ii) the "ideal protein concept", which is based on multiple studies conducted on protein and energy requirement. The application of these two concepts supports the conclusion that fish do not have a true requirement for protein but rather for a well-balanced dietary essential amino acids profile and the energy content associated (Cowey, 1995; Wilson, 2002).

1.1. Formulation of aquafeeds to the optimal digestible protein and digestible energy content

Formulation of feed for salmonids based on DP:DE ratio relies on the "protein sparing effect". Salmonids are carnivorous and 50 to 70 % of the energy required for metabolism during digestion is fuelled by oxidation of amino acids (Wood, 2001). However, salmonids also effectively utilize lipids as an energy source. In resting non-fed fish, lipids represent then the main fuel (35-68 %) followed carbohydrates (Wood, 2001). The protein sparing effect is, briefly, the increased utilization of the available dietary protein for conversion into muscle instead of energy production. High dietary inclusion of lipids enhances protein sparing and subsequently improves growth performances.

A typical diet for rainbow trout contains approximately 420 g protein and 23 MJ per kg feed to reach an optimal DP:DE ratio of 18. The optimal DP:DE ratio differs with fish species and size, primarily due to differences in expected growth performances.

As such, feed formulation is based on nutrient content rather than raw material source, which allows for a higher flexibility in recipe composition and is less sensitive to price variations. The purpose of this "open-formulation" is to maintain DP:DE at a constant level using blends of raw materials, aiming at maintaining good growth performances and price competitiveness of the feed. In open-formulation the next limiting factor, after the DP:DE ratio, is the amino acid profile to support high growth performances, feed efficiency, as well as low waste outputs (Bureau and Hua, 2010).

1.2. Dietary amino acid supply based on the "ideal protein" concept

FM typically fulfils amino acid requirements of carnivorous fish and comes close to the "ideal protein" in terms of amino acid composition. The "ideal protein" concept uses lysine as a reference amino acid, to express (as percantage of lysine) the requirement for all other essential amino acids. The amino acid profile fulfilling the requirement is assumed to maximize growth, feed utilization, nitrogen retention or other criteria related to amino acid utilization. The amino acid content should cover the requirements for all physiological functions. In this concept, the requirement is not on the protein itself (i.e. the origin) but rather on the amino acid composition of the digestible protein fraction.

Like any other animals fish have dietary requirement for several amino acids, named indispensable or essential amino acids. EAA cannot be synthetized *de novo* by the body relatively to needs and have to be provided by the diet (Wu, 2009). Rainbow trout have dietary requirement for ten EAAs to sustain potential growth and feed utilization (Table 1).

The majority of EAAs in plant feedstuffs are either deficient or in excess in comparison with FM as shown by Médale and Kaushik (2009). Inclusion of various protein sources in aquafeeds introduces the notion of limiting EAA(s), i.e. the EAA(s) supply in the smallest amount relatively to requirements (Table 1). In plant protein feedstuffs, methionine and lysine are often the first two EAAs that become limiting, and additional supply is required to achieve good growth performance.

Aquafeeds are commonly supplemented with CAAs in order to preserve the optimal EAA composition. CAAs are highly concentrated single amino acids (99 %) which are highly digestible (close to 100%), and are manufactured by chemical processing or fermentation. These additives are added in powder form to the meal mix before processing. Despite some technical difficulties associated with CAAs (leaching, degradation in the gastrointestinal tract or during the process etc.), supplementation has remained a necessity in order to secure a balanced dietary EAA profile.

Table 1: Digestible essential amino acid requirement (% DM of the feed) for rainbow trout (*Oncorhynchus mykiss*). Source: NRC (2011)

		Weight class		
		0.2-20 g	20-500 g	500-1,500 g
Amino acid	Abbreviation	% diet DM[a] (20 MJ DE)		
Arginine	Arg	1.91	1.77	1.62
Histidine	His	0.83	0.77	0.69
Isoleucine	Ile	1.12	1.19	0.98
Leucine	Leu	2.26	2.11	1.78
Lysine	Lys	2.47	2.31	1.92
Methionine + Cystine	Met+Cys	1.32	1.23	1.10
Phenylalanine + Tyrosine	Phe+Tyr	2.49	2.33	1.82
Threonine	Thr	1.77	1.63	1.60
Tryptophan	Trp	0.43	0.4	0.42
Valine	Val	1.90	1.76	1.64

[a] Dry Matter

Dietary supplementation with one or a mixture of CAAs improves the nutritional value of the feed through enhanced feed intake, feed and protein utilization and growth, compared to diets devoid in one or more EAA(s). However, the performance of fish fed these diets often does not equal that which can be obtained by fish fed a FM based diet. Aquafeeds with inclusion of plant protein and CAA supplementation are used under the assumption that the protein source and the physical form of EAAs (free or protein-bound) do not affect the amino acid bioavailability.

2. Dietary parameters affecting amino acid availability

In fish nutrition, amino acid availability is typically estimated from the digestible protein fraction. Digestibility coefficients of nutrients are calculated as the differences between intake (feed content) and loss (faecal content) using either the addition of an indigestible marker to the feed (such as yttrium oxide, Y_2O_3 or chromium oxide, Cr_2O_3) or by direct analysis of collected faecal matter.

2.1. The limitation of using digestibilities to estimate amino acid availability

The digestible fraction of a protein raw material is highly related to its nutrient content, its source (e.g. plant or marine origin, CAAs) and degree of refining. These factors influence the content of anti-nutritional factors (ANFs) such as fibres, protease inhibitors or phytic acid, which are known to disturb the digestive and absorptive processes. Therefore, prior to integration of a new raw material into a recipe, the digestible protein and energy fractions need to be determined, as well as the value for each amino acid.

The use of digestibility coefficients presents several limitations in the estimation of feed derived amino acid availability. Digestibility does not take into account the amount of amino acids directly used by the cells of the intestinal wall (Brosnan, 2003; Fang et al., 2010) which influences the true bioavailability for metabolic pathways, or the release of endogenous amino acids in the intestinal lumen. Furthermore the digestibilities do not reflect the absorption patterns, known to depend on dietary content (Bogé et al., 1979), form (i.e. protein bound or free; Dabrowski and Dabrowska, 1981), but also on the raw material composition (Larsen et al., 2012). Despite several disadvantages, measurement of amino acid digestibilities remains a useful tool to balanced dietary profile as close as possible to requirement.

Measurements of plasma amino acid profiles during the digestive process may serve as another indicator of amino acid availability. As mentioned previously, CAAs are highly digestible additives that are present in a free form (e.g. not bound to a protein), and therefore do not require an enzymatic degradation to become available for absorption. As a result, CAAs may be available for absorption across the gut wall earlier in the digestive process than amino acids that are protein-bound (Fig. 1).

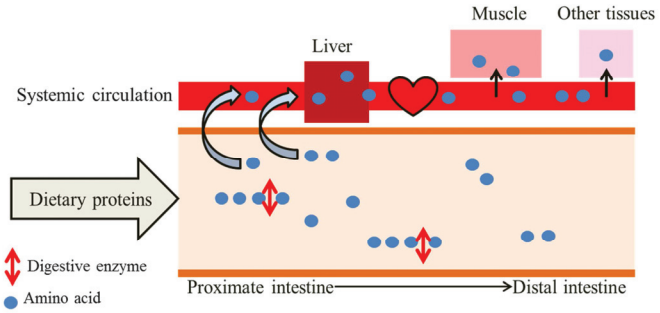

Fig. 1: Schematic view of the digestion process and absorption

Variations in plasma concentrations during the digestion of a meal indicate the amount available after passage of the gut wall and liver, and also reflect uptake patterns. However, endogenous amino acids are also found in the plasma (exchange between tissues; Brosnan, 2003), and with circulation times of less than a minute in rainbow trout (Davies 1970), plasma concentrations at a given time point may not exclusively reflect the amino acid uptake from feed. Nevertheless, the plasma levels during digestion provide supplementary information about the uptake pattern and the timing of free amino acid available for tissue.

2.2. Effect of crystalline amino acid supplementation on plasma amino acid profile

Poorer performance recorded for fish fed diets supplemented with CAA compared to “all protein-bound” diets are commonly considered to be caused by the temporal changes in availability at protein synthesis site (Cowey and Sargent, 1979; Thebault, 1985; Zarate and Lovell, 1997; Zarate et al., 1999; Dabrowski et al., 2003; Bodin et al., 2012) induced by the faster uptake of CAAs during the digestive process.

Amino acids are mainly transported in the plasma fraction of the blood; however a considerable fraction of amino acids are also found within the erythrocytes, which may hold 20 to 60% of the total amino acid content of the blood (Ogata and Arai, 1984). This is especially true for non-essential amino acids (NEAA), of which concentrations may be two to three-fold higher in the red blood cells than in the plasma. The free amino acids present in the plasma fraction are assumed to be the readily available for tissue uptake, and these are therefore commonly considered when investigating pattern changes, rather than whole blood content.

A wide range of studies have investigated how dietary parameters affect plasma amino acid profiles during digestion. Recently, Larsen et al. (2012) showed that feeding rainbow trout with plant based diet affected postprandial plasma amino acid profiles compare to the group fed a fish meal based diet. The nitrogen-free fraction of the vegetable diet was higher than in the fish meal diet, likely due to the utilization of low refined plant meal. In **Paper I**, the utilization of plant protein concentrate did not affected plasma amino acid profiles. Altogether these results suggest that the nitrogen-free faction (ANF) of a raw material, related to the degree of refining, is likely to affect amino acid uptake pattern and consequently plasma profiles rather the than the origin *per se*. The amino acid composition of the diet seems to be a major factor determining plasma profiles (Schumacher et al., 1995; **Paper I**). Walton and Wilson (1986) found positive correlations between plasma amino acid levels and dietary content at each sampling period in rainbow trout fed casein diets. A similar relationship was observed in **Paper IV** between plasma methionine concentrations at several sampling times when rainbow trout were fed plant based diets with increasing levels of crystalline methionine ($r = 0.84\text{-}0.94$, $P<0.001$). Typically, in rainbow trout fed CAAs under optimal temperature conditions, plasma concentrations of supplemented AAs show steep rates of increase until peaking approximately 12 h after feeding, followed by a decrease to reach fasting level between 24 to 36 h (Walton and Wilson, 1986; Murai et al., 1987; **Paper I** and **IV**). The speed with which CAAs are absorbed appears to exceed the capacity of tissue to metabolize them on the first passage (Cowey and Walton, 1988), resulting in premature and excessively higher peak concentrations compared to protein-bound amino acids (Schuhmacher et al., 1997; Ambardekar et al., 2009). Additionally, EAAs deriving from crystalline supplementation induce high plasma levels for a longer period of time compared to protein-bound amino acids (**Paper I** and **IV**).

Even though a wide range of dietary parameters have been shown to affect plasma amino acid profiles, very little quantitative information is available. Plasma amino acid concentrations respond to dietary levels in a linear manner (Walton and Wilson, 1986; **Paper IV**) or follow a broken-line relation (Bae et al., 2011, 2012). The effects of the dietary amino acid forms are usually discussed by comparison between dietary treatments, often using ANOVA models (Ambardekar et al., 2009). **Paper II** demonstrates that there is a combined effect of the dietary level and form of an EAA on postprandial plasma profile. The applications of generalized additive models, as shown in **Paper II**, allow more flexibility in analysing the response compared to linear models, which do not always reflect biological variations. The paper shows that

postprandial variations in methionine plasma concentrations could largely be explained (74 %) by dietary level and form (free, coated or protein-bound). Additional models showed that plasma methionine concentrations and dietary form affected, to different extents, postprandial variation of other EAAs plasma concentrations (accounting for 28 and 54 % of the variance). The results demonstrate for the first time that dietary changes, affecting availability of one EAA, significantly alters the overall plasma profile.

The major role of the free amino acid pool in the plasma is to deliver amino acid to tissues in adequate proportion (Robinson et al., 2011) in order to maintain organ homeostasis (Fafounoux et al., 2000), and support protein synthesis. Amino acid intake is one of the main processes responsible for preservation of whole-body protein and amino acid dynamic homeostasis. Maintaining the free pool of amino acid is essential in order to preserve amino acid functions (Panserat and Kaushik, 2010), such as the regulation of gene expression (Fafournoux et al., 2000; Jousse et al., 2004), but also modulate hormonal signals. Insulin is a particularly interesting hormone in the case of fish because amino acids are stimulators of the insulin excretion, having a stronger effect than glucose (Andoh, 2007; Seiliez et al., 2008), the main insulinotropin in mammals. Moreover, in fish, insulin is able to regulate plasma amino acid levels, by stimulating amino acid uptake by the tissues (Plisetskaya et al., 1991), in addition to having a hypoglycaemic effect (Andoh, 2007; Polakof et al., 2010). However, the magnitude of the insulinotropic effect differs between amino acids. Arginine, alanine, methionine and serine have been shown to induce higher in insulin levels compared to the other AA investigated (e.g. glycine, lysine, histidine) in barfin flounder, *Verasper moseri* (Andoh, 2007). Conversely, D isomers of arginine and alanine had no effect on insulin plasma levels. The amino acids insulinotropic role could also vary depending on species and the dose applied (Plisetskaya et al., 1991).

Dietary parameters affecting plasma free amino acid pool are also known to affect the amino acid ability to fulfil the diverse biological. The following part aims at describing the effect of dietary amino acid supply on biological functions involving amino acids as both substrate molecules and signalling factors at the gene expression level.

3. Effect of dietary changes on amino acid role as substrates molecules and signalling factors

The utilization of molecular technics, such as measurement of enzymes activities or gene expression, has been implemented to better understand the metabolic responses to dietary changes in fish. The RT-PCR method, to measure gene expression will be presented, followed by a description of the target of rapamycin (TOR) pathway, through which amino acid are able to regulate gene expression and protein synthesis.

3.1. A method to measure gene expression: real time quantitative PCR

The quantitative RT-PCR (Real Time – Polymerase Chain Reaction) is a fast and sensitive method, which quantifies the expression of a target gene relative to the expression of a reference gene. The messenger RNAs (mRNAs), extracted from a tissue sample (e.g. liver or muscle), are converted into complementary DNA (cDNA) during the process of reverse transcription. During the PCR, a part of the target cDNA is amplified using specific primers (forward, 5' and reverse, 3') and a DNA-polymerase (enzyme). Fluorescent probes (TaqMan®) are added to the reaction to quantify the number of DNA synthetized after each amplification cycle (real time). TaqMan® probes are oligonucleotides that contain a fluorescent reporter dye on one end (the 5' base) and a quencher located on the other end (the 3' base). These dual-labelled probes are designed to hybridize to a complementary region of the target sequence of the cDNA (produce of the reverse transcription step). When irradiated, the excited fluorescent reporter dye transfers energy to the quencher molecule rather than fluorescing. In the polymerisation process of a target cDNA, the 5' exonuclease activity of polymerase cleaves the probe and releases the fluorescent dye from the quencher and the Fluorescent Resonance Energy Transfer (FRET) no longer occurs. The decoupling results in the increase of fluorescence intensity that is proportional to the number of the probe cleavage cycles. The resulting fluorescence is then measured to estimate the amount of DNA produced per amplification cycle. A PCR usually comprise 30 to 40 cycles in order to complete the several phases. At start, reagents are in excess, and templates and products are in low concentrations enough not to compete with primer bindings. The amplification proceeds then at constant, exponential rate, which the most efficient phase of the reaction. The number of cycles necessary to obtain a threshold level of fluorescence (so called threshold cycle, CT) gives an estimation of the relative amount of cDNA present in the sample. Each cycle comprise similar steps, starting by denaturation of the DNA (melting) under heat treatment (94-98 ºC), followed by annealing of the primers to the single-stranded DNA template. Finally, the elongation step where the DNA polymerase synthetizes the new DNA strand, complementary to the DNA template starting from the primers.

To improve the quality of the results produced during a PCR, the primers should be chosen on two different exons, part of the sequence remaining in the final, mature version of the mRNA. In addition, to avoid pollution from "natural" DNA in the initial sample, DNase can be applied prior to start the reverse-transcription. Several experimental procedures allow for verifying the repeatability and reproducibility of the manipulation. The PCR efficiency (E) is derived from the standard curve slope, drawn using at least 5 dilution points (triplicate for dilution) of a pool of all samples. The efficiency ($E= 10^{-1/slope}$) assess the quality of the analysis, and should be between 1.9 and 2.1 to reflect an efficient hybridisation of the primers with the target genes.

The processing of data resulting from RT-PCR analysis requires utilization of reference genes and individuals to allow for relative comparison. Reference or house-keeping genes are genes designate

sequences that are constantly expressed, no matter the experimental conditions. For a given sample, the expression of the targeted gene is then calculated relatively to the expression of the reference gene. In the present thesis, elongation factor (EF1α) and β-actin were used as reference genes. The analysis of large sample set requires the analyses to be performed in several distinct plates. Utilization of a control sample, repeated in each chips, allow for the comparison of all the results. The method described by Pfaffl (2001) integrates the PCR efficiency (E), reference gene and control sample and was therefore chosen for the data analysis in the present thesis.

The RT-qPCR allows for measuring accurately the expression level of targeted genes and has been used to demonstrate that amino acids are able to affect the number of mRNA transcript in a given sample (Bustin, 2000). The next part will described one of the cellular pathways sensitive to nutrients (Avruch at al., 2009) and shown to be activated by amino acids able to regulate gene expression at translation step, the TOR pathway.

3.2. Amino acid regulation of gene expression through the TOR pathway

Several molecular mechanisms, triggered by nutrients, are present in the cells in order to respond to nutritional changes. Amino acids are able to regulate gene expression *via* several pathways, such as the TOR cascade. The TOR pathway is a major mechanism involving protein and protein-complexes that regulates initiation and elongation of mRNA translation into proteins (Crespo et al., 2002). Amino acids, associated with hormonal signals (insulin and/or insulin-like growth factors, IGFs), stimulate a cascade of protein phosphorylation (Fig. 2).

Once activated, the TOR complex initiates protein synthesis by activating, among other proteins, the ribosomal protein S6 through phosphorylation of the specific kinase S6K1. S6 is a 40S subunit of ribosome, responsible for binding met-tRNA and ribosomal subunits together, and then to the mRNAs to initiate protein synthesis. Additionally, TOR inhibits 4EBP1 (inhibitor of protein synthesis by competitive binding to elongation factor) by phosphorylation, releasing the elongation factor (elF4E) involved in the initiation of the mRNA translation into proteins.

In rainbow trout hepatic and muscular cells, the TOR pathway is also under hormonal control. Insulin activates the phosphorylation of the protein kinase B (Akt), mediated by a membrane receptor and several intermediary of the phosphatidylinositol family and phophoinositide kinases (Castillo et al., 2006; Seiliez et al., 2008; Lansard et al., 2010). IGFs trigger the same molecular mechanisms as insulin in muscular cells to promote muscle development, protein synthesis and to reduce protein degradation (Castillo et al., 2006; Cleveland and Burr, 2011; Fuentes et al., 2013).

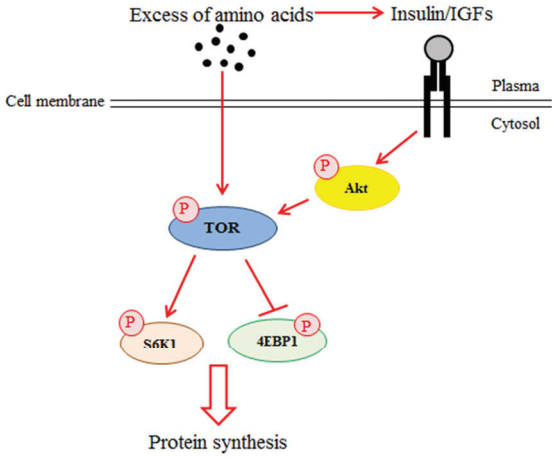

Fig. 2: Simplified scheme of the TOR pathway and activation by amino acids and insulin. Insulin or insulin-like growth factor (IGF) activates the protein kinase B (Akt) through a chain-phosphorylation. The phosphorylated form of Akt can in turn phosphorylate the target of rapamycin (TOR) complex (central protein-complex of the TOR pathway) in association with amino acids. TOR stimulates the phosphorylation of ribosomal protein S6 kinase-1 (S6K1) and binding protein (4EBP1), which activates initiation factor and releases elongation factor, respectively

TOR is a nutrient-sensitive pathway, modulated by the nutritional status. Under feeding condition, several intermediates of the pathway are activated in different tissues. In rainbow trout hepatocytes, the phosphorylated fraction of Akt, TOR and S6 were significantly higher for re-fed individuals compared to fasting fish, though without affecting the respective total levels of the intermediates (Lansard et al., 2009). Similarly, Seiliez et al. (2008) demonstrated that feed intake induced activation of the TOR pathway by enhancing phosphorylation and/or activity of Akt, TOR, S6K1 and 4EBP1 in rainbow trout muscular cells. These results suggest that the activation of protein synthesis in diverse tissues after meal ingestion (McMillan and Houlihan, 1988) is, at least partially, under the control of TOR, which has a central role in transducing the signal from nutrient derived from the digestion of the feed.

Undoubtedly, the mRNA level does not reflect the actual protein content nor the phosphorylation and/or activity status. However, changes in dietary methionine profile affected the level of TOR transcript, possibly affecting the capacity to up-regulate protein synthesis.

In addition to being activated by feed intake, the response of TOR pathway is modulated by the dietary composition, where e.g. increasing the carbohydrate dietary fraction at the expense of protein negatively affected the TOR signalling pathway in both muscle and liver cells of rainbow trout (Seiliez et al., 2011; Skiba-Cassy et al., 2013a).

However, the dietary raw material source (marine *vs.* plant based products) did not alter phosphorylation of Akt, TOR and S6K1 (Lansard et al., 2009). In species presenting relatively high protein requirement compare to other vertebrates, dietary protein level, rather than the origin seems important to activate protein synthesis. Then within the protein fraction, the amino acids do not contribute equally in activation of the TOR pathway. Among all, leucine has a unique functional role in the regulation of mRNA translation though the activation of TOR pathway (Kimball and Jefferson, 2006). The activation of TOR pathways by leucine was found to be insulin-dependent in mammal skeletal muscles (Kimball and Jefferson, 2006), and in rainbow trout hepatocytes (Lansard et al., 2011). Conversely, the hepatic TOR signalling pathway was neither activated by the presence of lysine or methionine *in vitro* (even in the presence of insulin; Lansard et al., 2011) nor by dietary methionine content *in vivo* (Skiba-Cassy et al., 2003b). However, **Paper III** shows that the expression of the TOR protein was up-regulated by increasing dietary level of methionine, yet not affected by the raw material composition of the diet (Table 2; Fig 3). Amino acids can regulate gene expression through other molecular pathways, conjointly or independently of insulin action.

Table 2: Composition of the plant protein based (PP) or fish meal based (FM) diets with two levels (1 and 2) of dietary methionine (0.94±0.04 % DM and 1.29±0.01 % DM, respectively)

Diet	**PP 1**	**PP 2**	**FM 1**	**FM 2**
Ingredient formulation (g 100g^{-1} feed)				
Fish Meal	8,00	8,00	25,80	44,80
Krill Meal	2,00	2,00	2,00	2,00
Soya Protein Concentrate	10,00	10,00	6,70	3,20
Pea Protein Concentrate	36,50	36,50	24,40	11,60
Wheat	18,90	18,90	18,90	15,80
Fish Oil	11,00	11,00	10,80	11,10
Rapeseed Oil	11,00	11,00	10,80	11,10
MONO Ca P	1,10	1,10	-	-
L-Histidin	0,10	0,10	-	-
Yttrium	0,05	0,05	0,05	0,05
D-L Methionine	0,32	0,65	-	-

Amino acid deficiency results in increased accumulation of free tRNA that binds and activates the general control non-depressible protein 2 (GCN2) kinase (Jousse et al., 2004). In mammals, the GNC2 monitors intracellular amino acid concentrations (Taylor, 2014), leading to global decrease of protein synthesis (Jousse et al., 2004; Wu, 2009). In rainbow trout fed a methionine deficient diet, the GCN2 pathway was up-regulated compare to individuals fed a balanced diet, while no differences on the phosphorylation level of Akt and TOR were measured (Skiba-Cassy et al., 2013b).

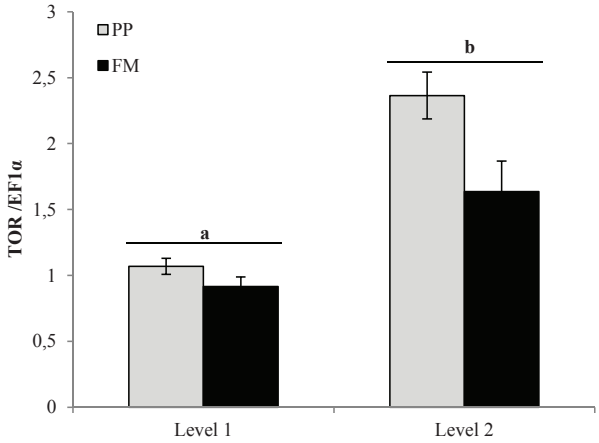

Fig. 3: Effect of methionine dietary level in plant protein based (PP) or fish meal based diet (FM) on the relative expression of TOR in rainbow trout hepatocytes. Methionine levels were reached by crystalline supplementation in the plant based diet or by increasing the fish meal content in the fish meal based diet. Level 1 corresponds to 0.94±0.04 % DM and level 2 to 1.29±0.01 % DM. The results marked with different letters differ significantly (two way ANOVA, *P*<0.05)

Amino acids are, through activation of the TOR pathway, likely to be major factors responsible for the up-regulation of protein synthesis after ingestion of a meal (McMillan and Houlihan, 1988). However, a description of the molecular mechanism sensing amino acid availability and responsible for activating the pathways at the cellular level is still scarce (Taylor, 2014).

3.3. Regulation of growth by amino acids: measurement of production variables and molecular mechanism

Determining the dietary effect on growth in commercial species is commonly achieved by measuring classic production variables related to growth efficiency (e.g. weight gain, specific growth rate or thermal growth coefficient), feed and nutrient utilization (e.g. feed conversion ratio, protein efficiency, nitrogen retention). In this way, during growth trials the final effect of raw materials, nutrient composition, or single nutrients on general performances can be estimated. However, the molecular mechanisms determining the metabolic response are rarely investigated simultaneously, and often remain unexplained. In addition, the conventional approach considers nutrients as substrates for molecule synthesis or energy production, and rarely accounts for their role as signalling factors.

In commercial species, such as salmonids, changes in the expression of the genes involve in the multiple, complex mechanisms responsible for growth provide valuable information on the pathways accounting for the results commonly measured with classic production variables. Description of the molecular pathways

triggered by dietary factors, gives additional understanding on the mechanisms involved to achieved growth and optimal feed utilization.

3.3.1. Regulation of the somatotropic axis as a major factor of growth

The somatotropic axis is an important regulator of growth, through stimulating muscle development and protein synthesis, while inhibiting protein degradation (Fuentes et al., 2013). The general organization of the somatotropic axis includes growth hormones (GH) secreted by the pituitary gland, the growth hormone receptors (GHRs), insulin-like growth factors (IGF-I and IGF-II), IGF receptors and IGF-binding proteins (IGFBPs). GHRs are responsible for transducing the signal from circulating GH into the cells (Gabillard et al., 2006). In the liver of rainbow trout, two sequences coding for GHR were found: GHR-1 and GH-2, shown to be differently regulated by temperature and nutritional status (Gabillard et al., 2006). IGFs are expressed in several organs, yet the main site for IGF-I synthesis is the liver (Reinecke, 2006). IGFs are polypeptide hormones, structurally similar to insulin and to some extent exert insulin-like effects. The IGF-I and IGF-II have50 % similarities in their sequences, but their physiological roles differ, as well as their expression pattern depending on tissue. IGFs have autocrine, paracrine and endocrine effects, the latter being modulated by binding's protein, IGFBPs. Indeed, the circulating IGFs are principally bound to IGFBPs, and less than 1% of the plasma IGF-I are present in the free form (Reinecke, 2006). So far six binding proteins (-1, -2, -3, -4, -5 and -6) have been identified in fish (Kelley et al., 2006) differing, among other things in their molecular size (measured in Dalton) and affinities to IGF-I and IGF-II (Reinecke, 2006).

The regulation of the somatotropic axis relies in part on complex interactions between its components that may either exert positive or negative feedback on each other. For example, circulating GH, enhances the hepatic expression and plasma level of IGF-I in a dose-dependent manner (Funkenstein et al., 1989; Niu et al., 1993; Duan et al., 1993), and has been found to correlate with growth rates in several fish species (Reinecke and Collet, 1998). GHR might take part in integrating the changes of GH plasma levels, and participating in the regulation of IGF-I (Saera-Vila et al., 2007; **Paper III**). Conversely, circulating IGF-I ensure a negative feedback control on GH secretion (Pérez-Sánchez et al., 1992). In fish growth performance and feed efficiency are often correlated with plasma IGF-I concentrations (Reinecke and Collet, 1998; Cleveland and Burr, 2011), likely liver-derived (Snyder et al., 2012). Gómez-Requeni et al. (2005) further proposed that the reduced growth rate measured in their trial with rainbow trout could be due to lower availability of free fraction of IGF-I, but rather to total plasma content; supporting the fact that IGFBPs modulate the regulatory role of IGFs (Reinecke, 2006).

In fish as in mammals, the GH-IGF axis is also regulated by the nutritional status (Renaville et al., 2002; Chauvigné et al., 2003). The expression of gene involve in the GH-IGF axis and their plasma levels were shown to be affected by feeding ration (Pérez-Sánchez et al., 1995; Metón et al., 2000), diet composition (Metón et al., 2000) and raw materials (Gómez-Requeni et al., 2005); reflecting growth performance results.

In a study on Atlantic salmon fed diets with different lysine levels (low, medium and high), Hevrøy et al. (2007) showed that the hepatic expression of genes involved in the GH-IGF axis (GHR, IGF-I and IGFBP-1) were up-regulated in individuals fed the high-lysine diet compare to the fish eating a deficient feed. IGF-I plasma levels were not affected, yet still growth performances were improved. Dietary methionine levels were shown to have limited effect on hepatic expression of GHR, IGF-I and IGFBP-1in Atlantic salmon (Espe et al., 2008).

Table 3: Performance results for fish fed the plant based (PP) or fish meal based (FM) diets containing two levels (1 and 2) of methionine (0.94±0.04 % DM and 1.29±0.01 % DM, respectively)

Diet	PP 1	PP 2	FM 1	FM 2
Apparent digestibility				
Protein	92.29±0.25	92.15±0.64	90.25±0.25	88.51±0.29
Methionine	95.43±0.52	96.53±0.29	94.27±0.89	94.83±0.28
Performance parameters				
Initial weight (kg)	8.34±0.36	8.12±0.43	8.11±0.34	8.05±0.34
Final weight (kg)	17.52±0.44	15.81±1.26	23.15±1.03	22.75±1.29
Weight gain (kg)	9.18±0.64	7.69±0.86	15.04±0.77	14.69±1.03
Feed intake (kg)	7.89±0.54	6.56±0.67	13.71±0.61	12.80±1.09
Feed intake (% biomass)	1.86±0.14	1.65±0.08	2.79±0.05	2.68±0.12
Specific growth rate	2.01±0.15	1.80±0.09	2.84±0.06	2.80±0.09
Feed conversion ratio (g/g feed)	0.86±0.01	0.85±0.01	0.91±0.01	0.87±0.01
Protein efficiency ratio (g/g crude PI)	2.78±0.02	2.81±0.03	3.08±0.02	2.84±0.05
Energy efficiency ratio (g / J)	50.6±0.4	50.5±0.5	51.0±0.4	50.4±0.8

In contrast, changes in the expression of GHR-I, IGF-I, IGFBP-1 in rainbow trout were induced by increasing dietary methionine or by changing the raw material composition (**Paper III**; Fig. 4). Hence, the diet presenting the lowest growth performances after six weeks (Table 3) of feeding also resulted in the lowest levels of mRNA transcript for GHR-I, IGF-I and IGFBP-1. Gradual increase of dietary methionine, to and above requirement levels, led to increased hepatic expression of GHR-I, IGF-I and IGFBP-1.

The simultaneous increased in IGF-I and IGFBP-1 with dietary methionine level, could result in higher total plasma content of IGF-I, however not necessarily in free form (Gómez-Requeni et al., 2005; Hevrøy et al., 2007). This could explain why neither the expression level of IGF-I nor IGFBP-1 was correlated with SGR, while that of GHR-I was. The GH-IGF axis is a major factor of growth that modulates the muscle development and protein accretion (Fuentes et al., 2013). The regulation of the somatotropic axis by amino acid availability could partially explain differences in growth performances and feed utilization in response to changes in dietary amino acid level and form (Fig. 4). The results further suggest that the components are differently regulated by dietary changes, at least at the transcriptional levels, supporting the existence of complex functions and relationship.

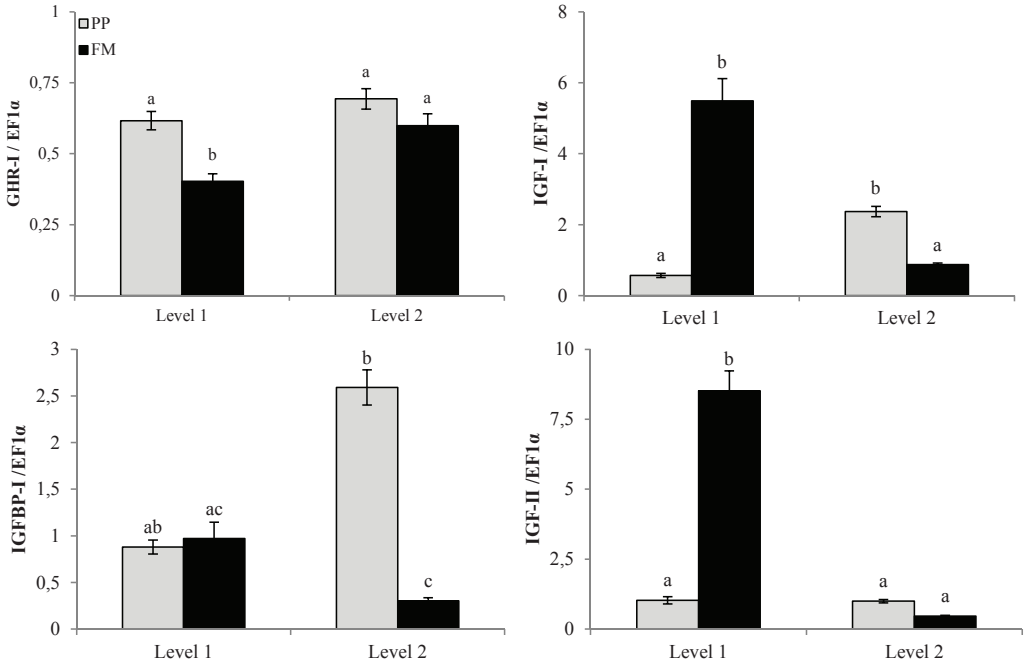

Fig. 4: Effect of dietary methionine supplied at two levels in plant protein based (PP) or fish meal based (FM) diets on the hepatic relative expression of GH-R, IGF-I, IGF-II and IGFBP-1. Methionine levels were reached by crystalline supplementation in the plant based diet or by increasing the fish meal content in the fish meal based diet. Level 1 corresponds to 0.94±0.04 % DM and level 2 to 1.29±0.01 % DM. The results marked with different letters differ significantly (one way ANOVA test, *P*<0.05)

3.3.2. Regulation of protein turnover by amino acids

The continuous process of protein synthesis and degradation in cells is termed intracellular protein turnover (Wu, 2009). Protein synthesis is the process of building endogenous protein using the pool of free amino acids. In contrast to other nutrients (lipids and glucose) there is no internal storage of amino acids *per se* as the primary function for deposition protein is growth and not storage of amino acids (Brosnan, 2003). The degradation of endogenous protein to obtain free amino acids is done at the expense of the organism. Indeed, proteins are the most expensive molecules to synthetize (Smith et al., 1999), and for a growing fish the cost is estimated to range between 11 to 24 % of the energy expenditure at basal metabolism, and for as much as 42 % of the metabolic cost of digestion (Wood, 2001). In addition, as proteins are essential components in the body, they require a continuous, expensive maintenance process (constantly synthetized and degraded, i.e. protein turnover). As suggested by the ability of amino acids to regulate protein synthesis through molecular pathways (Kimball and Jefferson, 2006), the lack of one amino acid does not stop protein synthesis solely due to the impossibility of completing the sequence but also by down-regulating of the

process. The existence of up-front controls, preventing the start of protein synthesis that could not be achieved appears logical, especially when considering the energetic cost of the process.

Amino acids, in addition to modulating protein synthesis through the TOR pathway, are able to affect protein degradation pathways at the transcriptional level (**Paper III**). Protein degradation occurs through four pathways: i) ubiquitin targeted digestion of protein by proteasome, ii) transport to lysosomes and digestion through the action of cathepsin, iii) apoptosis and digestion following the caspase cascade and finally, iv) digestion through the use of calpains (Capn), i.e. non-lysosomal intracellular calcium dependent proteases (Overturf and Gaylord, 2009; Cleveland and Burr, 2011). Protein degradation has been shown to be the predominant pathway influencing protein deposition in humans (Fereday et al., 1998) but also in rainbow trout (Cleveland and Burr, 2011). In an experiment comparing three rainbow trout strain, Salem et al. (2005) showed the fastest growth rate was achieved by the family presenting the highest expression of calpastatin long and short sequences (CAST-L and -S, respectively), inhibitor Capn proteases, in the muscle while no differences in the expression of the proteases was measured. This supports that the protein degradation and its regulation are important factors of growth.

White muscle is the largest reservoir of the body proteins, i.e. muscle proteins represent 50% of the body protein in juvenile rainbow trout (Ballantyne, 2001). Protein synthesis rate in the muscle is much lower than in the liver, reflecting the different roles of these organs, the latter being central for metabolism (Carter and Houlihan, 2001). However white muscle retains protein far more efficiently than other tissues, e.g. 60 % compared to less than 10% for liver as shown by Houlihan et al. (1988). Growth achieved by protein accretion in the muscle is a factor of both synthesis and degradation, largely under the control of the nutritional status of the animal.

In rainbow trout under starvation, protein synthesis rates decrease and proteolysis is enhanced by increased protease activities and a concomitant decrease in their inhibition (Salem et al., 2007). The mobilization of endogenous proteins to maintain the free amino acid pool appeared to occur by a synchronized down-regulation of protein biosynthesis and increased proteolytic activities, at least of the calpain cascade and ubiquitin pathway. Conversely, feeding enhances protein synthesis; less than two hours after meal ingestion, a peak of protein synthesis was registered in rainbow trout hepatic cells (McMillan and Houlihan, 1989). Nutritional factors, such as ration and raw material origin, also affect protein deposition through differential regulation of the degradation pathways in rainbow trout (Cleveland and Burr, 2011; Snyder et al., 2012). **Paper III** shows that the transcription of specific genes related to hepatic protein degradation (proteasome 20 delta, Capn 1, CAST-L and CAST-S) was largely correlated to dietary methionine level. However, no information is available on the muscle protein turnover even though, amino acids, in association with IGF-I modulate protein synthesis and degradation through the TOR pathways (Kadowaki and Kanazawa, 2003;

Fuentes et al., 2013). Gradual increase of dietary methionine content induced a significant increase in hepatic expression of TOR conjointly with an improved growth and protein utilization in rainbow trout (**Paper III**).

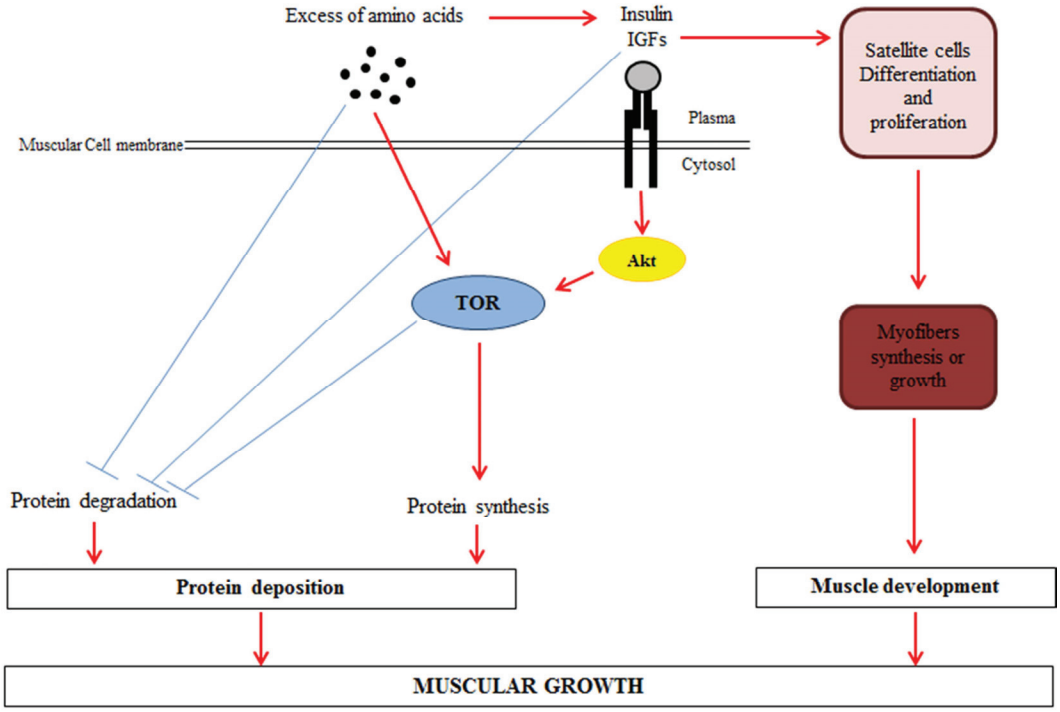

Fig. 5: Schematic summary of muscular growth regulation by amino acids though protein deposition (decreased degradation and increased synthesis) and muscle development. Insulin or Insulin-like growth factors (IGFs) together with amino acids activate the target of rapamycin (TOR) pathway which in turn stimulate protein synthesis and inhibit protein degradation. Additionally, IGFs activate satellite cell recruitment and differentiation into myocytes. Myocytes will then fuse together to create new fibres (hyperplasia) or fuse with already existing myofibers (hypertrophy), resulting in muscular growth

There is increasing evidence that amino acids are able to modulate growth, not only because they are precursors of protein synthesis, but also because they are able to regulate major functions though nutrient sensitive pathways. Amino acids can be utilized as an energy source, or their carbon skeletons can also serve for *de novo* synthetize of glucose and lipid depending on whether there are glucogenic or ketogenic amino acids.

3.4. Adaptation of intermediary metabolism to dietary changes: regulation of gene expression by amino acid and measurement of metabolic indicators

The intermediary metabolism accounts for all the reactions required in the synthesis of cellular components from nutrients and its regulation is closely related to the availability of nutrients and energy, i.e. dependent on both nutritional status and overall dietary nutrient composition (Seiliez et al., 2011; Skiba-Cassy et al., 2013a). Amino acid catabolism is a highly integrated part of the intermediary metabolism in the production of energy (ATP), metabolic intermediates (pyruvate, acetyl-CoA, α-ketoglutarate, oxaloacetate), reductive power (NADPH) and results in the excretion of nitrogenous wastes. The liver is the main organ for amino acid catabolism, reflected by the high protein turnover, large ammonia production (50-70 % of the total ammonia), and the large free amino acid pool (Van der Thillart and Van Raaij, 1995). Ammonia and urea are the two main nitrogen compounds (approximately 60 and 30 % of the total nitrogen, respectively) excreted by fish (Kajimura et al., 2004). As a first step of catabolism, amino acids undergo a deamination: removal of the amino group which is either converted into ammonia or transferred to a keto acid.

$AA_1 + \alpha\text{-ketoA}_2 \rightarrow \alpha\text{-ketoA}_1 + AA_2$

The resulting α-keto acids may be utilized as an energy source after conversion in the citric acid cycle (TCA). The point of entry into the TCA cycle depends on the amino acids (Fig. 6). The TCA cycle lead to production of reductive power (such as $NADPH+H^+$ or $FADH_2$, responsible for carrying electrons from one reaction to another) and molecules of ATP, used to transfer cellular energy. The TCA cycle is a major crossroads of the metabolism, producing numerous intermediates further used in nutrient synthesis, e.g. malate used for glucose synthesis. As a result, the TCA links the metabolism of lipid, glucose and amino acids together and to energy production.

The enzymatic machinery involved in amino acid catabolism is quite comprehensive, and often measurements of the transcription of only a few specific enzymes is used to investigate the metabolic status in response to dietary changes. The enzyme glutamate dehydrogenase (GDH) is part of the major amino acid catabolic pathways, where deamination of amino acids leads to formation of glutamate, further deaminated by GDH to produce α-ketoglutarate (intermediate of the TCA cycle; Fig. 6). Alanine aminotransferase (ALT1) catalyses the synthesis of pyruvate from alanine. Through this reaction, alanine is an important carbon donor for glucose synthesis (Gonzáles et al., 2012). Aspartate aminotransferase (GOT2) is another transaminase using aspartate and α-ketoglutarate to produce oxaloacetate (intermediate of the TCA cycle). Finally, glutamine synthetase 1 and 2 (GLS01 and GLS02, respectively) eliminate free ammonia by producing glutamine from glutamate.

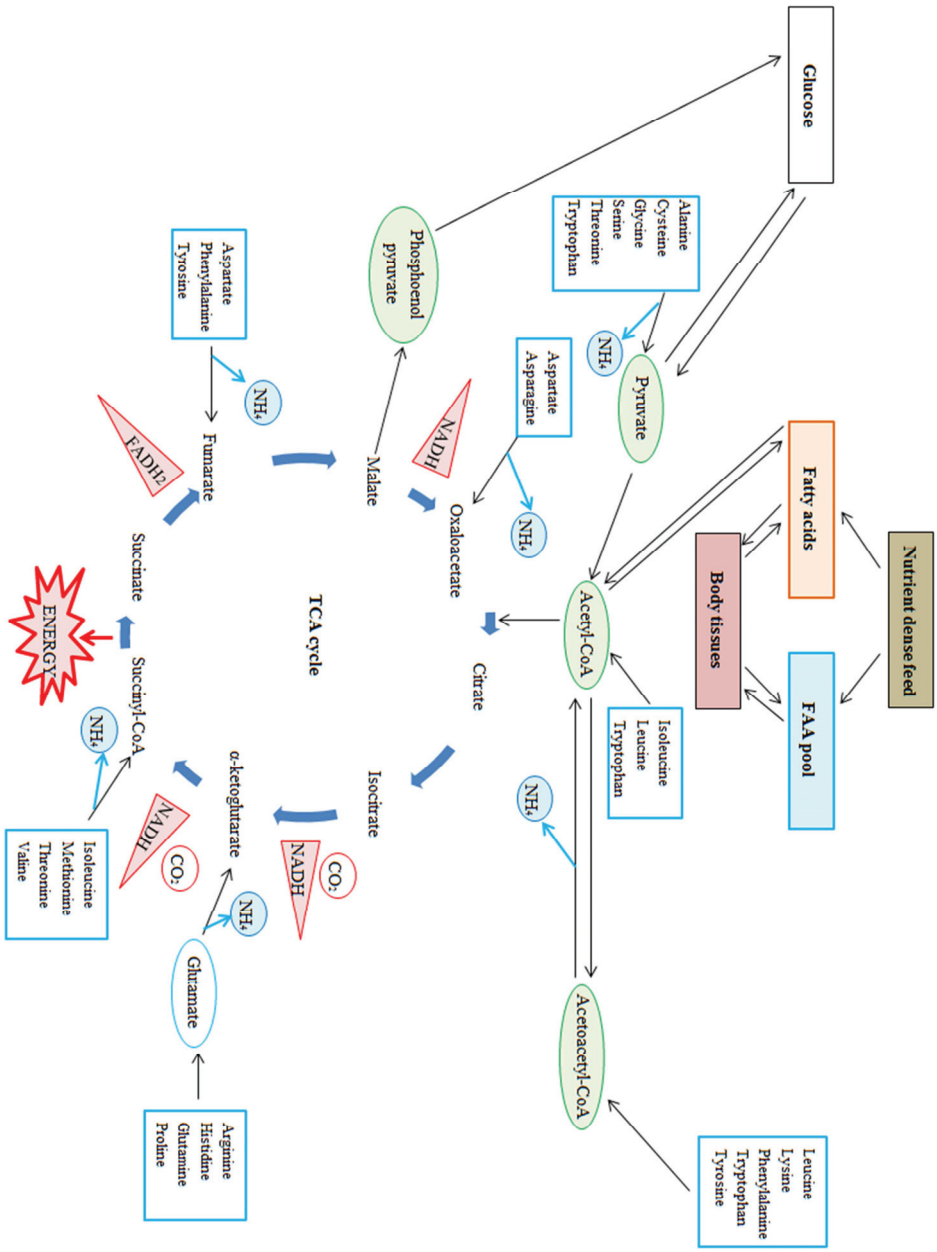

Fig. 6: Simplified scheme of the intermediary metabolism. In blue: amino acids and nitrogen molecules; in green: metabolic intermediates; in red: energy and reductive power

3.4.1. Dietary factors affecting the utilization of amino acids for energy production

Carnivorous fish have a high dietary requirement for protein compare to other vertebrates but are highly efficient at utilizing dietary protein for growth (Bowen, 1987). During digestion protein synthesis rates increase to promote the utilization of amino acid in anabolic functions (McMillan and Houlihan, 1989). However the large amino acid intake also leads to increased catabolism and ammonia excretion (Kajimura et al., 2004; **Paper I**, **IV**; Fig. 7). In rainbow trout fed to satiation on an amino acid balanced diet, 50 to 70 % of the energy required for metabolism is provided by amino acid catabolism, while only 14 to 30 % of the energy is derived from amino acid oxidation in resting, non-fed fish. In the latter group, lipids represent the main fuel (35-68 %; Wood, 2001).

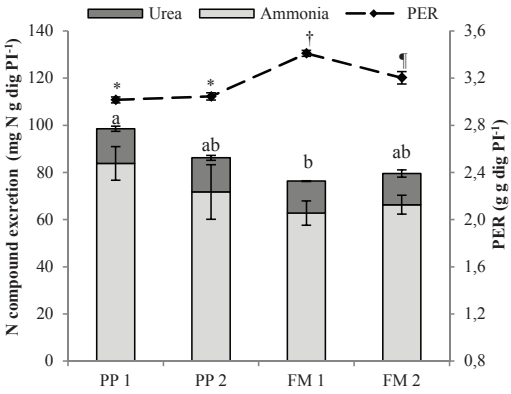

Fig. 7: Nitrogen excretion and protein efficiency ratio (PER) calculated per digestible protein intake (dig PI) for diets based on plant protein (PP) or fish meal (FM). Methionine levels were reached by supplementing crystalline in the PP diets or by increasing the fish meal content in the FM diets. Level 1 corresponds to 0.94±0.04 % DM and level 2 to 1.29±0.01 % DM. Different letters indicate significant changes in the nitrogen excretion (sum of ammonia and urea) and PER marked by different symbols differ significantly (one way ANOVA, $P<0.05$)

The gene expression profile of hepatic cells in rainbow trout under feeding conditions reflect a switch from lipid to amino acid utilization for energy production compare to fish undergoing a fasting period (Seiliez et al., 2011). In addition, the expression of genes encoding for enzymes participating in the amino acid catabolism appears to be linked to the protein content of the diet (Seiliez et al., 2011; Skiba-Cassy et al., 2013a) but also its amino acid profile (Skiba-Cassy et al., 2013b; **Paper IV**).

Rainbow trout fed unbalanced diets in terms of EAA requirement, typically have an increased amino acid catabolism, resulting in lower nitrogen retention and higher ammonia excretion (Green and Hardy, 2008; **Paper I**, **IV**). Interestingly, the hepatic expression of genes coding for enzymes of the amino acid catabolism did not reflect the amino acid utilization in response to changes in dietary methionine levels (Skiba-Cassy et al., 2013b; **Paper IV**) no matter the raw material matrix (Fig. 8). The expression of ALT1 and GDH seemed to respond positively to methionine intake, not reflecting the results on ammonia excretion (Fig. 7 and Fig. 8).These results reflect the complexity of amino acid catabolism and the factors involved in its regulation. In addition, metabolic intermediates derived from the breakdown of amino acid are also utilized for *de novo* lipogenesis or gluconeogenesis.

Amino acids can be divided in two groups, ketogenic or glucogenic, depending on their ability to serve as precursors of lipid or glucose synthesis respectively (Fig. 6). Leucine and lysine are the only two amino acids strictly ketogenic, i.e. their degradation do not lead to net glucose production. Carnivorous fish typically have a low dietary requirement for carbohydrate even though glucose is an essential nutrient for several tissues (e.g. central nervous system).

3.4.2. Regulation of *de novo* synthesis of glucose by amino acids

Carnivorous fish have a limited ability to utilize dietary carbohydrates, likely due to an inadequate regulation rather than inexistent and/or inactive uptake or metabolic pathways (Polakof et al., 2010). As a result, fish present a prolonged hyperglycaemia after ingestion of a diet rich in carbohydrates (Enes et al., 2009) despite the presence of insulin (Seiliez et al., 2008).

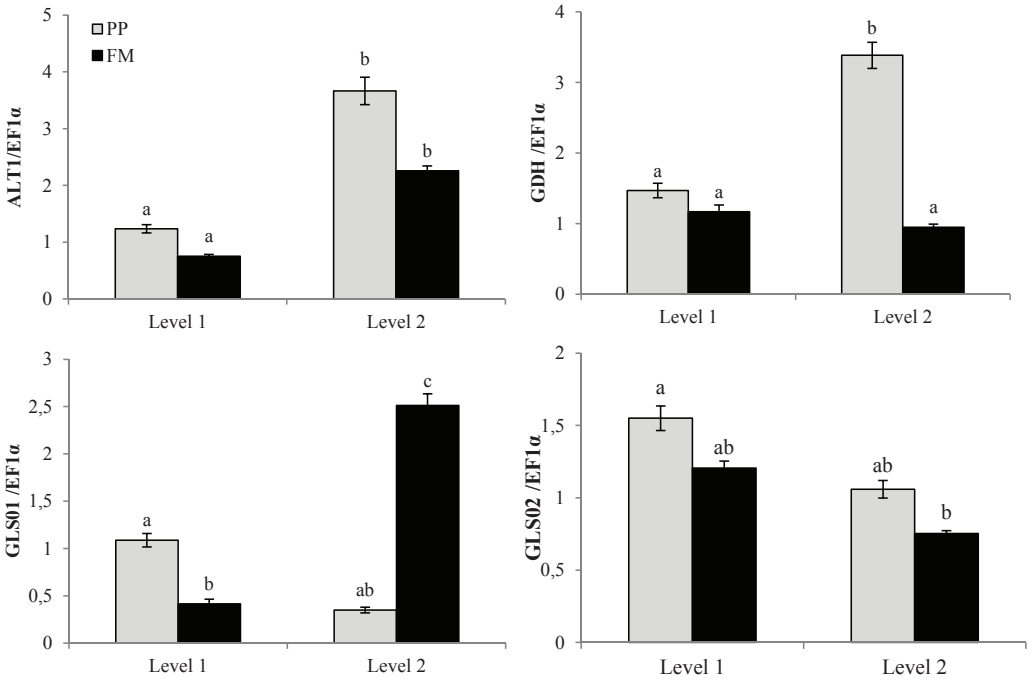

Fig. 8: Relative gene expression coding for hepatic enzymes related amino acid catabolism: alanine amino transferase (ALT1), glutamate dehydrogenase (GDH), glutamine synthase 1 and 2 (GLS01 and 2 respectively) given for diets based on plant protein (PP) or fish meal (FM). Methionine levels were reached by supplementing crystalline in the PP diets or by increasing the fish meal content in the FM diets. Level 1 corresponds to 0.94±0.04 % DM and level 2 to 1.29±0.01 % DM. The results marked with different letters differ significantly (one way ANOVA test, *P*<0.05)

In teleost fish, glucose has a low insulinotropic effect (compared to several amino acids; Andoh, 2007), which in turn has a mild regulating effect on plasma levels of glucose. As a result, tissues and organs have a lower capacity to take up dietary glucose in comparison to other vertebrates (Enes et al., 2009). Glucose metabolism seems to rely strongly on endogenous, *de novo* synthesis from pyruvate (two pyruvates to produce one molecule of glucose). The gluconeogenesis pathway is regulated by the activity of the key enzymes phosphoenolpyruvate carboxylase (PEPCK), fructose-1,6-biphosphatase (FBPase) and glucose-6-phosphatase (G6Pase) catabolizing three different steps (Skiba-Cassy et al., 2012; Fig. 9).

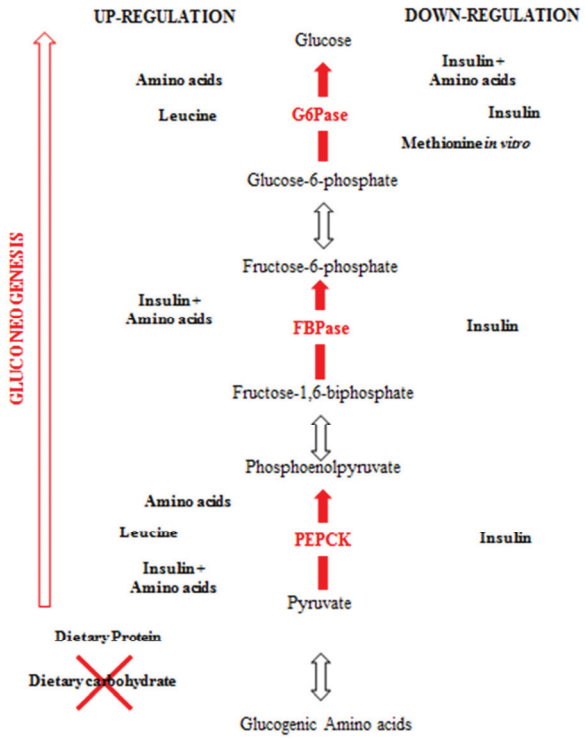

Fig. 9: Major steps of the glucose synthesis (gluconeogenesis) pathway, presented with intermediates, key enzymes (red font) and regulators (bold). PEPCK: phosphoenolpyruvate carboxylase; FBPase: fructose-1,6-biphosphatase (FBPase); G6Pase: glucose-6-phosphatase (from results published by Lansard et al., 2010, 2011 and Paper IV)

Nutritional status and dietary composition are able to regulate hepatic glucose metabolism. In fasting periods the net utilization of glucose as energy source is reduced, by down-regulated glycolysis (degradation of glucose into pyruvate; Dai et al., 2013). Under these conditions carbohydrates still fuel a significant share of the metabolism, as the overall energy requirement is reduced compare to a digestive state (Wood, 2001). In contrast glucose appears to be constantly synthetized, as reflected by the high expression levels of PEPCK

and FBPase no matter the nutritional status (fed *vs.* fasting) or the dietary carbohydrate content (Skiba-Cassy et al., 2012). In rainbow trout, the regulation of gluconeogenesis is controlled by protein intake (Kirchner et al., 2003a) and/or individual amino acids, supporting the importance of amino acids as substrates to provide glucose to the organism. The protein content of a diet has been shown to significantly affect PEPCK expression (Skiba-Cassy et al., 2013a) likely through the action of individual amino acids, with agonist hormonal signal (Lansard et al., 2011). Similar to PEPCK, the expression of FBPase is down-regulated when dietary protein content decreases (Kirchner et al., 2003a), and under the control of amino acid (Kirchner et al., 2003b), likely in concert with insulin (Lansard et al., 2010). G6Pase is known to catabolize the last step of gluconeogenesis, and protein and amino acids intake have been shown to induce its transcription (Kirchner et al., 2003b; Lansard et al., 2010). Taken together, these results suggest that fish do not rely on dietary glucose, but rather appear to be dependent on glucose synthetized *de novo* from amino acids derived from digestion of proteins.

Furthermore, *in vitro* studies of rainbow trout hepatic cells have revealed that gluconeogenesis is differentially regulated by individual amino acids and their concentrations. Using culture cells, Lansard et al. (2011) showed that single amino acids have different abilities to regulate genes expression of the gluconeogenesis in rainbow trout hepatocytes. Leucine alone was able to regulate the transcript level of PEPCK and G6Pase to a similar extent as an amino acid mixture. In contrast, methionine and lysine required the addition of insulin to mimic the effect of the EAA mixture of gene expression of glucogenic enzymes (Fig. 9).

In a similar experimental design, the hepatic gene expression of PEPCK and G6Pase responded in a dose-dependent manner to increasing concentrations of a mixture of amino acids (1-fold and 4-fold) compare to an untreated culture (Lansard et al., 2010). Conversely mRNA levels of FBPase were not affected, likely due to the absence of insulin. **Paper IV** showed that *in vivo*, increasing levels of methionine in rainbow trout diets up-regulated the gene expression of FBPase while the mRNA level of both PEPCK and G6Pase were unaffected by the dietary treatments. The effect on the overall gluconeogenic capacity remains uncertain, however, a linear response of FBPase expression to dietary methionine level suggests that methionine is able to regulate the expression of key enzymes in *de novo* glucose synthesis. Methionine can be used as substrate in the gluconeogenic pathways directly, or indirectly as a precursor for cysteine synthesis, which in turn is utilized to produce pyruvate (Fig. 6 and Fig. 9).

Further results showed that depending on the raw material composition (plant protein or fish meal), the gene expression of PEPCK, FBPase and G6Pase were differently regulated (Fig. 10). Rainbow trout fed six weeks with diets supplemented with methionine to requirement (0.94±0.04 % DM), either as crystalline methionine (PP 1) or by incorporating fish meal (FM 1) showed no significant differences in their gene expression levels. Addition of fish meal, and therefore methionine to the diet in excess of requirement (1.29±0.01%

DM) induced an apparent increase of gluconeogenesis capacity (FM 2), while the effect was opposite when methionine was added as crystalline (PP 2; Fig. 10). At higher methionine level, the diet composition had an opposite effect on the gene expression of gluconeogenic enzymes depending on the protein source (Fig. 10). These results support the fact that excess of methionine alters the capacity for gluconeogenesis and suggest that other factors present in the raw materials modulate the pathway.

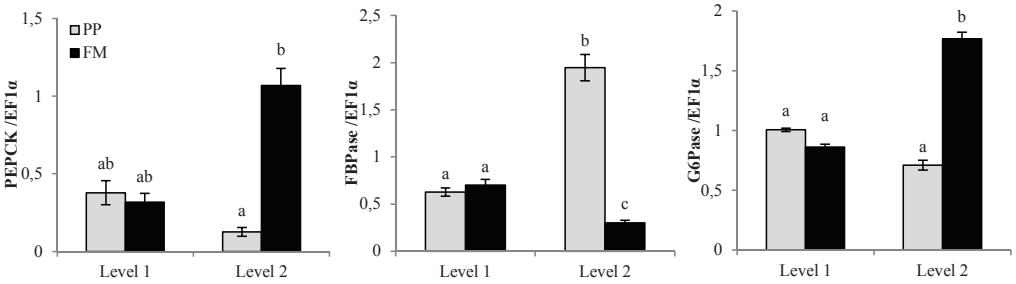

Fig. 10: Relative gene expression of the key enzyme of gluconeogenesis of fish fed diets based on plant protein (PP) or fish meal (FM). Methionine levels were reached by supplementing crystalline in the PP diets or by increasing the fish meal content in the FM diets. Level 1 corresponds to 0.94±0.04 % DM and level 2 to 1.29±0.01 % DM. The results marked with different letters differ significantly (one way ANOVA test, *P*<0.05)

The constant expression of gene related to *de novo* glucose synthesis and the lack of regulation by the nutritional status in rainbow trout (Skiba-Cassy et al., 2012) suggests that gluconeogenesis is an important pathway to provide glucose to the organism. Dietary carbohydrate intake has no effect on regulating gluconeogensis, while individual amino acids are able to regulate the transcription of key glucogenic enzymes (Skiba-Cassy et al., 2012), supporting their important role as carbon donors for endogenous glucose production. Amino acids are also precursors for the synthesis of metabolic intermediates, further utilized for lipid synthesis. Similarly to that of gluconeogenesis, amino acids act as regulation factors at the expression of several genes coding for enzymes involved in lipid metabolism (Lansard et al., 2010, 2011).

3.4.3 Regulation of lipid metabolism

Lipid is the ideal energy storage unit; it has a high energy content per mole, a low mass density due to the fact that lipid does not bind to water. Fish are able to synthesize lipid though *de novo* lipogenesis (Tocher, 2003). *De novo* lipid synthesis is achieved by converting acetyl-CoA (two carbons) into fatty acid using reducing power (NADPH) and subsequent addition of malonyl-CoA units (obtained from acetyl-CoA carboxylation; three carbons) :

$$\text{Acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH} + 14\ H^{+} \longrightarrow \text{16:0 fatty acid} + 7\ CO_2 + 8\ \text{CoA} + 14\ NADP^{+} + 6\ H_2O$$

Acetyl CoA can be obtained from catabolism of either amino acids or carbohydrates. The breakdown of five specific amino acids leads directly to acetyl CoA synthesis: leucine, lysine, phenylalanine, tyrosine and

tryptophan. Pyruvate originating from amino acids (alanine, cysteine, glycine, serine and threonine) or glycolysis of glucose is used for acetyl CoA formation. In addition, lipid synthesis has a high requirement for reductive power (see equation above), which can be obtained in three processes: i) oxidation step of the pentose phosphate pathway catabolized by glucose-6-phosphate dehydrogenase (G6PD), ii) oxidation of malate to pyruvate by malate dehydrogenase, and iii) through the TCA cycle (Fig. 6).

The enzyme fatty acid synthase (FAS) catalyses the successive steps of the lipogenesis and is highly expressed in lipogenic tissues, i.e. the liver and adipose tissue depending on the feeding status (Lansard et al., 2009; Skiba-Cassy et al., 2009). The dietary composition, e.g. raw material origin (Panserat et al., 2008; Lansard et al., 2009), carbohydrate:protein ratio (Seiliez et al., 2011) or amino acid profile (Espe et al., 2010), affects hepatic gene expression and/or activity of FAS in different manners. Nevertheless, single amino acids do not have a similar effect on stimulating of FAS transcription; leucine is able to mimic the regulation of a pool of amino acids while lysine and methionine only has a moderate although significant effect (Lansard et al., 2011). The regulation of FAS transcription by amino acids associated with insulin is achieved through the TOR pathway (Lansard et al., 2010, 2011; Dai et al., 2013).

G6PD is another enzyme of interest, catalysing a reaction resulting in reductive power synthesis (NADPH), an indispensable cofactor of *de novo* lipid synthesis. In rainbow trout, feeding induced G6PD expression (Skiba-Cassy et al., 2009, 2013a) compare to fasting individuals similarly to that of FAS. However, the gene expression of G6PD measured in rainbow trout fed diet with different levels of crystalline methionine did not reflect the transcription levels of FAS (negatively correlated to dietary methionine content) nor the differences in HSI measurements (**Paper IV**). Individuals fed adequate or excess levels of methionine presented a significant higher expression of G6PD compare to fish fed deficient diets. The results suggest that the mechanism regulating the expression of FAS and G6PD are differently modulated by dietary amino acid intake. In fish, as in mammals, the expression of FAS is induced by insulin and amino acid through the TOR pathway (Skiba-Cassy et al., 2009; Lansard et al., 2010; Dai et al., 2013). Although insulin infusion induced the expression of G6PD in the liver of rainbow trout (Polakof et al., 2010), its regulation of the expression is likely independent of TOR (Dai et al., 2013).

In an opposite process of lipogenesis, endogenous fatty acids are catabolized to provide energy, resulting in the synthesis of acetyl CoA and reductive power in a reaction named β-oxidation. The carnitine palmitoyltransferase 1 isoforms a and b (CPT1a and CPT1 b, respectively) are major enzymes in lipid oxidation, and respond to nutritional status and dietary changes as a mean of metabolic adaptation. Under fasting conditions or low dietary protein intake, lipid oxidation is up-regulated likely to provide energy for maintenance and metabolism (Skiba-Cassy et al., 2013a). The expression of CPT1 was higher in fasted rainbow trout compared to a re-fed group, and down-regulated by increasing dietary protein content (Seiliez et al., 2011; Skiba-Cassy et al., 2013a), likely though TOR (Dai et al., 2013). In general, published results

support the fact that under conditions of protein limitation or deprivation, energy production to support metabolism relies on endogenous lipid mobilization.

Amino acids are important substrates for lipid synthesis (Tocher, 2003) and the dietary amino acid profile is also able to influence lipid metabolism. Salmonids fed diets deficient in methionine presented an increased hepatic capacity for lipid synthesis and no changes the apparent oxidation (Espe et al., 2010; **Paper IV**). Hepatic FAS activity and gene expression were induced by the unbalanced methionine supplementation in salmon and trout, respectively (Espe et al., 2010; **Paper IV**), both resulting in increased HSI. In salmonids as in other fish, the liver is an important site of lipid synthesis and export (Tocher 2003). Lipid synthesis, for energy storage, appears to be the preferred strategy to utilize excess amino acid resulting from a limiting single EAA. Interestingly, gradual increase of dietary methionine content (from deficient to excess) lead to simultaneous decrease of FAS gene expression, reflected in HSI results and nitrogen excretion patterns during digestion in rainbow trout (**Paper IV**). In addition, a large excess of dietary methionine (1.29 % dry matter) induces hepatic gene expression of CPT1a without affecting feed utilization (FCR, PER and EER; **Paper III**) in comparison to fish receiving adequate dietary methionine level. The results suggest that feeding rainbow trout with an excess of crystalline methionine in a plant based diet might enhance protein deposition, endogenous lipid mobilization for energy production, and decrease lipogenesis.

Feeding rainbow trout with an excess of dietary methionine resulted in different expression patterns of genes involved in lipid synthesis depending on the raw material matrix. Individuals fed plant based diet supplemented with crystalline methionine above requirement showed a significant reduction in FAS expression while the fish fed fish meal based diets had lower transcript levels of G6PD (Fig. 11).

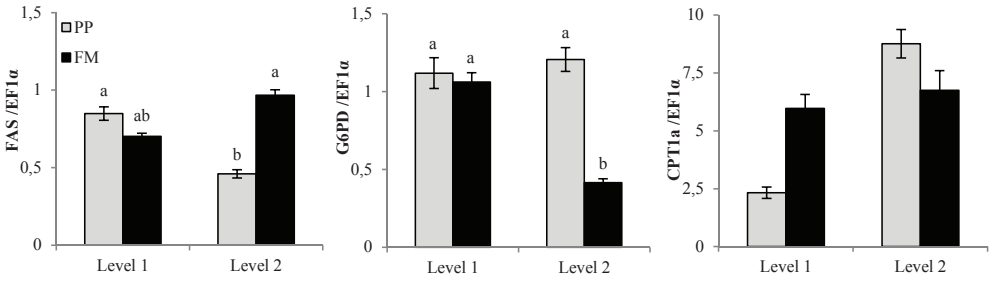

Fig. 11: Relative expression of gene coding for enzyme involved in lipid metabolism in fish fed diets based on plant protein (PP) or fish meal (FM). Methionine levels were reached by supplementing crystalline in the PP diets or by increasing the fish meal content in the FM diets. Level 1 corresponds to 0.94±0.04 % DM and level 2 to 1.29±0.01 % DM. The results marked with different letters differ significantly (one way ANOVA test, $P<0.05$)

The general lack of differences in the gene expression profiles between fish fed adequate level of methionine either as crystalline or protein-bound form could be related to the sampling time. Gene expression of enzymes involved in the intermediary metabolism changes during the digestion (Skiba-Cassy et al., 2013a,b),

in likelihood as a result in variation of amino acid availability. The significant effect of dietary methionine level and form on the relative expression of several genes related to amino acid catabolism, gluconeogenesis and lipid metabolism was believed to be related, at least in part to the observed variations in plasma concentrations. In **Paper IV**, postprandial plasma methionine levels and gene expression were measured on different individuals and at different time, at 20 h and 24 h after meal ingestion, respectively. However, in a concern to push forward the understanding on the methionine form effect, the expression of the genes related to intermediary metabolism were correlated to the average plasma methionine concentrations obtained in **Paper IV** (Table 2).

Table 4: Results of Pearson's correlation test of relative gene expression in rainbow trout fed four diets containing either plant protein and crystalline or fish meal supplemented at 0.94±0.04 or 1.29±0.01 % dry matter with i) dietary methionine level and ii) plasma methionine concentration 20 h after meal ingestion (nmol ml^{-1}). Significant correlation (P<0.05) are marked in bold

	vs. Dietary methionine (% DM)		Significant correlation with dietary crystalline methionine (**Paper IV**)	*vs*. Plasma methionine concentrations (nmol ml-1)	
	r	p-value		r	p-value
Amino acid catabolism					
ALT1	0.667	**<0.001**	**yes**	0.668	**<0.001**
GDH	0.330	0.099	**yes**	0.736	**<0.001**
GOT2	0.170	0.416	no	0.223	0.284
GLS01	0.277	0.180	**yes**	-0.312	0.129
GLS02	-0.543	**0.004**	no	0.007	0.971
Gluconeogenesis					
PEPCK	0.204	0.308	no	-0.306	0.129
FBPase	0.279	0.197	**yes**	0.665	**<0.01**
G6Pase	0.268	0.205	no	-0.382	0.066
Lipid metabolism					
FAS	-0.211	0.323	**yes**	-0.477	**0.019**
CPT1a	0.415	**0.039**	no	0.197	0.345
G6PD	-0.238	0.253	**yes**	0.265	0.201

The expression of GDH, FBPase and FAS responded linearly to gradual increasing of dietary crystalline methionine levels (**Paper IV**), but not after including the fish meal baseddiets (Table 2). However, the expression of these genes responded in a linear manner to plasma methionine concentrations supporting the fact that, more than responding to dietary level, the transcription of several genes could be modulated by plasma amino acid concentrations. Molecular mechanism such as TOR, might be able to sense amino acid availability in and out of the cells (Taylor, 2014) and modulate the metabolic response accordingly, in order to optimize amino acid utilization.

Main results and discussion

Hypothesis 1: Diet recipe (raw material and amino acid content) influences postprandial plasma amino acid patterns and protein utilization.

The results presented in **Paper I** suggest that the utilization of refine plant protein raw materials itself does not alter plasma amino acids profiles in fish. Rather, the postprandial plasma concentrations of essential amino acids reflect the dietary content. Plasma concentrations were, however affected by crystalline supplementation, leading to differences the utilization of essential amino acid. Conversely, the plasma levels of non-essential were only marginally affected by the choice of raw materials and their respective dietary levels.

Conclusion: partly **rejected**, partly **accepted**. Dietary amino acid level and form, rather than the protein origin seems to be predominant factors affecting plasma pattern.

Hypothesis 2: Changes in the supplementation of one essential amino acid level and form affect postprandial plasma amino acid profiles.

Dietary methionine level and form significantly affected plasma concentrations during digestion of a single meal in a predictive manner. Using statistical modelling it was shown that 74 % of the variances in postprandial plasma methionine concentrations could be explained by just a few dietary parameters (**Paper II**). In addition, the effect of changes in dietary methionine form and plasma concentrations was demonstrated to affect postprandial plasma concentrations of the other essential amino acids.

Conclusion: **accepted**. Dietary changes in methionine affect the overall plasma pool of free essential amino acids.

Hypothesis 3: Dietary methionine level and protein source (plant or fish meal) affect growth performances at a molecular level through modulating the transcription of genes of the somatotropic axis and in protein turnover.

Dietary methionine level and protein origin significantly affect growth performances and feed utilization. Fish fed plant based diets with crystalline methionine showed significantly reduced feed intake and specific growth rates compared to that of fish fed similar level of methionine in fish meal based diets (**Paper III**). The expression of several genes coding for molecules related to the somatotropic axis and protein turnover responded in a linear fashion to dietary methionine levels, reflecting overall growth performances. The transcriptional levels of GHR-I and IGF-I were strongly correlated to methionine content, as were enzymes related to protein degradation (Prot 20D, Capn 1, CAST-L and CAST-S).The dietary raw material source,

plant or fish meal significantly altered the expression of genes related the GH-IGF axis (Fig 4) while protein turnover was affected to a lower extent (data not shown).

Conclusion: **accepted**. Dietary methionine level and raw material souce affect growth and feed utilization, likely by modulating the somatotropic axis and protein turnover at the transcriptional level.

Hypothesis 4: Dietary methionine level and protein source (plant or fish meal) affect intermediary metabolism at the transcriptional level, resulting in different amino acid utilization.

The expression of several genes involved in the intermediary metabolism showed a correlation to dietary methionine levels, reflecting the results of the nitrogen excretion and HSI. In the case of methionine deficiency, FAS expression was up-regulated, while HSI and ammonia excretion increased, compared to a fish fed a diet supplemented to adequate level. In the case where one essential amino acid is limiting for the completion of protein synthesis, lipid synthesis seems to be the preferred strategy to utilize the amino acids. The overall expression of genes involved in amino acid catabolic pathways seems up-regulated in the case of excess of methionine, as well as FBPase. This could be a mechanism triggered in response to increasing of amino acid level occurring during the digestion of a meal, to maintain plasma free amino acid pool (**Paper IV**). The raw material source significantly modulated the response to dietary methionine level on the hepatic intermediary metabolism at the transcription level as well as postprandial plasma methionine concentrations. The results suggest that the expression on several genes involved in the intermediary metabolism could be modulated by plasma methionine profile, as a result of dietary composition and methionine level.

Conclusion: **accepted**. Methionine and/or the balance of dietary profile are able to regulate intermediary metabolism at the transcription level, and affect optimal amino acid utilization. Plasma availability might be a factor in the regulation of gene expression by amino acids.

Conclusion and future perspectives

The importance of amino acid profiles in fish diets, rather than the origin of proteins (e.g. marine or plant), is already well established (Wilson, 2002) and utilized in aquafeed formulation. The effect of plant proteins and crystalline amino acids on growth performances is commonly attributed to changes in uptake patterns and availability in the plasma (Larsen et al., 2012; Bodin et al., 2012). The level and form of dietary essential amino acids affect postprandial plasma profiles (**Paper II**); however the raw material effect is likely due to the quality of the product (e.g. antinutritional content, degree of refining; **Paper I**).

During digestion, the rapid availability of amino acids derived from crystalline amino acids compared to protein-bound ones results in temporal mismatch at the site of protein synthesis, leading to decreased growth and increased ammonia excretion (Bodin et al., 2012). The present results showed that, in addition to the rapid appearance in the plasma, methionine from a crystalline source also reached much greater concentrations, and remained elevated for up to 36 h after meal ingestion. The mechanisms responsible for these variations and possible counter measures to delay uptake, warrant further examination.

Additionally, changes in dietary level and form of methionine affected plasma levels of other essential amino acids. Future work should be aimed at clarifying whether this observation is caused by changes in utilization, or if other regulatory mechanisms are in play.

Amino acids are involved in growth and metabolism, not only as precursors but also as signalling factors. The combined effects of changes in dietary amino acid profiles are measurable with classic production variables (**Paper III, IV**). The lower growth, feed and protein utilization, measured in response to deficient dietary amino acid profile (Green and Hardy, 2008) is likely the results of the modulation of the pathways by amino acids (**Paper II, IV**). Indeed, several amino acids (termed functional amino acids) are able to trigger molecular mechanism to regulate gene expression of enzymes involved in physiological functions (Seiliez et al., 2008; Lansard et al., 2010, 2011). However, studies on amino acid regulation of gene expression in muscular cells are necessary to understand the mechanism involved in growth. Muscular development and protein turnover are important factors accounting for biomass deposition.

The regulation of hepatic intermediary metabolism is closely related to dietary amino acid profile. While the expression of certain genes is under the control of absolute content or dietary amino acid profile, others could respond to circulating plasma levels. The metabolic changes in response to dietary composition and methionine level, measured at the transcriptional level and using production variables (**Paper III, IV**), could be affected by the variations in plasma amino acid profiles (**Paper I, II, IV**). Further investigations are required to verify the existence of such relationships.

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Paper I: Effect of plant proteins and crystalline amino acid supplementation on postprandial plasma amino acid profiles and metabolic response in rainbow trout (Oncorhynchus mykiss)

Effect of plant proteins and crystalline amino acid supplementation on postprandial plasma amino acid profiles and metabolic response in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The use of aquafeeds formulated with plant protein sources supplemented with crystalline amino acids (CAAs) is believed to influence amino acid (AA) uptake patterns and AA metabolic fate. Oxygen consumption and ammonia excretion rates were measured in rainbow trout (468.5 ± 86.5 g) force fed 0.75% of their body mass with a diet based on either i) fish meal (FM), ii) pea protein concentrate (PPC), or iii) pea protein concentrate supplemented with histidine, lysine, methionine and threonine (PPC+) to mimic FM AA profile. The specific dynamic action and nitrogen quotient (NQ) were calculated for 48 h of the postprandial period. In parallel, plasma AA concentrations were measured in blood samples withdrawn from the caudal vein before and then 2, 4, 6, 8, 12, 20, 32 and 48 h after feed administration. The unbalanced diet PPC had a significantly higher NQ compare to FM (0.29±0.09 and 0.18±0.04, respectively) and plasma profiles of essential AAs reflected the dietary deficiencies. Supplementation with CAA in diet PPC+ resulted in an intermediary NQ (0.21±0.04) and significantly affected plasma AA profiles; presenting greater and faster rises followed by sharp decreases compare to FM. The strongest effect was observed for methionine, presenting 3-fold higher concentrations at peak time for PPC+ compared to FM (297.0±77.0 and 131.8±39.0 nmol ml^{-1}, respectively). The differences in AA availability and metabolic profile in the pea diets compared to the FM diet were believed to be caused by an unbalanced dietary AA profile and CAA supplementation, rather than inclusion of plant protein concentrate.

Key words: Essential amino acid · Non essential amino acid · Ammonia excretion· Respirometry· SDA

Abbreviations:

AA: amino acid
ACC: acclimation
ANF: anti-nutritional factor
CAA: crystalline amino acid
DM: dry matter
EAA: essential amino acid
FM: fish meal
NEAA: non-essential amino acid
NQ: nitrogen quotient
PI: protein intake
PPC: pea protein concentrate
SDA: specific dynamic action
TAN: Total ammonia nitrogen

Introduction

Fish meal (FM) is the preferred protein source in the feeds for farmed carnivorous fish because of its high protein and micronutrient content, favourable amino acid (AA) composition and good palatability (Gatlin III et al. 2007). FM production relies on pelagic marine fisheries, from stocks that are currently either fully or over exploited (FAO 2012), and is a commodity that has tripled in price over the past 10 years. The production of aquafeeds to support increased production of farmed fish must therefore reduce dependency on FM and increase the use of alternative feed resources (Gatlin III et al. 2007; Bendiksen et al. 2011). Terrestrial animal and plant material, fish by-products and algae have been widely studied as possible substitutes for FM in aquafeeds. Several plant products have high availability, competitive prices and good nutritional value, which make them suitable candidates as FM replacers; successful partial replacement of FM with plant materials has been documented in many studies (Bórquez et al. 2011; Burr et al. 2012; Collins et al. 2012; Güroy et al. 2012; Slawski et al. 2012).

However, protein sources differ in their AA profiles (Médale and Kaushik 2009). Plant protein sources are often deficient in one or more essential amino acids (EAAs), and their use as replacements for FM requires supplementation with specific AAs to satisfy the nutritional requirements of the species. This is commonly achieved using crystalline amino acids (CAAs), which improves growth performance and feed utilization compared to fish fed unbalanced diets (Espe et al. 2006; Gaylor and Barrows 2009). Yet, plant based diets supplemented with CAAs still lead to lower growth performances compare to FM based diets (Davies and Morris 1997; Bodin et al. 2012). Protein origin (marine or plant) and whether AAs are provided as protein bound or in free form (i.e. crystalline) are commonly assumed to affect AA uptake kinetics during the digestive process (Cowey and Sargent 1979; Thebault 1985; Zarate and Lovell 1997; Zarate et al. 1999; Dabrowski et al. 2003; Larsen et al. 2012). Differences in uptake patterns of bound and free AAs change the temporal availability and lead to a mismatch at the site of protein synthesis. This change in the postprandial AA profile in the plasma is believed to lead to increased AA catabolism (Cowey and Walton 1988; Larsen et al. 2012).

The aim of the current study was to investigate the effects of fully replacing FM with pea protein concentrate (PPC) on AA uptake patterns and metabolic profile in rainbow trout (*Oncorhynchus mykiss*) in a short-term trial. Pea protein concentrate is known to be a suitable protein source for rainbow trout, being highly digestible (Thiessen et al. 2003; Collins et al. 2012) and containing reduced levels of antinutritional factors (ANFs) compare to other legumes or unrefined pea meal (Liener 1994; Francis et al. 2001; Thiessen et al. 2003; Øverland et al. 2009).

The study was conducted in two consecutive parts. The first part examined the effects of the diets on the specific dynamic action (SDA) and total ammonia excretion (TAN) in individual fish during digestion. Ammonia is the major end-product of AA catabolism, and is mainly excreted across the gills (Kajimura et al. 2004). TAN provides a quantitative measure of AA oxidation and deamination, while SDA provides

information on the metabolic cost of ingestion, digestion, absorption and assimilation of a meal (McCue 2006; Secor 2009). Nitrogen excretion, previously used to assess the fate of dietary protein (Cowey 1995), was tested in association with SDA as a possible alternative to the long and tedious growth trials. To identify possible causative mechanisms for changes in TAN excretion rates and SDA, a second part of the study examined the effects of the different diets on the postprandial plasma AA profiles, by blood sampling at several time points during the digestive process.

Materials and methods

Fish, husbandry and diets

Rainbow trout (468.5 ± 86.5 g) were obtained from a commercial fish farm (Funderholme, Denmark). The fish were counted (100 individuals per tank) and weighed into three fiberglass tanks (700 l). Each tank was fed 0.75% of the biomass per day on either a FM diet or a pea protein based diet (ACC; Table 1) for a minimum of three weeks. The purpose of using the ACC diet was to accustom the digestive system of the fish to plant protein. FM and CAAs were included in the ACC diet to ensure voluntary feeding during the acclimation period. The fish to be fed the FM diet during the experiment received the FM diet (one tank), while fish to be fed the plant protein diets (PPC and PPC+; Table 1) during the experiment received the ACC diet (two tanks). Fish were fed from 08:00 to 14:00 h using automatic belt feeders, and maintained under a 16:8 h light:dark photoperiod.

The experimental diets were designed to differ in protein source (Table 1) and AA profile (Table 2); the PPC and PPC+ diets contained only plant based protein sources. Diet PPC+ was supplemented with histidine, lysine, methionine and threonine in crystalline form (Evonik Industries AG, Hanau, Germany) to mimic the AA profile of the FM diet (Table 2). Replacing FM with plant based ingredient affected the proximate composition of the diets (Table 1). With the exception of arginine and phenylalanine, the PPC diet had a lower content of all EAAs compared to the FM diet, methionine being the most deficient (Table 2). The FM, ACC and PPC+ diets were designed to meet the AA requirement of rainbow trout (NRC 2011). A vitamin and mineral premix was supplemented in all diets to avoid any deficiency other deficiency than AA in PPC.

Experimental set up and sampling

Metabolic responses

Prior to SDA experiments, feed was withheld for 48 h. One fish per acclimation tank (i.e., three individuals) was anaesthetized in phenoxyethanol (0.25 ml l^{-1}), weighed and isolated in a non-transparent PVC tube (50 cm long and 10 cm in diameter) for 24 h acclimation. Then the three individuals were force fed 0.75% of their body mass with the one of the experimental diets FM, PPC or PPC+ diet using a stainless steel gavage.

Following feed administration, the fish were transferred to individual respirometric chambers of a known volume (50 cm long, 13 cm in diameter) as described by Skov et al. (2011). In brief, both ends of the respirometric chambers were covered in black opaque plastic and placed under opaque material to minimize visual disturbance. Oxygen consumption was determined for 48 h after force feeding using intermittent respirometry. Each chamber was connected to a flush pump, delivering oxygenated water (16°C; 130% O_2 saturation; 600 l h^{-1}), and a recirculating pump (recirculating the water within the chamber at 300 l h^{-1}). Pumps were controlled by computer software AutoResp (Loligo Systems, Denmark) and programmed to run in 30 min loops (9 min flush, 1 min wait and 20 min measurement). Fibre-optic oxygen probes were positioned in the recirculation loop of each chamber to measure oxygen concentration (every second). The values were used to calculate oxygen consumption for each period with AutoResp software.

Ammonia excretion was determined by analysing water samples obtained at the beginning and end of the 20 min respirometric measurement periods occurring at time 0 (i.e., just after force feeding), 2, 4, 6, 8, 12, 20, 32 and 48 h after the meal administration. Water samples were immediately frozen at -20 ºC until analysis.

At the end of the 48 h measuring period the fish were removed from the chamber, killed by a blow to the head followed by spinal transection, and the intestine was dissected out to verify whether the feed had been digested. The protocol was repeated to obtain a minimum of four replicates for each dietary treatment.

Plasma amino acids

In the second part of the experiment, the three acclimation tanks were fasted for 48 h and nine fish per tank were anaesthetized (0.25 ml l^{-1} phenoxyethanol) and weighed. One individual per tank was directly sacrificed and blood was sampled from the caudal vein (T0). The remaining 24 fish were subsequently force fed one of the experimental diets (FM, PPC or PPC+; 0.75% body weight). The fish were then confined in individual, non-transparent PVC tubes as described above. Blood samples were then obtained at 2, 4, 6, 8, 12, 20, 32 and 48 h after force feeding. At each sampling time one fish per treatment was removed from its chamber and killed by a blow to the head. Within the following minute, a blood sample was obtained from the caudal vein using a 1 ml heparinised syringe. Blood samples were centrifuged for 10 min at 3000 rpm at 5ºC. Plasma was transferred to a new vial and frozen at -80ºC until analysis for the content of AAs. The protocol was repeated to obtain eight replicates per time point for each dietary treatment.

Chemical analysis and data treatment

Feed analysis

Feed samples were homogenized (Krups Speedy Pro homogenizer) and analysed for dry matter and ash (Kolar 1992), crude protein (ISO 2005; assuming crude protein = 6.25×Kjeldahl nitrogen), fat (Bligh and Dyer 1959), and phosphorus (ISO 1998). The dietary AA composition was determined by a commercial laboratory (Eurofins Denmark A/S, Glostrup, Denmark).

Specific Dynamic Action (SDA)

The individual standard metabolic rate (SMR) was calculating using the 10 lowest oxygen consumption values for each fish recorded at the end of the 48 h period, assumed to be the values at rest. The SDA was calculated using a custom made script developed by Denis Chabot (Institute of Maurice Montagne, Quebec) for the statistical software R (R Core Team 2013) and expressed as mmol O_2 per kg body mass (mmol kg^{-1}) from which SMR was subtracted.

Ammonia excretion

Water samples were thawed overnight at 3°C, and analysed in duplicate using a colorimetric method for ammonia determination (DS 1975). The changes in ammonia concentrations were used to calculate the ammonia nitrogen excretion rate (N_{exc}) for each fish at each time point (mmol kg^{-1} h^{-1}).

$$Nexc = \frac{\Delta[N] \times Vresp}{\Delta t \times BM}$$

where Δ[N] is the change in ammonia nitrogen concentration (mmol l^{-1}), Vresp is the volume of the respirometer chamber excluding the fish (l), Δt is the time period (h), and BM is the body mass (kg).
The ammonia excretion rates for each fish were plotted against time to create an ammonia excretion profile. A curve was fitted to the data (TableCurve 2D v. 5.01, Systat Systems Inc., USA) and integrated to calculate the total ammonia excretion over the 48 h period (mmol kg^{-1}).

The total ammonia excretion at fasted state ($N_{basal\ exc}$) was calculated in a similar manner and withdrawn from the total ammonia excretion of the fed state to obtain ammonia excretion deriving only from the ingested meal, i.e.:

$$TAN = \int_0^{48} Nexc\ fed\ - \int_0^{48} Nbasal\ exc$$

As ammonia is considered to be the major end product of nitrogen compounds oxidation (Kajimura et al. 2004), the contribution of other nitrogenous compounds was not considered when determining total nitrogen excretion.
The nitrogen quotient (NQ) for each fish was obtained by dividing the TAN value by the SDA. The theoretical share of protein used as energy for metabolism (%) was subsequently derived from NQ using the standard value 0.27, accounting for NQ when protein in the only fuel metabolized (Van Der Thillart and Kesbeke 1978).

Plasma amino acids

Plasma samples were thawed at room temperature and deproteinised (diluted 4:1 in distilled water, 4:1 in 20% sulfosalicylic acid and centrifuged 20 min at 9000 rpm at 5ºC) and analysed for AA composition according to the method described by Larsen et al. (2012). In brief, the samples were derivatized with Waters

AccQ Tag reagent (Waters Corporation, Milford, Massachusetts, USA) according to the manufacturers instructions, and diluted 1:4 in sodium acetate buffer (0.1 mol l^{-1} sodium acetate, 30 ml l^{-1} acetonitrile (60%), pH 6.2). Identification and quantification was performed using an uHPLC system (Flexar FX-10, PerkinElmer Inc., Waltham, Massachusetts, USA), and using gradients of the sodium acetate buffer (describe above) and acetonitrile (60%) to separate the different AAs on the chromatographs. A standard AA mixture supplemented with glutamine and hydroxyproline (Sigma-Aldrich, St. Louis, Missouri, USA) was used to identify and quantify the 18 AAs given in Table 2.

The Chromera Flexar v3.2.0. 4847 software (PerkinElmer Inc., Waltham, Massachusetts, USA) was used for data recording and analysis. Plasma AA concentrations (μmol ml^{-1}) were subsequently plotted against time (h).

Statistics

Data (mean±SD) were analysed for statistical differences using one-way ANOVA and followed by the Holm–Sidak multiple comparison procedure. A probability of $p<0.05$ was considered significant in all analyses, which were carried out using SigmaPlot v. 11 (Systat Systems Inc., USA).

Results

Metabolic responses

Ammonia excretion increased significantly during feeding and excretion rates presented different profiles depending on the diets (Fig. 1). Peak excretion rates were reached 8 h after feeding of the FM and PPC dietary treatment groups, compared to 12 h after feeding of the PPC+ group. Peak values were similar for fish fed diet FM and PPC+ (0.6 mmol kg^{-1}) as well as their TAN for the 48 h period (Table3). No statistical differences for TAN and SDA were found between treatments ($p=0.073$ and $p=0.377$, respectively). However it is worth noticing that the unbalanced diet (PPC) presented the highest TAN (9.5 ± 1.4 mmol kg^{-1}) and the lowest SDA values (34.2 ± 8.3 mmol kg^{-1}).

Conversely, NQ values and the derived contributions from protein to fuel metabolism were significantly affected by the dietary treatments (table 3). Digestion of the PPC diet resulted in a significant higher NQ compared to the FM diet. Supplementation with CAA in the PPC+ diet decreased the share of protein used to fuel metabolism and resulted in an intermediary value to FM and PPC.

Plasma amino acid profiles

The postprandial plasma profiles for the EAAs non-supplemented or supplemented for each of the three diets are shown in Fig. 2 and 3, respectively. Substitution of FM with PPC did not influence the plasma profiles of arginine, isoleucine, leucine, tryptophan, valine (Fig. 2 a, b, c, e and f) nor histidine (Fig. 3 a). Hence, the plasma profiles of these six EAAs displayed largely similar variations over time and reached similar concentrations during the 48 h period. The plasma profiles for phenylalanine, lysine and threonine in fish fed the FM and PPC diets were displaced along the y-axis reflecting largely the differences in dietary content (Fig. 2 d and Fig. 3 b and d, respectively). Methionine was the EAA most influenced by substituting FM with PPC (Fig. 3 c), with concentrations decreasing throughout the measuring period in diet PPC, and being significantly lower ($p<0.05$) than in the FM group at 8, 12, 20, 32 and 48 h after force feeding.

Supplementation with CAAs affected plasma profiles of both supplemented and non-supplemented EAA in fish fed diet PPC+. With respect to supplemented EAAs (Fig. 3), plasma concentrations were generally higher than in fish fed the unbalanced diet (PPC), and the differences remained significant over time ($p<0.05$). Despite similar dietary level (Table 2), the plasma concentrations of methionine for PPC+ and FM diets revealed significant differences during the course of digestion (Fig. 3 c).

Raw material and dietary amino acid content did not have a clear effect on the non-essential amino acid (NEAA). Plasma concentrations and variations for alanine, aspartate, glutamate and glutamine were similar regardless of raw material and dietary content (data not shown). Differences in plasma profiles of cysteine and glycine seemed to reflect the differences in dietary content (Fig. 4 a and b), whereas this was not the case for proline, serine and tyrosine (Fig. 4 d, e and f). Plasma concentrations of hydroxy-proline were higher in FM diet than for the two plant based diets (Fig. 4 c). It should be noted that cysteine was difficult to quantify due to the very low concentrations.

Discussion

Metabolic response as indicator of protein utilization

Ammonia excretion is related to the ratio of digestible protein: digestible energy, dietary AA balance (Green and Hardy 2008), and protein synthesis (Lied and Braaten 1984; Bonaldo et al. 2011). Protein synthesis and protein turnover are major metabolic components of the SDA response (Brown and Cameron 1991a), and differences in dietary protein content may explain in part the smaller magnitude of the SDA response in the PPC diet. However, coupled with the larger ammonia excretion rates of the PPC diet, our findings support previous results showing that rainbow trout fed a diet with suboptimal AA profile have less efficient nitrogen retention compared to fish fed a balanced diet (Green and Hardy 2008).

Conversely, supplementation with CAAs to satisfy nutritional requirement has been shown to improve the retention of nitrogen for somatic growth rather than oxidation for metabolic energy (Green and Hardy 2008). Although not significant, the present results seem to indicate an increased nitrogen excretion in fish fed a plant based diet with a balanced AA profile (PPC+) compare to that of fish fed the FM control.

The simultaneous measurement of SDA and ammonia excretion allowed for calculations of the NQ, providing information on the fraction of metabolism that was fuelled by protein. The NQ is usually applied to fish in a steady state (i.e., non-feeding) which differs greatly from a digestive state where protein oxidation increases during the processing of a meal, fuelling 50-70% of the metabolism. In non-fed fish, protein oxidation only supplies 25% of the energy requirements of a fish (Wood 2001). Deficiency in one or more EAAs will render other AAs in excess and lead to increased rates of catabolism. The deficiency of methionine in the unbalanced PPC diet in all likelihood led to an increased catabolism or interconversion of other AAs (Carter and Houlihan 2001), which accounted for the increase in TAN excretion and higher NQ values.

As the magnitude of the SDA response is sensitive to dietary protein content (Jobling and Davies 1980; Secor 2009), it should be noted that the FM based diet had a higher protein content than the PPC diets (Table 1). However, results obtained in a short term trial, using few individuals provided data that was in line with previous published work (Green and Hardy 2008). The complete substitution of FM with plant protein products and supplementation with CAAs in the diets for carnivorous species implicate lower growth, feed utilization and a switch towards AA catabolism (Bonaldo et al. 2011; Jalili et al. 2012).

Effects of protein source on AA plasma profiles

Previous studies have indicated a temporal displacement of EAA plasma profiles when replacing FM with a plant protein source (Yamamoto et al. 1998; Larsen et al. 2012; Wacyk et al. 2012). This was not evident in the present study, possibly due to the use of high quality raw materials, e.g. plant protein concentrates with little or no ANF and fibres, which are common in unrefined plant products (Francis et al. 2001; Gatlin III et al. 2007).

The present study suggested that postprandial EAA plasma profiles are strongly affected by dietary EAA content. This is consistent with earlier studies (Plakas et al. 1980; Dabrowski 1982; Schuhmacher et al. 1995) showing a positive correlation between EAA levels in the plasma and their respective dietary inclusion levels. Hence, EAAs that were similar in content in the FM and in the plant based diet resulted in similar plasma profiles (i.e., arginine, histidine, leucine, isoleucine, tryptophan and valine). With the exception of methionine, EAAs that differed in content between the two diets (i.e., lysine, phenylalanine and threonine) showed comparable variations over time but with shifts in plasma concentrations, reflecting the differences in dietary content. The fish fed the unbalanced plant-based diet had consistently lower plasma methionine concentrations compared to the FM diet, likely reflecting the large dietary deficiency. Hence, using high

quality refined protein sources, postprandial plasma EAA profiles appear to rely on dietary content and not on protein origin.

Consistent with earlier findings, plasma concentrations of the majority of NEAAs were negligibly influenced by the dietary profiles (Plakas et al. 1980; Dabrowski 1982; Schuhmacher et al. 1995). Plasma concentrations of cysteine, hydroxy-proline, and tyrosine may have been influenced by the availability of their precursors in the diets (i.e., methionine, proline and phenylalanine, respectively). This was exemplified by the high plasma concentrations of phenylalanine in fish fed diet PPC and PPC+ were accompanied by high tyrosine plasma concentrations despite low dietary content of the latter.

Consequences of CAA supplementation on plasma AA profiles

The aim of the experimental design was that the FM and PPC+ diets contained similar levels of EAA which, with the exception of histidine, was achieved (Table 2). The addition of crystalline EAA to mimic the FM AA profile caused large variations in the plasma AA profiles. Previous studies have shown that plasma AA levels in fish fed CAA tend to reach higher concentrations, and peak earlier than when supplied at comparable dietary levels through intact protein (Yamada et al. 1981; Murai et al. 1987; Schuhmacher et al. 1997; Ambardekar et al. 2009). The results for histidine, lysine and threonine in the present study are consistent with these observations. The early occurrence of a peak in plasma levels is likely the result of rapid uptake of dietary CAA, because, in contrast to protein-derived AA, there is no requirement for protease to hydrolyse the peptide bonds before AAs become available for intestinal absorption (Cowey and Walton 1988).

Methionine supplied in crystalline form also displayed a tendency to accumulate in the plasma and to remain elevated for prolonged time periods compared to supplemented histidine, lysine and threonine. The reason for this is not evident, but may relate to the fact that crystalline methionine, as common practice in the feed industry, was supplied as a D-L mixture. D-isomers cannot be utilized for protein synthesis before they have been racemized into the L-isomer in an enzymatic reaction (Fiedman and Levin 2011). Salmonids are considered able to synthesize L-methionine from D-methionine (Sveier et al. 2001), and therefore utilize efficiently D-L mixture of methionine for growth (Kim et al. 1992; Sveier et al. 2001). The plasma methionine measured in the present experiment might have consisted of D-methionine accumulation caused by a preferential tissue uptake of L-isomers (Brown and Cameron 1991b).

Considering that dietary levels of lysine, methionine and threonine were similar in the FM and PPC+ diets, the present study showed that plasma levels of EAAs are affected to a high degree by the form in which they are supplemented; e.g. as whole protein (i.e. bound) or in a crystalline (free) form. Blood plasma is the route of delivery for AAs to the tissues for protein synthesis (Robinson et al. 2011) and the changes in free AA concentrations could explain, at least in part, the NQ results observed. Nevertheless, further investigations should be directed specifically at determining the cause of the accumulation of methionine in the plasma

during the digestion of diet supplemented with crystalline D-L mixture and the overall effect this may have on the fate of AAs. Additionally, there is growing evidence that also in fish AAs are involved in the regulation of metabolic pathways (Lansard et al. 2010, 2011). The effect of plasma AA profile, resulting from dietary changes (level and form), on their signalling role warrant further investigation. A distortion of the message carried by AAs could be a component of the difficulties encounter when fish are fed diet containing alternative protein sources and supplemented with CAAs.

Conclusion

The present study showed that a plant based diet deficient in three EAA (lysine, methionine and threonine) resulted in areduced availability of all three in the plasma during the digestive process, and lead to a higher share of protein oxidation for metabolism compared to a fish meal based diet. This was alleviated by supplementing CAA to a level apparently satisfying the nutritional requirements of the fish. As an effect, the availability of AA was improved (higher postprandial plasma concentrations) and the fraction of AA deaminated and oxidized (lower NQ value and share of protein used to fuel metabolism) was reduced, probably resulting in an enhanced protein synthesis.

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Table 1 Recipes and proximate composition for the three experimental diets (FM, PPC and PPC+) and the acclimation diet (ACC)

	FM	PPC	PPC +	ACC
Ingredient formulation (g 100 g^{-1} feed)				
Fish meal	62.0	0.0	0.0	9.8
Pea protein concentrate	0.0	48.3	44.4	37.2
Wheat	11.5	27.6	27.9	26.4
Fish oil	25.5	21.3	21.7	21.9
Monocalcium Phosphate	0.0	1.72	1.86	1.1
L-Lysine	0.0	0.0	1.03	0.87
D/L-Methionine	0.0	0.0	0.78	0.66
L-Threonine	0.0	0.0	0.44	0.37
L-Histidine	0.0	0.0	0.88	0.74
Vitamin and mineral premix*	0.99	0.97	0.97	0.98
Proximate composition (g 100 g^{-1} feed)				
Protein	44.7	37.5	39.1	38.5
Fat	34.1	28.3	28.3	28.6
Moisture	6.2	7.3	6.8	6.8
Ash	8.2	4.2	4.5	4.5
Phosphorus	1.22	1.04	1.04	0.96

*Balance to meet the requirements recommended by NRC (2011)

Table 2 Dietary amino acid content (% dry matter) and difference relatively to the fishmeal diet (FM). The amino acid supplemented as crystalline in PPC+ and ACC are highlighted in grey

Amino acid (AA)	Abbreviation	Diet composition (% DM[a])				% difference relative to FM diet[d]	
		FM[b]	PPC[c]	PPC +	ACC	PPC	PPC +
Essential AAs							
Arginine	Arg	2.50	2.76	2.76	2.62	10.30	10.15
Histidine	His	0.92	0.84	1.44	1.42	-7.95	57.63
Isoleucine	Ile	1.79	1.59	1.59	1.63	-11.00	-11.04
Leucine	Leu	3.21	2.85	2.83	2.81	-11.29	-11.68
Lysine	Lys	3.24	2.40	3.17	3.10	-25.81	-2.08
Methionine	Met	1.16	0.40	1.11	1.10	-65.97	-4.65
Phenylalanine	Phe	1.71	1.91	1.90	1.83	11.86	11.37
Threonine	Thr	1.80	1.31	1.71	1.65	-27.42	-5.30
Tryptophan	Trp	0.47	0.35	0.34	0.36	-26.37	-27.36
Valine	Val	2.13	1.73	1.76	1.78	-18.59	-17.27
Sum essential AAs		18.93	16.14	18.62	18.30	-14.73	-1.63
Non-essential AAs							
Alanine	Ala	2.65	1.51	1.50	1.59	-42.88	-43.27
Aspartate	Asp	3.80	3.84	3.80	3.68	1.02	0.10
Cysteine	Cys	0.42	0.38	0.38	0.38	-7.85	-8.92
Glutamate	Glu	5.71	5.76	5.84	5.59	0.77	2.30
Glycine	Gly	2.55	1.44	1.44	1.53	-43.68	-43.38
Proline	Pro	1.90	1.64	1.71	1.60	-13.83	-9.94
Serine	Ser	1.85	1.81	1.80	1.70	-2.18	-2.66
Tyrosine	Tyr	1.47	1.33	1.30	1.28	-9.82	-11.87
Sum non-essential AAs		20.35	17.70	17.78	17.35	-13.01	-12.61
Total (essential + non-essential)		41.77	36.51	39.05	38.25	-12.59	-6.51

[a] feed composition is given on a dry matter basis

[b] refers to a fish meal based diet

[c] refers to a pea protein concentrate based diet

[d] (content in PPC or PPC+ - content in FM) / content in FM

Table 3 Results of ammonia excretion (TAN), specific dynamic action (SDA) and nitrogen quotient (NQ) for the three experimental treatments (mean±SD). The results follow by a similar letter do not differ significantly (one way Anova, $p<0.05$).

	FM	PPC	PPC+
TAN (mmol kg^{-1})	7.2±1.6	9.5±1.4	8.9±1.5
SDA (mmol kg^{-1})	42.5±13.7	34.2±8.3	43.0±7.0
NQ[x]	0.18±0.04[a]	0.29±0.09[b]	0.21±0.04[ab]
% protein metabolized[y]	67.1±15.7[a]	109.0±34.5[b]	77.7±15.6[ab]
n[z]	6	5	4

[x] NQ was derived as TAN/SDA

[y] % protein metabolized was derived as NQ/0.27*100

[z] n, number of individuals

Fig. 1 Ammonia excretion rate (mmol kg^{-1} h^{-1}; mean±SD) during the digestion of a single meal given for the three dietary treatments

Fig. 2 Plasma profiles (nmol ml^{-1}; mean±SD) over time (h) for the essential amino acids that were not supplemented as crystalline amino acids in PPC+, (a) arginine, (b) isoleucine, (c) leucine, (d) phenylalanine, (e) tryptophan and (f) valine for the three dietary treatment after force-feeding of a single meal (T0)

Fig. 3 Plasma profiles (nmol ml^{-1}; mean±SD) over time (h) for the essential amino acids supplemented as crystalline amino acids in PPC+, (a) histidine, (b) lysine, (c) methionine and (d) threonine for the three dietary treatment after force-feeding of a single meal (T0)

Fig. 4 Plasma profiles (nmol ml^{-1}; mean±SD) over time (h) for the non-essential amino acids, (a) cysteine, (b) glycine, (c) hydroxy-proline, (d) proline, (e) serine and (f) tyrosine, for the three dietary treatment after force-feeding of a single meal (T0)

Fig. 1

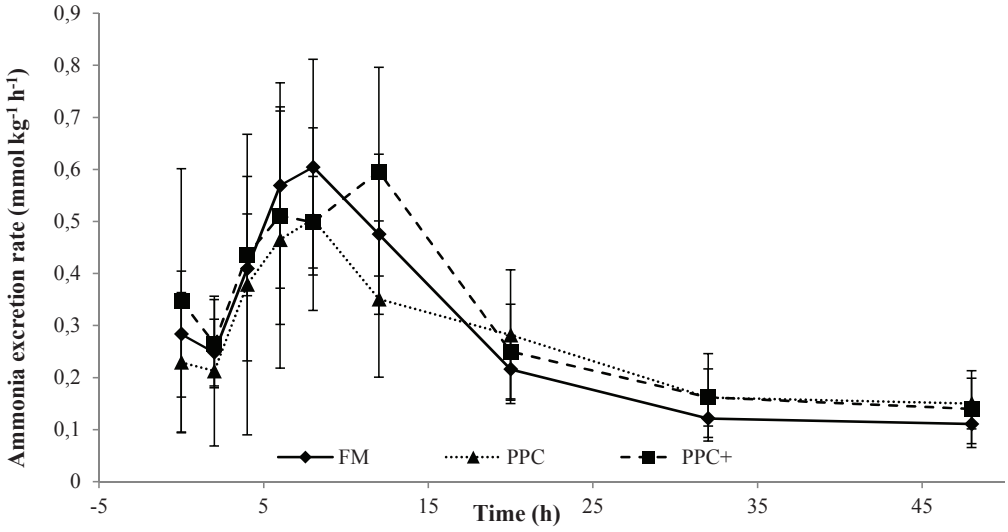

Ammonia excretion rate (mmol kg-1 h-1)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
-5
5
15
25
35
45
Time (h)
FM
PPC
PPC+

Fig. 2

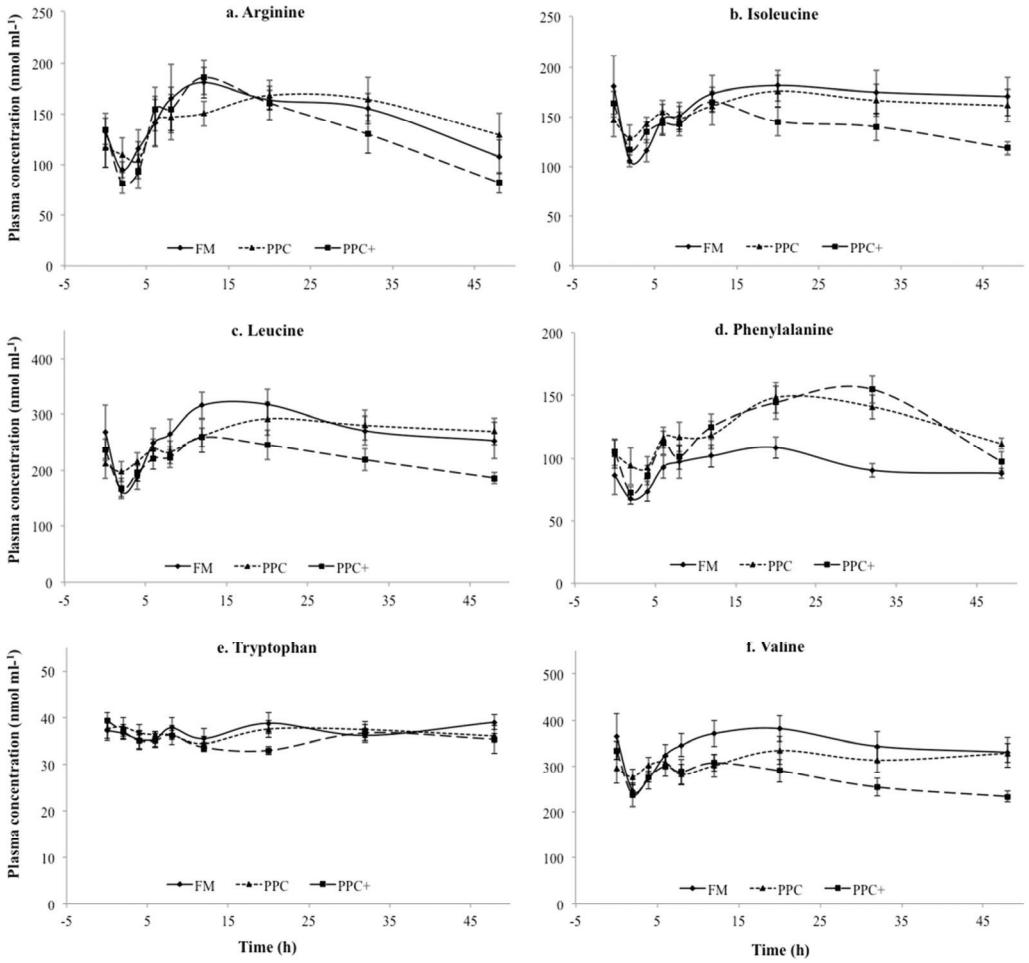

a. Arginine
b. Isoleucine
c. Leucine
d. Phenylalanine
e. Tryptophan
f. Valine
Plasma concentration (nmol ml-1)
Time (h)
FM
PPC
PPC+

Fig. 3

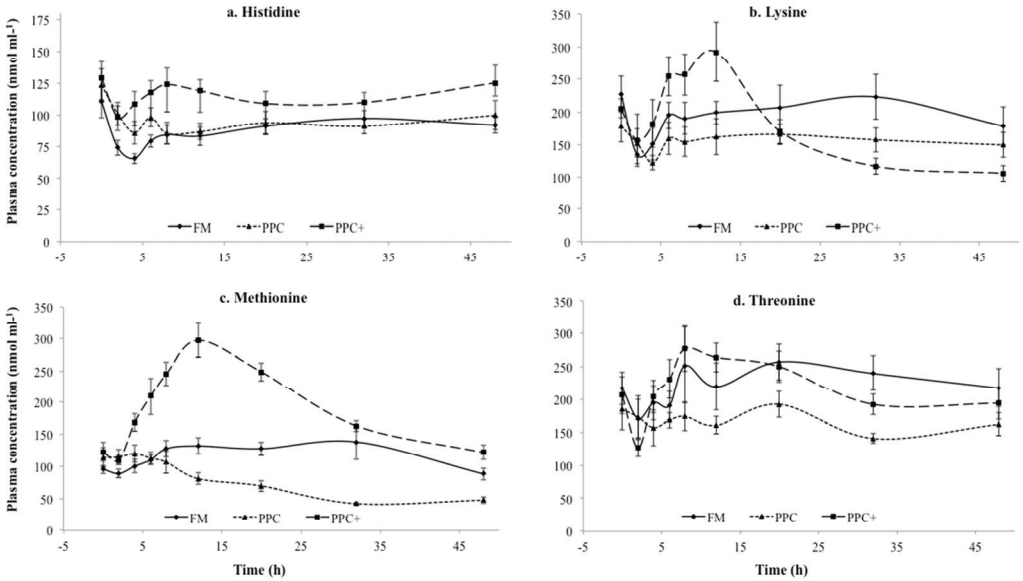
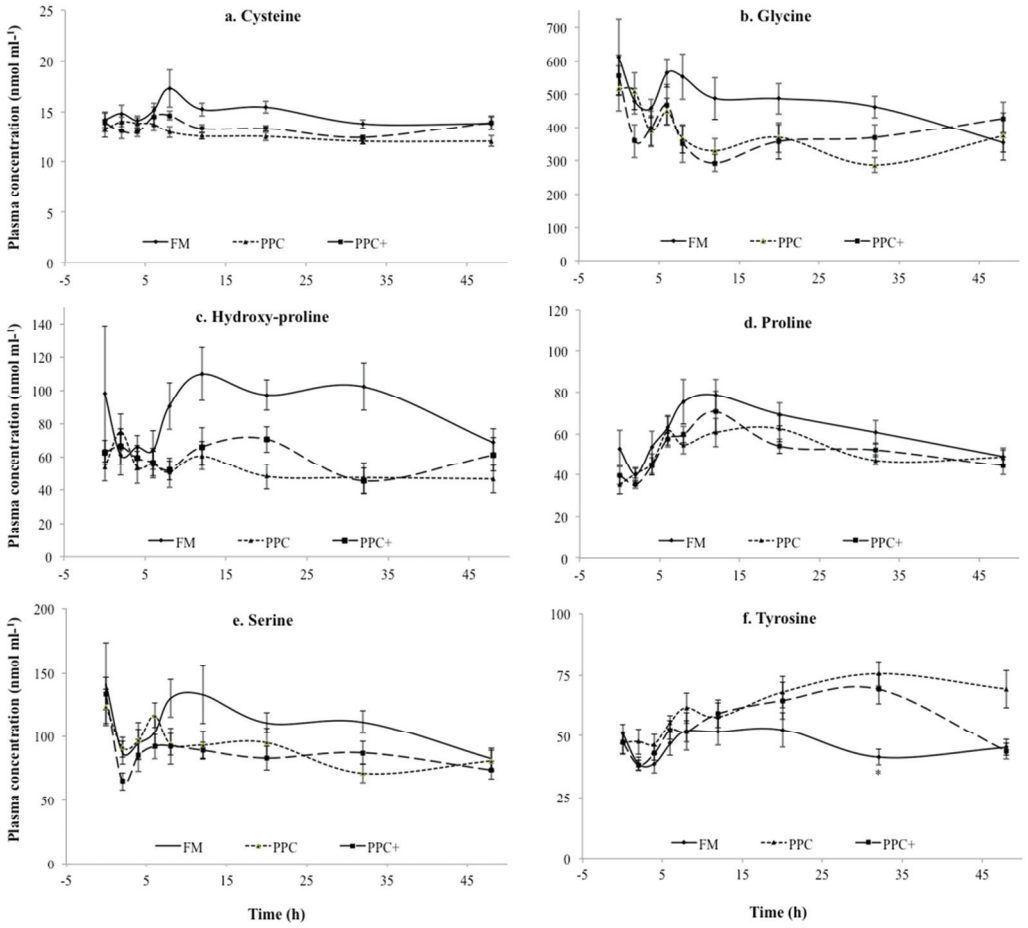

a. Histidine
b. Lysine
c. Methionine
d. Threonine
Plasma concentration (nmol ml-1)
Time (h)
FM
PPC
PPC+

Fig. 4


a. Cysteine
b. Glycine
c. Hydroxy-proline
d. Proline
e. Serine
f. Tyrosine
Plasma concentration (nmol ml-1)
Time (h)
FM
PPC
PPC+

Paper II: Modelling the effects of dietary methionine level and form on postprandial plasma essential amino acid profiles in rainbow trout (Oncorhynchus mykiss)

Modelling the effects of dietary methionine level and form on postprandial plasma essential amino acid profiles in rainbow trout (*Oncorhynchus mykiss*)

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Running title: Modelling plasma amino acid concentrations

Abstract

Aquafeeds formulation is susceptible to affect amino acid (AA) availability for metabolic functions. Statistical models were applied to quantify the effect of dietary methionine level (from 0.60 to 1.62 % dry matter) and form (free, coated or bound) on postprandial concentrations of plasma essential amino acid (EAA) in rainbow trout. Twelve diets were formulated with pea and soya protein concentrate or fish meal as the main protein ingredients; and were supplemented or not with increasing amount of either crystalline or agar coated methionine. Fish were acclimatized to one of the 12 diets for 6 weeks before postprandial plasma sampling (6 sampling points up to 36 h, 7 fish each time), further analysed for EAA content. Using generalized additive models, we show that (1) dietary methionine level and form explained 74 % postprandial methionine plasma variations, and that (2) the methionine dietary form and plasma concentrations significantly affected the plasma concentrations of the other EAAs. Finally linear model revealed a positive relationship (R^2>0.9) between plasma concentrations of the three branched chain AAs under the present experimental conditions. The results obtained add new information on the dietary effects on EAAs in the plasma availability and the interactions between them.

Keywords: crystalline amino acid, branched chain amino acid, plasma concentration, general additive model, linear model, fish feed

Introduction

The number of raw materials of terrestrial origin that are used as protein sources in salmonid feed have increased dramatically over the past 20 years, primarily due to the decline in fish meal availability and increase in price. Salmonids and other carnivorous species are consequently fed more sustainable feeds (Gatlin et al. 2007; Bendiksen et al. 2011) with a larger portion of protein originating from plant based products. The latter typically have a low content of certain essential amino acids (EAAs), especially methionine. Diets formulated using these raw materials become deficient in EAAs and require supplementation with, e.g., crystalline amino acids (CAAs) to achieve a satisfactory profile that meets dietary requirements. Despite CAA supplementation, plant based diets do not perform as well as fish meal based diets. A commonly accepted explanation for this is the effect of the amino acid (AA) source (protein or crystalline) on the plasma concentrations. Amino acids deriving from crystalline sources (free) reach the plasma earlier than the ones originating from proteins (bound), resulting in a temporal mismatch in availability at the site of protein synthesis (Thebault 1985; Zarate and Lovell 1997; Larsen et al. 2012; Rolland et al. 2014).

Common practice in evaluating the availability of AAs derived from feed is to measure their digestibility, i.e. the difference between dietary and faecal AA content. However, prior to reaching the systemic circulation and protein synthesis sites, dietary AAs are taken up through the intestinal wall, an important site for AA catabolism (Fang et al. 2010; Wu 2009). Efferent vessels of the intestinal circulation eventually empty into the hepatic portal vein, making AAs available to the liver, a major organ in terms of AAs catabolism. Only AAs remaining in the blood past the hepatic circulation can be considered available for metabolic processes in other tissues. Consequently, a large discrepancy is likely to exist between AA availability calculated from apparent digestibility and what is truly available for protein synthesis. In addition to their role in protein synthesis and as a metabolic fuel, AAs are involved in signalling pathways in various bodily functions (Wu 2009). Because they are versatile molecules acting as regulators in key metabolic pathways, the maintenance of their homeostasis is essential (Fafournoux et al. 2010; Panserat and Kaushik 2010). The free AA pool is influenced by the rate of AAs synthesized *de novo*, from turnover of endogenous protein, and from breakdown of dietary proteins. Dietary parameters affecting plasma AA concentrations are therefore likely to affect homeostasis and alter biological functions involving AAs (e.g., regulation of gene expression; Jousse et al. 2004; Lansard et al. 2010, 2011). Among AAs, methionine is of particular interest because, in addition of being nutritionally essential for rainbow trout (NCR, 2011) and limiting in plant based diets, it has a functional role in regulating metabolic pathways (Wu, 2013).

The present study aimed at quantifying the effect of dietary methionine level and form (free, coated and bound) on postprandial plasma concentrations of EAAs. For this purpose, rainbow trout (*Oncorhynchus mykiss*) were acclimatized to twelve fish- or plant-meal based diets for six weeks, the plant-meal diets being

supplemented with increasing levels of crystalline or bound methionine. A time series of postprandial plasma samples were subsequently obtained to test the effects of different dietary methionine levels and forms on AA profiles. Based on the diet formulations it was assumed that the variability observed in postprandial plasma AA concentrations was mainly due to the dietary treatment (i.e. AA dietary level and form of supplementation). To test this assumption the following three hypotheses were established:

Hypothesis 1 predicted that plasma methionine concentrations reflect dietary level and form as well as postprandial time of sampling. The hypothesis was based on the observation that plasma EAA concentrations in rainbow trout reflect dietary levels during digestion, i.e., showing a sharp increase in the first hours of the digestion of a meal and a subsequent gradual return to baseline levels (Schuhmacher et al. 1997; Karlsson et al. 2006; Rolland et al. 2014). Previous results also emphasized the effect of the form in which AAs are supplied in the feed. AAs supplemented in free form (crystalline) have been shown to reach the systemic circulation earlier than AAs bound as proteins (Murai et al. 1987; Cowey and Walton 1988; Schuhmacher et al. 1997; Ambardekar et al. 2009; Rolland et al. 2014). Conversely to that of EAAs, it has been found that plasma concentrations of non-essential amino acids (NEAAs) do not relate to dietary level (Walton and Wilson 1986; Schuhmacher et al. 1997; Rolland et al. 2014). NEAAs were therefore not considered in the present study.

Hypothesis 2 predicted that plasma concentrations of the other EAAs (apart from tryptophan) are influenced by dietary methionine level and form as well as postprandial sampling time. This hypothesis was based on the fact that the supply of one EAA affects the utilization of other AAs (Green and Hardy 2008; Rolland et al. 2014).

Finally, hypothesis 3 argued that plasma concentrations of branched chain amino acids (BCAAs) follow similar patterns during digestion, reflecting their use in common metabolic pathways. The fates of BCAAs are closely related to one another, potentially due to similarities in their chemical structure and the fact that they share common transporters, metabolic pathways, and functions (Morris 2006; Shimomura and Harris 2006). Hence, BCAAs are likely able to influence the requirements and kinetics of each other.

Previous studies modelling the relationship between plasma AA concentrations and dietary composition in fish are rare and have typically applied broken line models to account for the response to dietary AA levels (Park et al. 2005; Bae et al. 2011, 2012). In the present study, statistical models were used in an attempt to explain biological responses by allowing for the description of multifactorial relationships.

Material and methods

Diets

A total of 12 diets were designed to be iso-energetic and iso-nitrogenous and to have similar AA profiles except for methionine, which varied in both content and form (Table 1). The diets were produced by BioMar A/S as 4.5 mm pellets and labelled according to the source and level of methionine.

Nine of the diets (Free 0-5, Coated 3-5; Table 1) were made using an identical raw material matrix, differing only in methionine content and whether methionine was supplied as free (crystalline; Free 0-5) or coated (agar coating; Coated 3-5). The plant protein sources used (wheat gluten, soya protein concentrate, and pea protein concentrate) were selected for their high nutritional quality and low levels of anti-nutritional factors. To ensure high palatability, fish meal and krill meal were added at concentrations of 80 and 20 g kg^{-1} feed, respectively. The last three diets (Bound 3, 5, 6) contained protein bound methionine supplied from fish meal and the content of soya/pea protein concentrate was consequently reduced.

The ratio between soya and pea protein was kept constant in all diets except for Bound 6 which contained no soya -or pea concentrate (Table 1). No methionine was added to diet Free 0, which consequently contained the lowest concentration of methionine. Diets Free 1 to Free 5 contained increasing levels of crystalline D-L methionine (1.10, 2.09, 3.19, 4.29 and 6.50 g kg^{-1} feed), while diet Coated 3, Coated 4 and Coated 5 contained increasing levels of agar coated D-L methionine (2.61, 5.36 and 8.12 g kg^{-1} feed).

Experimental set up

The trial was run at BioMar's experimental facility in Hirtshals, Denmark using juvenile rainbow trout obtained from a commercial fish farm (Fulderholme, Denmark). The fish were acclimatized to the different experimental diets for six weeks, being fed *ad libitum* each day from 8:00 to 12:00 using automatic belt feed dispensers. The fish were exposed to a 12:12 h light:dark cycle and kept in fresh water at a temperature of 15.1±0.1 ºC. Oxygen (105±1%) and pH (7.55±0.06) levels were recorded daily. At the end of the acclimation period, feed was withheld for 48 h and 60 individuals per treatment were randomly selected and acclimatised for one week to hand feeding *ad libitum* for a 20 min period.

Due to the number of samples, the study was divided in two sets following the same protocol. For the first set, three replicates of 20 fish (individual mass 343.1±65.5 g) fed diet Free 0 to Free 5 were bulk weighed in 18 fiberglass tanks. For two days the fish were allowed to acclimatize to the new density while being hand fed *ad libitum* for 20 min. The fish were then starved for 48 h prior to the start of sampling. On the sampling day, the 18 tanks were hand fed 120 g within 20 min. The tank feeding periods were displaced by 10 min to provide time for blood sampling, which were obtained at 0, 3, 6, 12, 20 and 36 h after feeding. Furthermore, to minimize disturbances of the digestive process, the same tank was sampled only twice with an interval of

at least 12 h (i.e., sampling the same tank at time 0 and 12; 3 and 20; 6 and 36 h, respectively). The protocol was repeated for the coated and bound diets.

To minimize stress when taking blood samples seven fish per treatment were caught simultaneously at each sampling time and euthanized using an over dose of anaesthetic (phenoxyethanol, 1 ml l^{-1} for a minimum of three minutes). Upon removal from the anaesthetic solution, each fish was dealt a blow to the head, and blood was sampled directly from the ventricle. The spinal cord was severed and fish were dissected to verify the presence of food in the gastrointestinal tract. Blood samples from fish with an empty gastrointestinal tract were discarded and replaced. All blood samples were immediately centrifuged at 3000 rpm for 5 min, and the plasma fraction frozen on dry ice and subsequently stored at -80 ºC until analysis.

Sample analysis

Feed samples were homogenized (Krups Speedy Pro homogenizer) and analysed for dry matter and ash (Kolar 1992), crude protein (ISO 2005; assuming crude protein = 6.25×Kjeldahl nitrogen), lipid (Bligh & Dyer 1959), and phosphorus (ISO 1998). Dietary gross energy was measured using a bomb calorimeter (IKA-Calorimeter C7000, IKA Analysentechnik, Heitersheim, Germany) after drying of the diet samples for 48 h at 60 ºC. Finally, feed samples were analysed for EAA content (Larsen et al. 2012).

The EAA plasma concentrations were analysed after the samples were thawed at room temperature and deproteinised (adding 1:4 distilled water, 1:4 sulfosalicylic acid and centrifuged 20 min at 9000 rpm at 5 ºC). After deproteinisation, the samples were derivatized with Waters' AccQ Tag reagent according to the manufacturer instructions (Waters Corporation, Milford, Massachusetts, USA) and diluted 1:4 in a sodium acetate buffer (0.1 mol l^{-1} sodium acetate, 30 mL L^{-1} acetonitrile (60%), pH 6.2). Identification and quantification was performed using an uHPLC system (Flexar FX-10, PerkinElmer Inc., Waltham, Massachusetts, USA), using gradients of sodium acetate (0.1 mol l^{-1}) and acetonitrile (60%) to separate the different amino acids on the chromatographs. A standard amino acid solution (Sigma-Aldrich, St. Louis, Missouri, USA) was used to identify and quantify the content of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. The chromatographs for the different samples were analysed using the Chromera Flexar v3.2.0. 4847 software (PerkinElmer Inc., Waltham, Massachusetts, USA).

Data analysis

The original dataset was composed of 20 variables (dietary methionine form, sampling time, dietary level and plasma concentrations of each of the nine EAAs measured) from 504 individuals (fish). Statistical analysis was performed using R statistical software (R Core Team 2013).

Normality

All variables were tested for normality prior to further analysis. Variables were checked visually using histograms and QQ-plots, and statistically using the Shapiro test for normality where a $P>0.05$ was considered normally distributed. Logarithmic transformations were performed to obtain normality for data on histidine, methionine and threonine plasma concentrations, while square root transformations were performed to obtain normality for the data on arginine, phenylalanine and lysine plasma concentrations.

Principal Component Analysis (PCA) and selection of outliers

In order to describe the dataset and explore the possibilities of reducing the number of variables by identifying patterns, a Principal Component Analysis (PCA) was performed using the R package FactoMineR (Husson et al. 2013). The score (not shown) and loading plots were used to describe the distribution of the individuals and variables, respectively. A variances plot (not shown) was used to display the explained variance (%) by the components. Including components that could explain 90 % of the variance was considered sufficient.

Models: generalized additive model (GAM) and linear model (LM)

Linear and Generalized Additive Models, LMs and GAMs respectively (Hastie and Tibshinari 1990; Zuur et al. 2009), were applied to test the three hypotheses and clarify the nature of these relationships. GAMs are nonparametric or semi-parametric generalizations that allow for non-linear relationships between the response variable and multiple explanatory variables. Splines were used to summarize the trend of a response variable as a function of one or more predictor variables and replace least square fits in regressions. The non-parametric nature of splines means that it does not assume a rigid form for the dependence of the response variable on the predictor variable. Where linear models assume that a response is linear for each predictor, additive models assume that each predictor affects the response in a smooth way. The amount of smoothing is determined by the number of degrees of freedom applied to the smoothing spline function of each covariate. All covariates were included in the initial full model. The least significant covariates, as determined by their P value, were removed one at a time, until all covariates were significant ($P<0.05$; Zuur et al. 2009). The final models are then reduced versions of these full models. The analyses were performed using the R (R Core Team 2013) package "mgcv" (Wood 2011).

Model applied to test hypothesis 1: Form and level of dietary methionine affect postprandial methionine plasma concentrations in a predictive manner.

The following GAM aimed at dissociating the effect of dietary level and form on methionine plasma concentrations during digestion (Table 2). The full model was formulated as follows:

(1) **GAM (log Met ~ Factor(Met Form) + *s*(Dietary Met, k = 4) + *s*(Time, k = 4))**

where log Met denotes the log transformed methionine plasma concentrations, Met Form represents the three dietary forms (free, coated and bound; Table 2), Dietary Met are the levels of methionine provided in the different feeds, Time denotes the sampling time after meal ingestion, and *s* is an isotropic smoothing function, uniform in all orientations, used to define the smooth terms (thin-plate regression spline; Wood 2003, 2006). To simplify the interpretation of the results, the maximum degrees of freedom (measured as number of knots k) allowed to the smoothing functions were limited for the variables dietary methionine level and time (k = 4). A Gaussian distribution was used seeing as the methionine plasma concentrations were log transformed and followed a normal distribution. The identity link was applied to describe the relationship between the mean value of the dependent variable and 3 covariates.

Model applied to test hypothesis 2: The postprandial plasma concentrations of other EAA (arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine and valine) are affected by the dietary form and plasma concentrations of methionine.

The EAA plasma concentrations were tested individually as a response to their respective dietary content and the dietary form and plasma concentrations of methionine (Table 2). A significant correlation between dietary methionine levels and methionine plasma concentrations was established (R^2=0.592, P<0.001), and the dietary methionine level was therefore not included in the model. Furthermore, plasma EAA concentrations were assumed *a priori* to be influenced more by methionine plasma concentrations than methionine dietary level. The full model was formulated as:

(2) **GAM ($tEAA_i$ ~ *s*(Dietary EAA_i) + factor (Met Form) + *s*(log Met) + *s*(Time))**

where $tEAA_i$ denotes the transformed plasma concentration of the EAA in question (EAA_i = {Arginine; Histidine; Isoleucine; Leucine; Lysine; Phenylalanine; Threonine; Valine}), Dietary EAA_i defines the levels of the EAA_i provided in the different feeds, log Met represents the log-transformed methionine plasma concentrations, Time denotes the sampling time after meal ingestion, Met Form defines the three different methionine forms in the diets, and *s* is an isotropic smoothing function, uniform in all orientations, used to define smooth terms (thin-plate regression spline; Wood 2003, 2006). The maximum degrees of freedom allowed to the smoothing functions were limited for the variables dietary EAA level, methionine plasma concentration and time (k = 4). A Gaussian distribution was used as the methionine plasma concentrations were log transformed and followed a normal distribution. The identity link was applied to describe the relationship between the mean value of the dependent variable and 4 covariates.

Model applied to test hypothesis 3: BCAA plasma concentrations (isoleucine, leucine and valine) follow a linear relationship over time.

The following model was used to test how plasma concentrations of isoleucine, leucine and valine during digestion were related to each other (Table 2). A LM was used because the relationship between the three BCAAs is expected to be of a linear form. The LM was formulated as:

(1) **LM ($BCAA_1 \sim BCAA_2 + BCAA_3$)**

where $BCAA_1$, $BCAA_2$ and $BCAA_3$ refer to either of the three BCAAs.

Results

Feed composition

The proximate composition, energy content and EAA profiles of the experimental diets are presented in Table 1. The protein and energy content was quite similar in ten of the twelve diets (varying between 40.5-42.8% protein and 22.7-23.3 MJ kg^{-1}). Diet Bound 3 and Bound 6 deviated somewhat from the rest, Bound 3 being lower in both protein and energy (35.7% and 21.5 MJ/kg), and Bound 6 being higher in protein (46.4%). In all cases, all diets were considered to contain sufficient levels of protein and energy to support optimal growth of rainbow trout (NRC 2011). Regarding the EAA profiles, the diets differed primarily in lysine and methionine content, lysine varying from 2.8 % dry matter (DM) in Free 2 to 4.5% DM in Bound 6, and methionine varying from 0.6 % DM in Free 0 (consider deficient) to 1.6 % DM in diet Bound 6. In comparison, the official rainbow trout requirements of lysine and methionine are 2.3 and 0.7 % DM, respectively (NRC 2011).

Postprandial plasma amino acid concentrations

Table 3 presents the data as average (n=7) of the postprandial plasma concentrations for each of the nine EAAs, at the six sampling times for the 12 diets. The raw data constituted the main data matrix for the statistical analyses.

Principal Component Analysis (PCA)

The loading plots for several dimensions of the PCA displayed similar patterns for the variables Isoleucine (Ile), Leucine (Leu) and Valine (Val) as shown in figure 1a and 1b, and examined in hypothesis 3. No particular patterns were observed for the other EAAs, therefore their plasma concentrations were used in hypothesis 2 (Table 2).

Model results relating to hypothesis 1: Form and level of dietary methionine affect postprandial plasma concentrations of methionine in a predictive manner.

The variables Time, Dietary Form and Level had a significant effect ($P<0.001$) on methionine plasma concentrations (Table 4). The final GAM incorporated all initial terms and was able to explain 74.3 % of the variance observed in the methionine plasma concentrations. The diagnostic plots used in model validation indicated good fits of the model (data not shown). The plasma methionine concentrations increased sharply over the first 12 h (peak) and then decreased as shown by the effect of smooth term (Fig. 2a). Dietary methionine level up to approximately 1.1% DM affected linearly methionine plasma concentrations followed an asymptotic relationship at higher dietary concentrations. The plasma methionine concentrations and dietary level followed an asymptotic relationship where, above a dietary level of 1.1 % DM, the plasma concentrations did not increase linearly with a dietary increase (Fig. 2b).

Model results relating to hypothesis 2: The postprandial plasma concentrations of arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine and valine are affected by the dietary form and plasma concentrations of methionine.

All explanatory variables had a significant effect on the EAA plasma concentrations ($P<0.05$) apart from coated and bound forms of methionine on histidine ($P=0.49$ and 0.211, respectively), lysine ($P=0.372$ and 0.531, respectively), and valine ($P=0.766$ and 0.058, respectively). The models explained between 28.7 and 53.6 % of the variance (Table 5). The dietary levels of EAAs had a significant effect on their respective plasma concentrations (Table 5), however, no common patterns were observed between the EAAs (Fig. 3a-e and 4a-c). Methionine plasma concentrations similarly had a significant positive effect on all other EAA plasma concentrations ($P<0.001$; Fig. 3f-j), and almost linear effect on for the three BCAAs (Fig 4d-f). With respect to time, all plasma EAA concentrations increased during the first 12 h after meal ingestion, then decreased to initial levels at different rates (Fig. 3k-o and 4g-i). Arginine, isoleucine, leucine, phenylalanine and valine showed large plasma variations over time while histidine and threonine remained near constant.

Model results relating to hypothesis 3: BCAA plasma concentrations (isoleucine, leucine and valine) follow a linear relationship over time.

As shown in Table 6 all linear models presented strong relationships ($R^2>0.9$) and supported the results from the PCA where the variation of BCAAs plasma concentrations appeared to be closely related. Figure 5 presents a 3D plot of the relationship between the three BCAAs with an indication of the dietary methionine form. The graph illustrates that the plasma concentration of the BCAAs of individuals in the postprandial state follow a close-to-linear relation, irrespective of the dietary form and time of sampling.

Discussion

Hypothesis 1: Form and level of dietary methionine affect postprandial plasma concentrations of methionine in a predictive manner

In aquaculture, the increasing use of sustainable protein sources changes feed compositions beyond what can be considered natural for carnivorous fish. Plant-based raw materials and CAAs are typical ingredients known to challenge the nutritive value of a diet. However, these ingredients are so far essential for aquafeed production to remain viable both in terms of economic and environmental perspectives.
As previously shown for rainbow trout, plasma EAA profiles are influenced by the choice of raw materials (Larsen et al. 2012), dietary EAA levels (Plakas et al. 1980; Dabrowski 1982; Schuhmacher et al. 1995; Rolland et al. 2014), and the dietary EAA form (Yamada et al. 1981; Murai et al. 1987; Cowey & Walton 1988; Schuhmacher et al. 1997; Ambardekar et al. 2009; Rolland et al. 2014). In the present study, methionine was supplied in three different forms (free, coated and bound) at a wide range of inclusion levels (from 0.60 to 1.62 % DM) using highly digestible protein sources. The results showed that the plasma methionine concentrations in fish fed less than 1.1 % DM methionine responded linearly to the dietary content. At methionine inclusions beyond ~1.1 % DM, the tendency changed and plasma concentrations approached but did not completely reach a plateau at a dietary inclusion level of 1.62 % DM. Higher inclusion levels should be tested to identify if and when maximum saturation occurs. Whether or not this response can be interpreted as saturation kinetics or reflects an active physiological process in which plasma methionine is maintained below toxic levels requires further investigation. Methionine is the most toxic of the EAAs in chickens when ingested in excess (Katz & Baker 1975). Methionine toxicity not only reduces growth in rats (Stekol & Szarn 1962), but has also been shown to have adverse effects on nitrogen utilization in humans (Garlick 2006) and chickens (Harter & Baker 1978). However, to our knowledge, no study has evaluated methionine toxicity in rainbow trout or in any other fish species.

The statistical model used indicated that with the knowledge of only a few dietary parameters, it is possible to provide highly accurate estimates of postprandial methionine plasma levels at any given time. The unexplained variances in the model might well be due to dietary parameters not included in the model, and/or to endogenous contributions of methionine to the plasma. Dietary AA levels and the form in which they are supplied alter the efficiency with which protein is retained or utilized in rainbow trout (Bodin et al 2012). In the present study, we have shown how these two variables apparently affect available plasma concentrations of methionine. Considering that the nutrient levels in the plasma reflect what is available for metabolic functions (e.g. protein synthesis), further studies aiming at linking plasma EAA concentrations to the metabolic status of key tissues (e.g. liver) are necessary. Such studies could potentially provide an in-depth explanation on the effects of dietary parameters on growth and feed utilization in rainbow trout.

Hypothesis 2: The postprandial plasma concentrations of other EAA (arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine and valine) are affected by the dietary form and plasma concentrations of methionine

The second hypothesis tested to which extent the dietary form of methionine and its plasma concentrations influence the plasma profile of arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine and valine. In rainbow trout, plasma amino acid profiles have previously been shown to reflect the nutritional status (Navarro et al. 1997). The results presented here show that they also respond to their respective dietary levels as well as the methionine plasma concentrations during digestion.

In a study on non-fed rainbow trout, a significant correlation between the disappearance rate of AAs from the plasma and their molar proportion in the whole body protein was found (Robinson et al. 2011). Moreover, the authors proposed that plasma AA turnover was linked to body functions, e.g. plasma delivers AAs to tissues and organs in the required proportion to optimize protein synthesis. In that respect, changes in the plasma concentration of a single AA may exert an effect on the entire AA plasma profile. The present results are consistent with this, showing that methionine plasma concentrations significantly influence the plasma concentrations of all other EAAs tested. The response to plasma methionine and postprandial time of sampling for all EAAs followed similar trends. However, the explanatory variables (methionine form and an amino acid dietary level) tested in the model did not have equal effects on all EAAs. The free methionine form (crystalline) was the only category to have a significant effect on all EAAs tested. Interestingly, supplementation of AAs in the crystalline form is known to strongly affect their plasma profiles compared to protein bound AAs (Yamada et al. 1981; Murai et al. 1987; Cowey & Walton 1988; Schuhmacher et al. 1997; Ambardekar et al. 2009; Rolland et al. 2014). Additionally the present results showed that supplementation of a single EAA in the crystalline form affects the plasma profiles of other EAAs.

In general, the model in the current study explained between 30 to 50 % of the variances. The unexplained variances may be the result of differences in uptake pathways, other biological functions or dietary parameters not considered in the model and known to affect AA utilization (e.g. protein or energy content; Green & Hardy 2008). The intention of the model was, however, not to explain the relationship between dietary and metabolic changes but rather to illustrate that dietary manipulation of one EAA, in this case methionine, influences plasma concentrations of other EAAs such as arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine and valine.

Hypothesis 3: BCAA plasma concentrations (isoleucine, leucine and valine) follow a linear relationship over time

Isoleucine, leucine and valine possess an aliphatic side chain and have similar chemical properties, why they are grouped as BCAAs. They are quantitatively and qualitatively (Navarro et al. 1997) important nutrients,

being the most abundant EAAs in the plasma of rainbow trout (constituting 55-58%). In mammals, they account for approximately 35% of the EAAs found in muscle protein (Harper et al. 1984). The BCAA results showed that, apart from being significantly affected by dietary parameters and plasma methionine concentrations, there was a significant relationship between their individual plasma concentrations during digestion. Therefore, by knowing the plasma concentration of one BCAA it is possible to predict the concentrations of the other two.

BCAAs share common cell membrane transport systems (Morris 2006) and the two first metabolic enzymes (catalyzed by branched-chain aminotransferase and branched-chain ketoacid dehydrogenase complex, BCKDC, respectively). Therefore, they have been hypothesized to influence kinetics and requirements of each other (Cynober & Harris 2006). The model supports this hypothesis, and the interactions between BCAAs kinetics imply that studies investigating the requirement or effect of a single BCAA should consider the status of the two others.

In healthy rats, BCAAs have been found to stimulate muscle protein synthesis (Kobayashi et al. 2006). Administration of isoleucine and valine alone had no effect (Kimball & Jefferson 2006a), while leucine was found to be almost as efficient as a mixture of the three BCAAs (Kimball & Jefferson 2006b). Leucine acts on protein synthesis by up-regulating the binding step in translation initiation of mRNA but also by activating the mammalian target of rapamycin (mTOR) pathway (Kimball & Jefferson 2006b; Li et al. 2009). Nevertheless, the present study stresses the influence that BCAAs have on each other's plasma concentrations. Isoleucine and valine could therefore affect protein synthesis through influencing leucine plasma concentrations, explaining the lack of response when they are administered individually.

Conclusion

With the aid of generalized additive and linear models, the current study showed that a few dietary parameters (methionine level and form) have a significant effect on postprandial plasma methionine concentrations in rainbow trout. Furthermore, the models showed that the plasma methionine level and dietary form apparently affect the plasma concentrations of other EAAs including arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine and valine. Finally, the models showed that postprandial plasma concentrations of BCAAs (isoleucine, leucine and valine) no matter the diet. The present study adds valuable new information regarding the effect of diets on the dynamics of the EAAs in the plasma pool, as well as on how individual EAAs apparently influence each other.

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Table 1 Recipe, proximate composition (g kg-1 feed) and essential amino acid content (g kg-1 Dry Matter) of the 12 experimental diets

	Free0	Free1	Free 2	Free3	Free4	Free5	Coated3	Coated4	Coated5	Bound3	Bound5	Bound6
Ingredients formulation (g kg-1 feed)*												
Fish Meal	80,0	80,0	80,0	80,0	80,0	80,0	80,0	80,0	80,0	258,0	448,2	634,0
Krill meal	20,0	20,0	20,0	20,0	20,0	20,0	20,0	20,0	20,0	20,0	20,0	20,0
Soya Protein Concentrate	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	100,0	66,7	31,7	-
Pea Protein Concentrate	365,4	365,4	365,4	365,4	365,4	365,4	365,4	365,4	365,4	243,8	115,8	-
Wheat gluten	189,0	189,0	189,0	189,0	189,0	189,0	189,0	189,0	189,0	189,0	158,0	114,0
Fish Oil	110,0	110,0	110,0	110,0	110,0	110,0	110,0	110,0	110,0	108,0	111,0	112,0
Rapeseed Oil	110,0	110,0	110,0	110,0	110,0	110,0	110,0	110,0	110,0	108,0	111,0	112,0
Monocalcium Phosphate	10,6	10,6	10,6	10,6	10,6	10,6	10,6	10,6	10,6	-	-	-
L-Histidine	1,15	1,15	1,15	1,15	1,15	1,15	1,15	1,15	1,15	-	-	-
D-L Methionine	-	1,10	2,09	3,19	4,29	6,50	-	-	-	-	-	-
Coated D-L Methionine	-	-	-	-	-	-	2,61	5,36	8,12	-	-	-
Vitamin and mineral premix	0,94	0,94	0,94	0,94	0,94	0,94	0,94	0,94	0,94	0,79	0,68	0,68
Proximate composition (g kg-1 feed)												
Moisture	77,9	83,3	82,4	89,0	83,0	83,2	83,1	79,4	80,0	101,4	59,6	44,7
Protein	420,8	419,6	420,4	418,2	419,1	417,8	424,4	427,6	426,0	356,6	405,1	463,8
Fat	278,6	271,6	284,4	275,8	283,7	279,9	266,7	266,5	268,4	272,4	295,9	306,0
Phosphorus	10,2	10,3	10,2	10,2	10,1	10,1	10,5	10,3	10,4	10,4	14,1	18,2
Ash	48,0	47,8	46,7	46,3	46,3	46,4	47,9	48,1	47,8	62,4	83,7	106,2
Energy	233,3	231,4	232,6	230,3	232,4	232,4	228,9	230,8	227,2	214,7	227,6	232,9
Essential amino acid (g kg-1 DM)												
Arginine	33,93	34,17	30,36	35,54	37,05	34,72	37,58	29,63	29,40	30,10	31,75	31,54
Histidine	12,40	12,30	11,10	12,54	13,77	12,21	13,60	14,87	14,10	12,16	15,24	16,86
Isoleucine	17,99	18,99	17,82	19,76	20,31	20,69	21,53	23,29	22,85	18,11	21,39	23,51
Leucine	34,45	35,05	32,04	36,40	37,70	36,51	39,33	40,74	39,85	32,26	38,01	42,09
Lysine	29,08	30,92	28,22	30,57	30,82	32,35	33,43	34,54	33,24	31,59	39,53	44,52
Methionine	6,01	6,83	7,08	9,55	10,87	12,94	9,21	11,80	13,80	9,06	12,78	16,17
Phenylalanine	21,84	22,09	25,05	24,57	25,60	24,15	25,91	25,82	25,00	19,42	21,46	22,83
Threonine	16,57	16,97	14,90	17,31	17,91	17,20	18,49	21,08	20,95	17,10	20,54	23,46
Valine	18,67	19,61	19,26	20,12	20,87	21,37	22,64	24,06	23,95	20,35	24,24	26,71

*Fish meal: SA Super Prime (Peru); Krill meal: South American, 56 % protein ; Soya Protein concentrate: 60% protein; Pea Protein concentrate; 72 % protein; D-L Methionine: Evonik Industry AG, Germany; Coated D-L Methionine: D-L methionine povided by Evonik Industry AG, Germany and coated by Sparos, Portugal; Vitamin and mineral premix: is estimated to meet the requirements according to NRC (2012)

Table 2 Description of the variables used in the three different models

Model components	Abbreviation	Description		
Hypothesis 1				
Methionine	Met	Concentration of methionine in the plasma (µmol/ml)	Response variable	Continuous
Methionine level	Dietary Met	Dietary methionine content (% DM)	Explanatory variable	Continuous (smooth term)
Time	Time	Sampling time (hour)	Explanatory variable	Continuous (smooth term)
Methionine form	Met Form	Methionine form in the diet	Explanatory variable	Categorical 3 levels (Free, Coated, Bound)
Hypothesis 2				
Arginine	Arg	Plasma concentration of arginine (µmol ml-1)	Response variable	Continuous
Histidine	His	Plasma concentration of histidine (µmol/ml)	Response variable	Continuous
Isoleucine	Ile	Plasma concentration of isoleucine (µmol/ml)	Response / explanatory variable	Continuous
Leucine	Leu	Plasma concentration of leucine (µmol/ml)	Response / explanatory variable	Continuous
Lysine	Lys	Plasma concentration of lysine (µmol/ml)	Response variable	Continuous
Phenylalanine	Phe	Plasma concentration of phenylalanine (µmol/ml)	Response variable	Continuous
Threonine	Thr	Plasma concentration of threonine (µmol/ml)	Response variable	Continuous
Valine	Val	Plasma concentration of valine (µmol/ml)	Response / explanatory variable	Continuous
Methionine	Met	Plasma concentration of methionine (µmol/ml)	Explanatory variable	Continuous (smooth term)
Dietary EAA	Dietary EAA	Dietary content for the EAA tested as a response variable	Explanatory variable	Continuous (smooth term)
Time	Time	Sampling time (hour)	Explanatory variable	Continuous (smooth term)
Methionine form	Met Form	Methionine form in the diet	Explanatory variable	Categorical 3 levels (Free, Coated, Bound)
Hypothesis 3				
Isoleucine	Ile	Plasma concentration of isoleucine (µmol/ml)	Response / explanatory variable	Continuous
Leucine	Leu	Plasma concentration of leucine (µmol/ml)	Response / explanatory variable	Continuous
Valine	Val	Plasma concentration of valine (µmol/ml)	Response / explanatory variable	Continuous

Table 3 Postprandial plasma concentrations (nmol ml-1) of essential amino acids (EAA; mean±SD, n=7) measured for the 12 diets at 6 different time (hr) points after meal ingestion

EAA	Time	Free 0	Free1	Free 2	Free 3	Free 4	Free 5	Coated 3	Coated 4	Coated 5	Bound 3	Bound 5	Bound 6
Arginine	T0	176.1±89.0	214.5±48.0	194.3±74.9	194.3±92.4	116.6±22.9	154.0±57.5	116.5±39.8	179.3±63.3	212.7±100.0	144.9±40.6	157.5±25.3	156.1±51.8
	T3	228.4±60.7	197.4±67.7	244.9±71.0	187.1±66.3	243.8±78.5	234.9±61.2	201.2±43.7	244.5±71.2	208.7±42.8	151.3±57.0	224.4±44.4	145.6±41.8
	T6	234.1±69.0	282.0±83.5	308.6±58.8	316.1±92.8	367.4±116.7	382.0±74.3	316.3±72.8	289.6±85.5	335.8±123.3	238.6±44.1	311.4±88.1	220.5±27.7
	T12	303.2±74.1	406.3±66.3	357.4±49.8	343.9±65.0	368.9±54.4	425.4±96.2	387.2±61.3	338.9±46.7	306.2±78.8	280.7±59.3	221.0±44.3	282.1±66.6
	T20	257.9±62.1	291.0±60.3	298.9±65.8	246.0±78.3	230.7±59.5	280.7±89.8	237.2±77.9	244.2±83.2	301.0±79.5	169.5±60.6	210.1±53.9	301.3±41.4
	T36	275.1±46.4	180.4±88.3	254.1±90.9	180.9±74.8	208.1±84.5	189.2±57.2	105.0±37.7	162.7±80.0	211.1±74.9	134.2±60.2	139.6±54.0	134.6±28.3
Histidine	T0	150.6±30.9	173.4±21.5	171.7±26.6	182.8±39.6	120.1±23.0	122.4±18.6	114.9±52.3	168.8±28.7	183.6±51.4	165.3±30.8	197.6±50.1	185.6±31.3
	T3	143.0±24.7	136.5±23.1	123.7±23.8	114.6±25.2	141.5±33.1	122.1±11.4	138.2±26.1	145.1±28.5	140.6±28.6	129.0±23.8	172.9±22.9	147.9±20.2
	T6	144.5±29.9	168.2±25.2	174.0±16.9	171.3±45.3	210.7±50.0	218.8±27.1	194.2±49.3	184.6±36.4	188.2±53.0	142.6±20.8	218.6±55.0	193.6±16.4
	T12	148.4±36.0	171.0±32.3	177.3±25.7	179.5±18.8	266.3±74.8	236.9±49.8	283.3±32.0	229.6±36.4	195.2±44.3	179.0±30.7	179.1±16.0	221.0±39.2
	T20	147.1±29.1	155.5±41.4	190.1±44.6	164.2±46.6	139.8±31.1	167.7±45.4	182.3±33.8	157.6±26.4	188.1±29.6	162.2±33.3	188.3±33.4	265.1±29.7
	T36	219.7±39.3	167.3±29.9	194.2±35.7	155.1±16.7	145.5±22.8	130.0±15.3	140.6±22.2	155.6±39.7	164.0±22.0	165.6±29.6	157.2±16.9	172.1±30.8
Isoleucine	T0	123.2±49.2	139.9±47.2	150.8±54.5	187.7±60.8	105.1±29.2	86.0±15.2	98.0±33.5	186.4±30.8	184.3±58.7	142.7±27.9	156.9±35.7	156.6±47.1
	T3	219.2±32.1	180.6±37.5	271.6±58.6	182.0±69.0	238.2±50.6	227.2±20.9	217.1±21.6	213.6±44.8	173.0±28.5	160.1±35.4	206.3±38.0	215.7±58.2
	T6	200.6±56.9	180.2±37.7	251.4±51.1	215.8±57.0	252.8±96.9	211.8±18.0	247.8±57.8	247.2±61.6	243.5±57.3	222.8±41.9	265.2±76.7	302.0±50.7
	T12	180.1±56.7	273.2±66.5	251.4±26.5	202.7±28.7	263.5±52.9	269.2±62.2	214.0±45.9	247.5±67.7	200.9±45.7	154.8±31.9	167.8±13.4	233.4±41.0
	T20	261.6±43.4	273.0±24.0	222.6±58.1	211.0±22.1	166.0±26.4	233.1±36.1	156.3±34.0	172.8±47.9	159.4±39.6	195.9±35.3	180.3±16.8	203.9±23.9
	T36	186.5±39.1	179.3±63.5	206.3±48.5	202.1±57.3	162.2±43.4	148.4±29.4	117.8±38.0	123.9±29.9	148.1±37.0	133.8±31.4	146.9±27.2	143.2±24.1
Leucine	T0	195.8±85.4	226.7±81.3	241.2±82.5	299.3±106.2	152.6±38.1	130.9±26.0	144.4±56.0	296.3±58.5	274.0±94.9	218.1±45.5	236.4±53.7	225.1±56.7
	T3	343.1±49.8	290.8±58.9	430.0±84.3	301.1±112.8	390.0±91.8	358.6±45.0	336.2±36.1	327.5±71.9	271.8±28.6	245.0±50.7	330.4±63.3	331.2±84.3
	T6	319.8±108.5	285.7±74.4	423.6±93.9	355.1±99.1	426.4±166.7	354.3±31.9	411.2±97.7	423.8±108.8	398.1±100.3	356.8±61.1	449.5±137.8	517.7±72.7
	T12	279.9±90.6	458.1±125.8	425.3±50.0	340.6±48.2	463.4±86.5	496.4±102.7	394.0±72.5	453.4±109.7	355.1±82.2	262.6±47.5	283.0±26.7	382.2±77.4
	T20	434.2±76.4	451.7±38.8	373.4±98.6	353.0±36.6	279.4±51.7	394.0±64.7	256.7±48.8	289.1±84.9	266.2±59.5	304.6±46.8	297.9±28.6	330.5±43.4
	T36	302.2±59.9	280.3±103.3	324.2±75.4	312.1±91.0	256.8±69.6	235.9±47.7	188.8±58.6	198.6±49.0	235.9±61.8	200.8±48.9	223.7±44.8	218.2±38.9
Lysine	T0	167.4±80.3	216.0±74.6	180.4±73.9	228.5±88.0	111.4±43.8	132.1±51.0	135.9±83.5	231.1±86.0	303.0±182.4	235.5±76.7	334.9±113.5	356.5±140.9
	T3	230.8±67.8	196.0±42.8	227.4±54.0	196.2±59.4	188.9±68.9	174.7±31.7	226.5±74.0	243.3±62.7	159.2±33.3	168.9±69.1	304.3±79.7	277.8±61.4
	T6	189.8±50.8	276.3±69.2	251.6±54.8	279.4±87.0	292.7±116.9	272.7±72.6	283.0±93.8	277.0±93.5	335.5±116.4	274.5±59.5	412.1±114.9	403.2±77.8
	T12	281.4±104.2	344.9±98.6	322.1±66.0	301.9±59.7	380.1±79.1	432.8±108.7	377.1±117.2	381.4±59.4	228.6±82.0	266.0±89.1	260.1±46.2	521.6±57.3
	T20	286.5±74.0	319.1±97.7	335.6±116.1	220.8±88.5	196.3±81.7	294.5±90.9	199.6±77.4	212.3±76.2	267.1±94.8	264.9±105.6	313.1±65.6	495.8±91,7
	T36	429.1±93.5	234.1±109.7	277.4±34.4	239.8±74.7	196.7±104.8	193.7±57.2	133.8±29.0	188.9±76.6	188.6±87.0	177.3±49.3	269.1±93.7	315.4±79.5
Methionine	T0	63.3±14.8	59.6±11.4	101.4±33.3	131.6±30.1	121.9±25.3	182.4±47.0	70.1±29.8	158.1±62.4	240.0±68.0	107.2±15.2	118.8±22.2	118.4±29.4
	T3	58.5±14.8	83.7±23.4	118.2±21.7	142.8±38.8	236.1±52.8	383.3±50.4	160.9±50.0	329.0±122.8	373.5±130.0	96.0±25.3	165.9±34.3	149.9±32.8
	T6	56.8±26.4	102.7±26.9	180.0±43.0	218.8±66.8	415.0±83.9	655.9±99.8	211.1±66.6	371.6±90.6	512.3±233.9	145.1±18.7	236.4±71.4	265.7±41.7
	T12	66.9±16.3	145.0±37.7	228.1±33.8	362.0±70.2	570.1±143.5	769.1±129.6	210.4±60.7	403.7±92.2	480.2±150.0	133.4±17.7	176.9±38.5	283.1±62.6
	T20	49.4±9.9	86.1±19.0	207.2±71.5	259.1±94.7	289.2±107.0	434.8±164.4	122.0±32.6	261.1±91.2	507.5±126.9	105.7±35.6	199.5±56.9	398.5±106.5
	T36	49.1±6.5	68.2±18.7	99.4±23.3	140.2±24.6	155.8±34.0	241.3±55.8	81.8±16.2	169.7±41.4	220.6±72.2	92.2±13.3	126.0±30.7	146.1±18.7
Phenylalanine	T0	141.8±46.6	183.9±43.9	229.6±58.0	192.1±94.0	128.4±54.1	141.7±31.9	90.4±30.9	148.5±42.0	169.2±62.2	139.0±34.9	129.2±20.6	111.8±28.1
	T3	180.9±29.3	165.2±46.9	245.4±34.6	172.9±47.3	220.3±41.9	247.2±47.6	194.9±10.6	189.3±62.4	188.0±53.6	134.5±26.7	154.3±20.4	105.4±27.0
	T6	195.3±76.9	214.9±48.7	260.3±55.7	203.8±62.9	282.5±71.9	290.5±39.2	270.9±64.7	252.2±40.4	228.6±64.6	185.6±28.6	200.5±48.9	173.4±30.3
	T12	227.0±41.3	230.1±36.7	280.8±28.7	277.5±13.7	307.9±41.1	298.0±62.6	295.7±36.8	271.3±30.0	266.0±33.9	213.3±40.3	181.3±30.4	211.2±31.7
	T20	254.5±51.8	233.9±48.7	306.8±49.2	252.6±83.0	226.1±60.4	237.9±49.0	246.2±35.8	233.3±55.9	251.2±48.6	151.8±59.1	173.2±26.5	234.0±46.6
	T36	175.3±40.1	187.0±49.4	213.5±64.9	201.4±48.0	191.3±67.7	177.6±40.1	141.5±28.8	176.5±36.2	174.3±42.7	138.4±39.8	122.1±28.8	117.1±24.9

Threonine	T0	125.3±46.2	139.9±48.8	134.6±44.5	154.4±54.0	108.9±15.2	116.8±35.3	103.6±23.2	151.0±25.3	203.9±82.2	154.4±32.4	237.3±46.2	354.7±81.7
	T3	161.4±48.4	121.0±33.1	155.3±18.4	116.1±43.8	148.0±47.6	144.1±29.9	134.5±43.7	163.6±40.0	143.1±18.7	104.4±28.0	230.0±41.3	231.9±46.7
	T6	134.1±41.8	162.0±49.4	168.4±23.4	180.7±59.5	217.1±86.8	233.2±31.8	207.0±77.2	190.7±68.4	222.1±78.9	155.3±22.9	329.3±163.4	386.5±72.1
	T12	192.9±58.8	212.2±58.5	209.0±32.0	186.1±29.8	274.4±86.8	244.5±75.0	213.9±50.8	200.6±37.7	219.8±69.3	193.6±54.9	239.8±64.9	456.3±118.5
	T20	170.2±50.0	185.6±54.5	227.8±92.7	190.3±85.0	140.9±31.3	215.8±95.8	162.5±49.3	176.0±53.9	218.3±60.0	185.3±93.6	259.6±52.8	551.0±78.0
	T36	222.5±52.3	152.0±32.9	177.9±51.2	146.1±25.0	165.0±38.3	162.8±25.7	126.6±24.5	176.1±68.7	176.9±46.7	162.8±53.0	210.8±56.3	329.3±69.5
Valine	T0	267.6±83.5	296.0±90.5	329.4±105.2	404.0±96.8	227.4±54.0	187.6±33.5	196.3±65.9	380.9±73.0	372.4±117.0	344.2±71.0	337.9±69.5	348.9±77.2
	T3	423.8±48.4	375.6±48.3	517.5±62.4	375.8±97.5	451.0±66.3	430.2±33.4	408.4±36.9	405.9±65.4	341.7±46.6	341.2±52.5	428.1±72.0	456.7±118.1
	T6	411.0±107.7	368.8±63.0	488.0±95.7	436.8±78.8	490.6±162.7	424.2±29.2	470.5±102.4	501.1±106.0	474.5±111.6	458.5±64.8	567.1±156.1	642.5±79.5
	T12	405.4±117.1	517.2±142.5	498.1±35.2	439.4±55.0	543.6±98.1	590.2±92.0	506.4±101.1	526.5±96.8	413.3±104.7	338.6±55.8	377.9±41.6	523.7±93.3
	T20	532.0±95.2	536.9±58.5	503.4±134.4	422.0±70.3	339.7±42.1	457.3±82.3	340.9±50.9	357.8±89.5	350.3±60.0	422.4±81.0	423.7±48.8	537.0±55.8
	T36	394.0±64.7	349.3±115.3	393.9±65.1	380.1±90.2	325.1±77.3	295.0±43.0	242.7±61.7	253.7±53.3	288.3±70.4	267.1±47.2	290.0±61.7	304.1±65.0

Table 4 Generalized additive model (GAM) results for plasma methionine concentration in response to time, dietary level and form

Terms	Estimatea	Lowerb	Upperb	P value	Explained variance	R^2
Categorical term						
Methionine form - Free	1	0,95	1,053	<0.001		
Methionine form - Coated	0,789	0,722	0,863	<0.001		
Methionine form - Bound	0,513	0,465	0,566	<0.001		
Smooth term						
Time	4,036			<0.001		
Methionine level	2,724			<0.001	74,3	0,738

aThe estimate for the smooth terms are estimated degrees of freedom; bLower and upper values for the confident interval are calculated using the Estimate minus SE and plus SE, respectively. The SE is given by the model.

Table 5 Generalized additive model (GAM) results for variation of the plasma concentration given for each essential amino acid responding to its dietary level, methionine plasma concentration, dietary methionine from and time

EAA	Terms	Estimatea	Lowerb	Upperb	P value	Explained variance	R^2
Arginine	Categorical term						
	Methionine form - Free	0	-0,049	0,049	<0.001		
	Methionine form - Coated	-1,164	-1,373	-0,956	0.001		
	Methionine form - Bound	-3,253	-3,407	-3,099	<0.001		
	Smooth terms						
	Arginine dietary level	2,767			0,016		
	Methionine concentration	1,835			<0.001		
	Time	2,965			<0.001	52,9	0,519
Histidine	Categorical term						
	Methionine form - Free	1	0,965	1,036	<0.001		
	Methionine form - Coated	0,977	0,915	1,043	0,49		
	Methionine form - Bound	0,952	0,883	1,028	0,211		
	Smooth terms						
	Histidine dietary level	2,69			0,029		
	Methionine concentration	2,648			<0.001		
	Time	2,72			<0.001	28,7	0,272
Isoleucine	Categorical term						
	Methionine form - Free	0	-9,367	9,367	<0.001		
	Methionine form - Coated	-24,372	-43,033	-5,711	0,011		
	Methionine form - Bound	-31,563	-49,207	-13,919	<0.001		
	Smooth terms						
	Isoleucine dietary level	2,23			0,001		
	Methionine concentration	2,043			<0.001		
	Time	2,929			<0.001	30,3	0,290
Leucine	Categorical term						
	Methionine form - Free	0	-15,784	15,784	<0.001		
	Methionine form - Coated	-42,815	-73,597	-12,033	0,007		
	Methionine form - Bound	-60,277	-90,984	-29,570	<0.001		
	Smooth terms						
	Leucine dietary level	2,059			0,009		
	Methionine concentration	1			<0.001		
	Time	2,96			<0.001	36,6	0,355
Lysine	Categorical term						
	Methionine form - Free	0	-0,105	0,105	<0.001		
	Methionine form - Coated	0,183	-0,266	0,632	0,372		
	Methionine form - Bound	0,094	-0,373	0,560	0,531		
	Smooth terms						
	Lysine dietary level	2,931			<0.001		
	Methionine concentration	1,956			<0.001		
	Time	2,798			<0.001	34,4	0,330

Phenylalanine	Categorical term						
	Methionine form - Free	0	-0,030	0,030	<0.001		
	Methionine form - Coated	-1,393	-1,552	-1,235	<0.001		
	Methionine form - Bound	-5,933	-6,041	-5,825	<0.001		
	Smooth terms						
	Phenylalanine dietary level	2,784			0,007		
	Methionine concentration	2,606			<0.001		
	Time	2,83			<0.001	53,6	0,526
Threonine	Categorical term						
	Methionine form - Free	1	0,953	1,048	<0.001		
	Methionine form - Coated	0,874	0,797	0,958	0,004		
	Methionine form - Bound	1,134	1,036	1,241	0,007		
	Smooth terms						
	Threonine dietary level	2,652			<0.001		
	Methionine concentration	1,664			<0.001		
	Time	2,581			<0.001	52,0	0,510
Valine	Categorical term						
	Methionine form - Free	0	-16,121	16,121	<0.001		
	Methionine form - Coated	-5,118	-38,740	28,504	0,766		
	Methionine form - Bound	-29,148	-59,308	1,012	0,058		
	Smooth terms						
	Valine dietary level	2,916			<0.001		
	Methionine concentration	1			<0.001		
	Time	2,869			<0.001	41,5	0,404

aThe estimate for the smooth terms are estimated degrees of freedom; bLower and upper values for the confident interval are calculated using the Estimate minus SE and plus SE, respectively. The SE is given by the model.

Table 6 Results of the linear model (LM) for the variation of the plasma concentration for each the three branched chain amino acids responding to the two others

Variables	R^2	P-value
Isoleucine	0,9705	<0.001
Leucine	0,9722	<0.001
Valine	0,9184	<0.001

Figure captions

Figure 1 Results of the principal component analysis (PCA). Correlation between the original variables and the corresponding dimensions (Dim) of the PCA, with the respective variance explained (%), (A) Dim 4 *vs*. Dim 2, (B) Dim 6 *vs*. Dim 2. Black arrows show the distribution of plasma amino acid concentrations and dietary methionine level and form. The remaining variables (amino acid dietary contents), appear in greys arrows.

Figure 2 Effect of postprandial sampling time (a) and dietary methionine level (% Dry Matter) (b) on plasma methionine concentrations applied with a smooth term (k=4). Each point represents an individual fish. Postprandial time of sampling and dietary methionine level both affected significantly the methionine plasma concentrations ($P<0.001$).

Figure 3 Effect of methionine dietary level (Dietary level; % Dry Matter; a-e), plasma methionine concentration (Methionine; nmol ml^{-1}; f-j), and postprandial sampling time (Time; hr; k-o) on variations in other essential amino acids measured in the plasma applied with a smooth term (k=4) on their respective plasma concentrations. Each point represents an individual fish. All parameters add significant effects on EAA plasma concentrations ($P<0.05$).

Figure 4 Effect of methionine dietary level (Dietary level; % Dry Matter; a-c), plasma methionine concentration (Methionine; nmol ml^{-1}; d-f), and postprandial sampling time (Time; hr; g-i) on plasma variations in branched chain amino acids applied with a smooth term (k=4) on their respective plasma concentrations. Each point represents an individual fish.

Figure 5 Three dimensional graph representing the linear relationship in plasma concentrations (nmol ml^{-1}) of isoleucine, leucine and valine obtained from fish fed different forms of dietary methionine (black = free, white = coated and grey = bound).

Figure 1

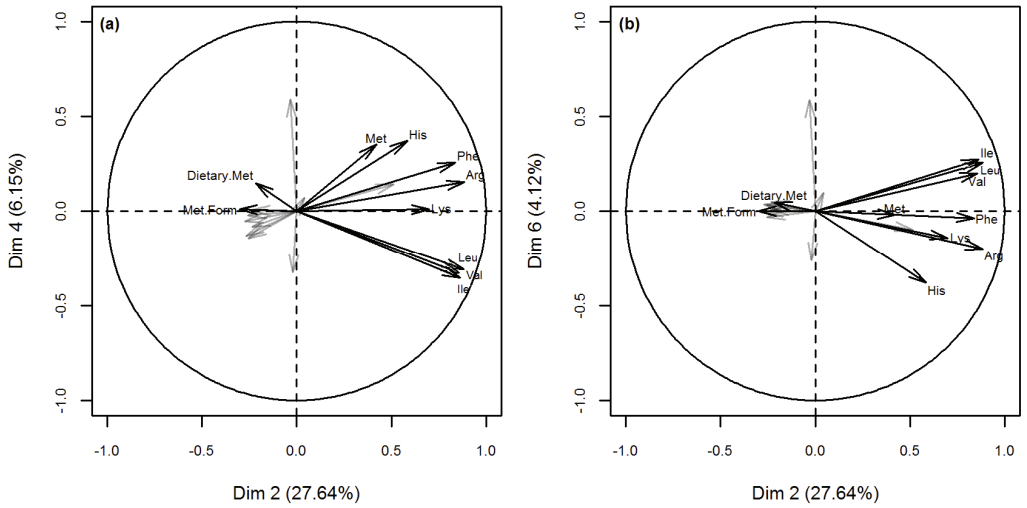

(a)
Met
His
Phe
Arg
Dietary.Met
Met.Form
Lys
Leu
Val
Ile
Dim 4 (6.15%)
Dim 2 (27.64%)
1.0
0.5
0.0
-0.5
-1.0
(b)
Ile
Leu
Val
Dietary.Met
Met.Form
Met
Phe
Lys
Arg
His
Dim 6 (4.12%)
Dim 2 (27.64%)

Figure 2

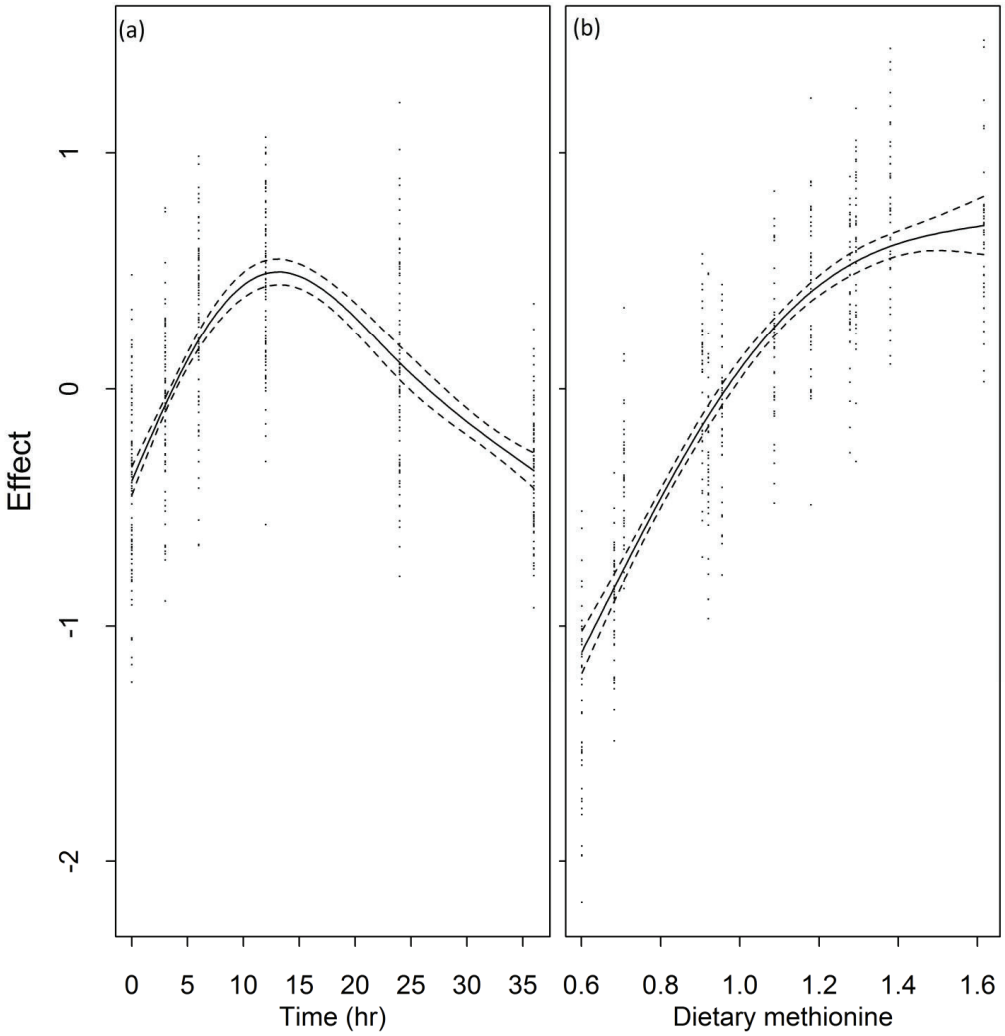

(a)
(b)
Effect
1
0
-1
-2
0
5
10
15
20
25
30
35
Time (hr)
0.6
0.8
1.0
1.2
1.4
1.6
Dietary methionine

Figure 3

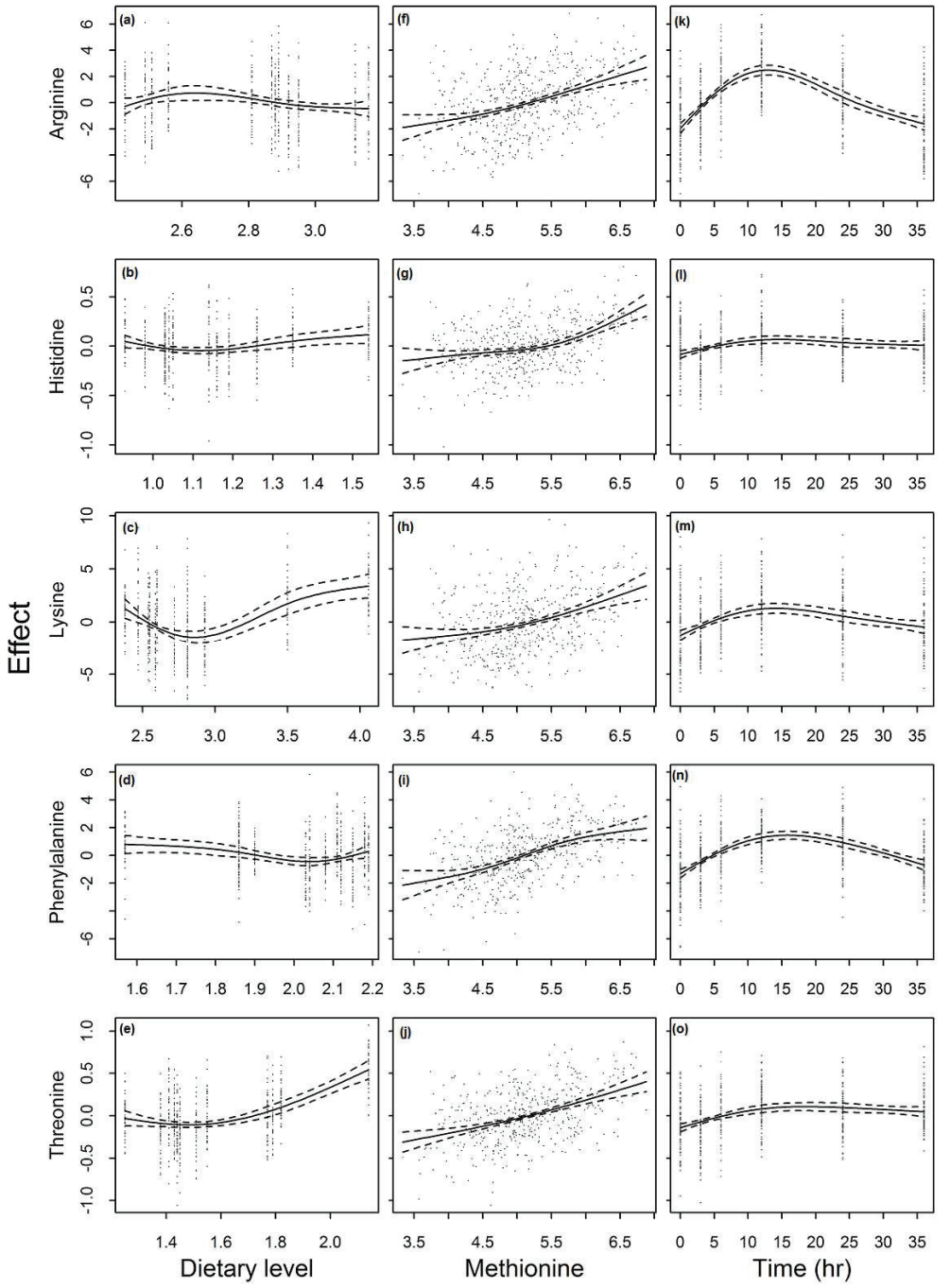

Effect
Arginine
Histidine
Lysine
Phenylalanine
Threonine
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
Dietary level
Methionine
Time (hr)

Figure 4

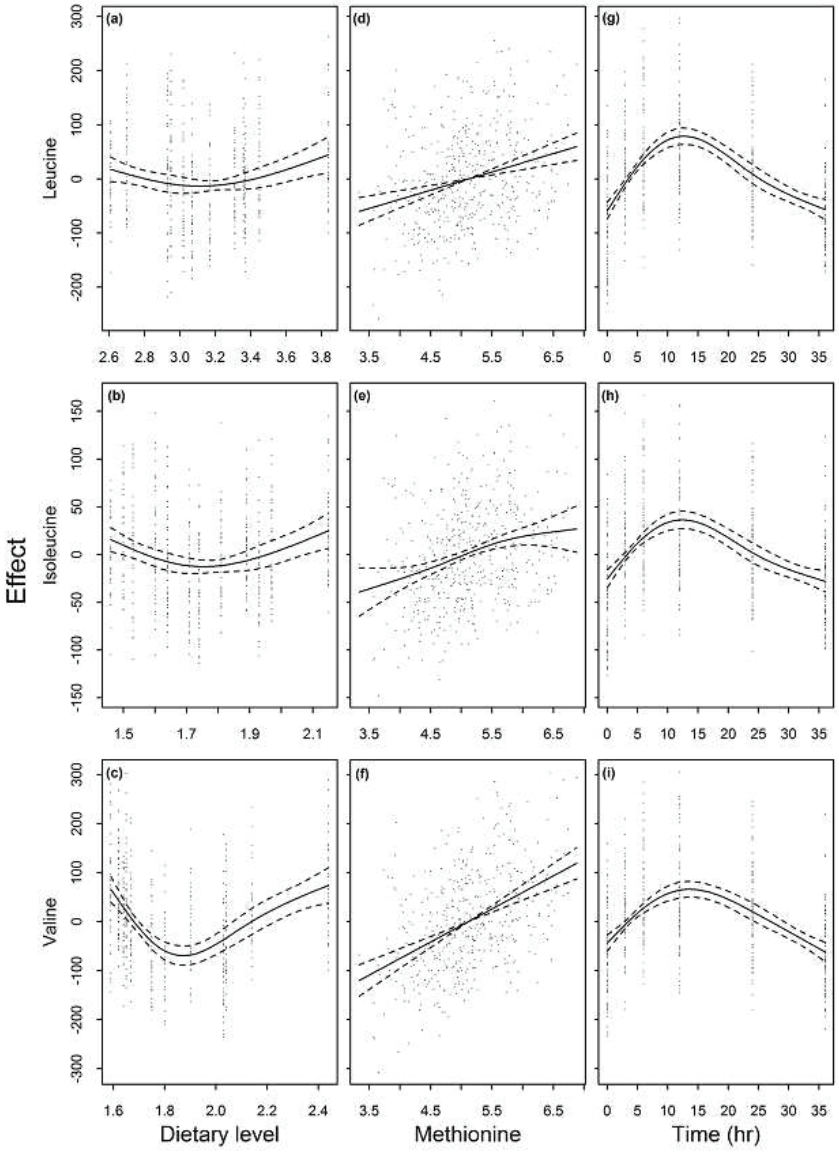
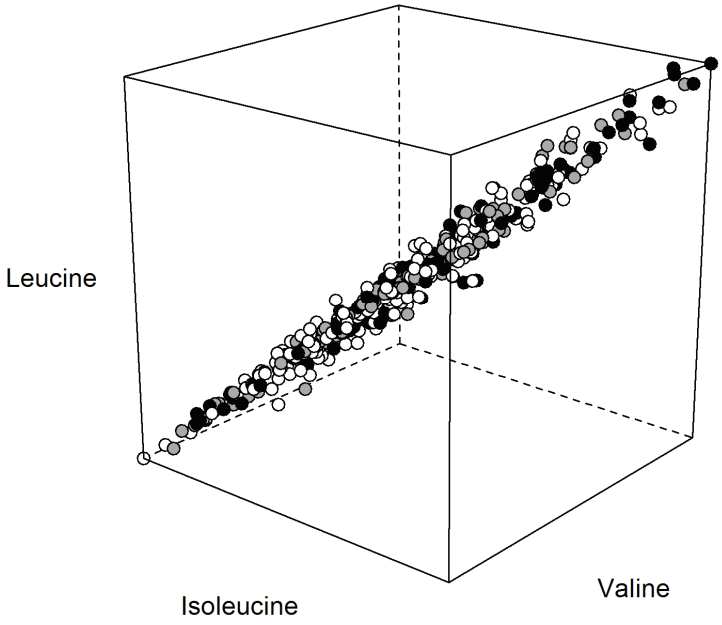

Effect
Leucine
Isoleucine
Valine
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Dietary level
Methionine
Time (hr)

Figure 5



Paper III: Dietary methionine level affects growth performance and hepatic gene expression of GH-IGF system and protein turnover regulators in rainbow trout (Oncorhynchus mykiss) fed plant protein-based diets

Dietary methionine level affects growth performance and hepatic gene expression of GH-IGF system and protein turnover regulators in rainbow trout (*Oncorhynchus mykiss*) fed plant protein-based diets

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Abstract:

The effects of dietary level of methionine was investigated in juvenile rainbow trout (*Oncorhynchus mykiss*) fed five plant-based diets containing increasing content of crystalline methionine (Met), in a six weeks growth trial. Changes in the hepatic expression of genes related to i) the somatotropic axis: including the growth hormone receptor I (GHR-I), insulin-like growth hormone I and II (IGF-I and IGF-II, respectively), and insulin-like growth hormone binding protein-1b (IGFBP-1b); and ii) protein turnover: including the target of rapamycin protein (TOR), proteasome 20 delta (Prot 20D), cathepsin L, calpain 1 and 2 (Capn 1 and Capn 2, respectively), and calpastatin long and short isoforms (CAST-L and CAST-S, respectively) were measured for each dietary treatment. The transcript levels of GHR-I and IGF-I increased linearly with the increase of dietary Met content ($p<0.01$), reflecting overall growth performances. The apparent capacity for hepatic protein degradation (derived from the gene expression of TOR, Prot 20D, Capn 1, Capn 2, CAST-L and CAST-S) decreased with increasing dietary Met level in a relatively linear manner. Our results suggest that Met availability affects, directly or indirectly, the expression of genes involved in the GH/IGF axis response and protein turnover, which are centrally involved in the regulation of growth.

Keywords: Fish; Crystalline amino acid; Somatotropic axis; Proteolytic pathways; TOR

1. Introduction

Commercial aquafeeds are nutrient dense diets formulated to provide the fish with a ratio of digestible protein to energy (DP:DE) considered optimal for growth performance (Kaushik and Cowey, 1991; Cho and Bureau, 2001). Recently, an increased awareness on the importance of amino acid (AA) profiles in high performance diets rather than protein origin has emerged. Although fish meal remains the preferred protein source for carnivorous fish species, economical and sustainability constraints drive a trend towards increased substitution with land-based protein sources which differ, among other things, in the amino acid profiles (Médale and Kaushik, 2009). Feed formulators may blend protein sources based on their AA content and supplement with crystalline amino acids (CAAs) to fulfil species requirements for essential amino acids (EAAs). However, several studies have shown that the fish do not utilize CAAs as efficiently as protein-bound AA, often resulting in reduced growth performance and poor feed utilization (Sveier et al., 2001; Bodin et al., 2012). A common perception in the literature is that there is a temporal mismatch in AA availability at the protein synthesis sites (e.g. Yamamoto et al., 1998; Dabrowski et al., 2003), possibly caused by a rapid intestinal uptake of CAAs compared to protein-bound AA (Schuhmacher et al., 1997; Ambardekar et al., 2009). Amino acid intake provides substrates for endogenous protein synthesis, and is also a metabolic system responsible for maintaining cellular homeostasis (Fafournoux et al., 2000). Because AA also serve as signalling molecules involved in essential physiological functions such as the regulation of gene expression (Fafournoux et al., 2000; Jousse et al., 2004), their homeostasis has to be precisely maintained (Fafournoux et al., 2000; Panserat and Kausik, 2010).

Dietary manipulations (protein and oil source, carbohydrate:protein ratio, AA supplementation) influence the expression of genes involved in intermediary metabolism (Seiliez et al., 2011; Wacyk et al., 2012; Skiba-Cassy et al., 2013), protein turnover (Overtuf and Gaylord, 2009; Snyder et al., 2012), and growth regulation (Gómez-Requeni et al., 2005; Hevrøy et al., 2007). Growth is largely under the control of the growth hormone/insulin-like growth factor (GH/IGF) system (Reinecke et al., 2005; Fuentes et al., 2013). In addition to the GH and IGFs, in many fish species including salmonids the GH/IGF (or somatotropic) axis also includes two growth hormone receptors (GHRs), IGF receptors and IGF-binding proteins (IGFBPs). Circulating GH, which is secreted by the pituitary gland, enhances the hepatic expression and plasma levels of IGF-I in a dose-manner relation (Funkenstein et al., 1989; Niu et al., 1993; Duan et al., 1993), and has been found to correlate with growth rates in several fish species (Reinecke and Collet, 1998). Conversely, circulating IGF-I ensures a negative feedback control on GH secretion (Pérez-Sánchez et al., 1992). The GHRs present in the liver of fish including the rainbow trout (*Oncorhynchus mykiss*) transduce the signal from circulating GH into the cells (Gabillard et al., 2006a). Consequently, GHRs play an essential role in integrating the changes of GH plasma levels, and in the regulation of circulating levels of IGF-I.

IGFs have been shown to promote muscular growth and development by stimulating myogenesis and cell differentiation, and by inhibiting apoptosis (Reinecke, 2006b). The liver is the main site for gene

expression of IGF-I (Duan et al., 1993; Duguay et al., 1994) and the major site of synthesis for circulating IGF-I and IGF-II (Plisetskaya, 1998). Plasma levels of both IGF-I and IGF-II have been correlated to hepatic mRNA levels, and reflected in body mass and growth rates in rainbow trout (Gabillard et al., 2003, 2006b). In salmonids, the hepatic gene expression and plasma levels of IGF-I have been shown to respond to nutritional status (Chauvigné et al., 2003; Hevrøy et al., 2011), ration size (Pérez-Sánchez et al., 1995; Metón et al., 2000), and diet macronutrient composition (Metón et al., 2000; Gómez-Requeni et al., 2005). IGFs circulate in plasma almost exclusively bound to binding proteins (IGFBPs), which also modulate the regulatory role of IGFs by increasing their half-life and protecting them from proteolytic degradation, among others. Six distinct IGFBPs families, homologous to mammalian IGFBPs (IGFBP-1, -2, -3, -4, -5, -6), have been characterized in fish. Because certain teleost lineages (such as salmonids) experienced four genome replications (4R), MacQueen et al. (2013) found 19 different genes coding for IGFBPs. As for IGFs, the liver is believed to be responsible for the endocrine response with only few IGFBP-1 and IGFBP-2 relatives carrying the hepatic message (IGFBP-1b paralogs 1 and 2, and IGFBP-2b1; Shimizu et al., 2011; MacQueen et al., 2013). IGFBPs are regulated differently by the nutritional state; the gene expression of IGFBP 1 and 2 being down-regulated in feeding state in fish. Though, IGFBP-1 displays the most reactive regulation in relation to nutritional condition (Jousse et al., 1998). In fish, IGFBP-1 is an inhibitor of IGF action and its expression is driven by protein deprivation, leading to growth defection (Straus et al., 1993; Gay et al., 1997; Kelley et al., 2001) and down-regulated in re-fed salmon (MacQueen et al., 2013). In mammals, IGFBP-1 is believed to be a key component involved in the growth limitation in response to protein deficiency and triggered, at the cellular level, by AA deprivation associated with hormonal signals (Jousse et al., 1998).

The GH/IGFs system is known to regulate protein synthesis and degradation through the target of rapamycin (TOR) pathway (Fuentes et al., 2013). The TOR cascade is under AA and hormonal control, and regulates gene expression and protein synthesis at the translation step. The regulatory roles of single AA differ, with leucine and methionine (Met) exerting a greater regulatory effect on gene expression than other AAs such as lysine (Kimball and Jefferson, 2006; Lansard et al., 2011). *In vivo*, the functional role of AAs is further influenced by hormonal signals and feedback mechanisms as well as by interactions with other nutrients, reflecting the animals overall nutritional status (Seiliez et al., 2011; Skiba-Cassy et al., 2013) and dietary AA profile (Espe et al., 2007; Hevrøy et al., 2007; Wacyk et al., 2012). The TOR pathway is regulated by successive phosphorylation of the different components and is therefore usually investigated at the protein level, using measurement of the phosphorylated state. However, the response in terms of gene expression of the TOR protein to increasing doses of a single AA has, to our knowledge, not been investigated previously.

Protein turnover is the net result of synthesis and degradation. Proteolysis is achieved through several pathways under the control of IGFs (Fuentes et al., 2013), involving different proteases including the i) ubiquitin pathways (proteasome 20 delta, Prot 20D), ii) lysosomal protein degradation (Cathepsine-L), and

the iii) calpain cascade involving calpain 1 and 2 (Capn 1 and Capn 2 respectively) and their inhibitors, the long and short isoforms of calpastatin (CAST-L and CAST-S, respectively). Protein degradation is an important factor in regulating protein turnover (Salem et al., 2005), sensitive to the nutritional status, the composition of the diet (Snyder et al. 2012; Belghit et al., 2013) but also Met deficiency (Belghit et al., 2014).

The increasing interest in investigating the GH/IGF system and protein turnover in farmed fish species reflects the need to understand the underlying factors altering their growth performance. In the present study, the hepatic gene expression of several components of the GH/IGF axis (GHR-I, IGF-I, IGF-II and IGFBP-1b), protein synthesis (TOR) and proteolysis (Prot 20D, Cathepsine-L, Capn 1, Capn 2, CAST-L and CAST-S) were determined in rainbow trout fed plant-based diets with different Met levels, supplied as crystalline form.

2. Material and methods

2.1. Fish and diets

Juveniles of rainbow trout were obtained from a commercial fish farm (Funderholme Dambrug A/S, Denmark) and kept in quarantine for three weeks in a recirculated freshwater system (exchange rate of 8% of the total water volume per day). During this period, the fish were hand-fed a commercial diet (BioMar A/S, Brande, Denmark) once per day to apparent satiation.

Five nutrient dense diets (4.5 mm extruded pellets labelled Met 1, Met 2, Met 3, Met 4 and Met 5) were designed using the same raw materials matrix, in order to obtain similar proximate composition. The diets were produced at the Tech Center, BioMar A/S, Brande, Denmark. The diets contained mainly plant protein ingredients (wheat gluten, soya protein concentrate and pea protein concentrate), with Met 1 used as the reference recipe (Table 1). The plant raw materials were all high quality products with respect to their nutritional composition (Davies et al., 1997; Thiessen et al., 2003; Collins et al., 2012). Diets Met 2 to Met 5 were designed using the reference recipe and increasing the supplementation of crystalline D-L Met (0.1, 0.3, 0.4, and 0.7 g 100 g^{-1}; Table 1). As per design, the diets were isoproteic and isoenergetic (41.9±0.1 g $100g^{-1}$ feed and 23.2±0.1 MJ kg^{-1} feed, respectively). The AA profile of all diets fulfilled NRC requirements (NRC, 2011) and differed in the Met content (Table 1).

2.2. Growth trial

Prior to the growth trial, fish were fasted for 48 h, and 1050 rainbow trout (116.8 ± 0.6 g; mean ± SD) were subsequently stocked in 700-L fiberglass tanks in a number of 70 fish per tank. The tanks were mounted in a separate, recirculating aquaculture system. Freshwater was replaced at a rate of 8 % of the total volume per day, and a 12:12 h light:dark photoperiod and temperature (15.1 ± 0.1 ºC) were maintained throughout the trial. For a period of six weeks, triplicate tanks per dietary treatment were fed in excess each

day from 8:00 to 12:00 using automatic belt feeders. Uneaten pellets were collected in swirl separators attached to the tanks and quantified immediately at the end of each feeding period.

The day prior to terminating the growth trial (and 24 h after the last feeding), seven individuals per treatment were randomly selected and sacrificed in a lethal dose of anaesthetic (phenoxyethanol, 1 ml L^{-1}). Individual body masses were recorded, and livers dissected while placing the fish on ice. The livers were stored in cryogenic plastic tubes and flash-frozen in liquid nitrogen. The samples were kept at -80ºC until further analysis on gene expression. To terminate the trial, feed was withheld for another 24 h and the biomass in each tank was recorded. No mortality was recorded during the trial period.

2.3. Chemical analysis

Feed samples were homogenized using a Krups Speedy Pro homogenizer and analysed for dry matter and ash (NMKL, 1991), crude protein (ISO, 2005; assuming crude protein = 6.25×Kjeldahl nitrogen) and lipid (Bligh and Dyer, 1959). Dietary gross energy was measured using a bomb calorimeter (IKA-Calorimeter C7000, IKA Analysentechnik, Heitersheim, Germany) after drying of the diet samples for 48 h at 60 ºC.

The EAA content of each diet was determined using the method described by Larsen et al. (2012), based on Rutherfud (2009). In brief, 9 mg of finely grinded pellets (4 replicates per diet) were transferred to a 5 ml vacuum hydrolysis tube (Thermo Scientific, Rockford, Illinois, USA) with 1.7 ml HCl (6 mol L^{-1}) containing 0.2% (v/v) phenol. Air vacuuming and nitrogen gas were alternatively applied (4 times, 1 min each) to insure oxygen removal, using a three-way stopcock. After an acid hydrolysis, 24 h at 110°C in a Reacti-Therm Heating Module (Pierce, Thermo Scientific, Rockford, Illinois, USA), the samples were spiked with an internal standard (nor-leucine; Sigma-Aldrich, St. Louis, Missouri, USA). The hydrolysis tubes were rinsed with 1 ml of water each and the HCl removed with vacuum centrifugation (CentriVap, Labconco, Kansas City, Missouri, USA). Finally the samples were re-dissolved using 3 ml sodium acetate buffer (0.1 mol L^{-1} sodium acetate, 30 ml L^{-1} acetonitrile (60%), pH 6.2) and derivatized with Waters AccQ Tag reagent (Waters Corporation, Milford, Massachusetts, USA) according to the manufacturer instructions. The samples were then further diluted 1:4 in sodium acetate buffer (describe above). Identification and quantification were performed using an uHPLC system (Flexar FX-10, PerkinElmer Inc., Waltham, Massachusetts, USA), and using gradients of the sodium acetate buffer (describe above) and acetonitrile (60%) to separate the different AAs on the chromatographs. A standard AA mixture supplemented with nor-leucine (Sigma-Aldrich, St. Louis, Missouri, USA) was used to identify and quantify the 10 AAs. The results are expressed as % dry matter (DM).

2.4. Gene expression analysis

Gene expression levels were determined by real-time qPCR (qRT-PCR) for targeted genes involved in the GH/IGF axis, TOR signalling pathway and protein degradation. The primer sequences as well as the accession number in the GenBank (http://www.ncbi.nlm.nih.gov/genbank) are given in Table 2.

Total RNA samples were extracted from liver samples using NucleoSpin® RNA (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's recommendations. In brief, the samples were homogenized, cells were lysed and the lysate filtrated. The RNAs binding conditions were adjusted before adding DNase followed by three washes. The purity (A_{260}/A_{280}) of the total RNA was tested using spectrophotometry (NanoDrop1000, Thermo Scientific) and visual inspection of products (S18 and S28) on a 1 % agarose gel. A quantity of 0.5 µg of the resulting total RNA was reversed transcribed into cDNA using AffinityScript qPCR cDNA synthesis kit (Agilent Technologies, California, USA) and oligo dT primers (DELTAgene™ Assays, Fluidigm Corporation, San Francisco, USA) according to the manufacturer's instructions, including a specific target amplification (hold at 95 ºC for 10 min, 14 cycles 95 ºC 15 s - 60 ºC 4 min and terminated by a hold period at 4ºC) and a step to remove unincorporated primers using Exonuclease I (New England BioLabs, MA, USA). Target gene expression levels were determined by quantitative qRT-PCR using 48x48 Dynamic Array™ IFC on a BioMark HD System (Fluidigm Corporation, San Francisco, USA). Primers are detailed in Table 2 and were designed to overlap an intron if possible using known sequences in rainbow trout nucleotide databases (GenBank and Signae). The qRT-PCR was carried out using SsoFast™ EvaGreen® Supermix with Low ROX (Bio-Rad Laboratories, CA, USA). The protocol was initiated at 95 ºC for 60 s for initial denaturation of the cDNA and a hot start of the hydrolysis probes. The procedure was continued by a two-step amplification programme (5 s at 96 ºC followed by 20 s at 60 ºC) repeated 30 times. Melting curves were systematically monitored (temperature gradient at 1 ºC s^{-1} from 60 to 95 ºC) at the end of the last amplification to confirm amplification reaction. Each run included gene duplicates, control sample and two types of negative controls; non-reverse transcriptase treated (i.e. not containing cDNA 'RT control') and no-template ('blank') samples. Elongation factor 1α (EF1α) and β-actin were employed as a non-regulated reference genes, as previously used in rainbow trout and analysed both in duplicate on each chip. No changes in EF1 or in β-actin gene expression were observed in our investigation (data not shown). The data analysis was started using Fluidigm® Real-Time PCR Analysis Software v.3.0.2 and BioMark HD Data Collection Software v.3.1.2. The relative quantification of target gene was performed using the mathematical model described by Pfaffl (2001). The relative expression ratio (R) of a target gene was calculated on the basis of real-time PCR efficiency (E) and CT deviation (ΔCT) of the average unknown sample versus average control sample, and expressed in comparison to the EF1α reference gene as the PCR efficiency was slightly better than for β-actin. PCR efficiency (E) was measured by the slope of a standard curve using serial dilutions of cDNA of a pool sample. PCR efficiency values ranged between 1.9 and 2.1.

2.5. Statistical analysis

All statistical analyses were performed using SigmaPlot v. 11 (Systat Systems Inc., USA). The data were tested for normal distribution using a Shapiro test and in case of failure, simple transformations (logarithm, reverse or square root) or combinations of them. When all data had passed the normality test, one-way ANOVA, followed by the Holm–Sidak multi comparisons procedure, was carried out. Correlations between data were examined using Pearson's Correlation test. Values are throughout presented as mean ± SD (SE for gene expression results presented in figures). A probability of $P<0.05$ was considered significant in all analyses.

3. Results

3.1. Growth performance and feed utilization

The effects of increasing the dietary crystalline Met concentration significantly affected all performance parameters determined (Table 3). Feed intake (FI) increased with increasing dietary Met level ($P=0.044$); however no statistical differences using a post-hoc test was found between diets. Final weight (Wf) and specific growth rate (SGR) were significantly improved by adding Met equivalent to 0.96 and 1.09 % DM (Met 3 and Met 4, respectively) compared to the largely deficient diet Met 1. Further increasing the level of dietary Met (1.29 % DM, Met 5) eliminated any significant effects on Wf and SGR. Increasing dietary Met to 0.96 % DM (Met 3) significantly improved FCR, protein efficiency ratio (PER) and energy efficiency ratio (EER) compared to diets below current recommendations (*i.e.*, Met 1 and Met 2), whereas there were no further improvements at higher Met inclusion levels (*i.e.*, Met 4 and Met 5).

3.2. Gene expression

3.2.1. GH/IGF axis

The results for the gene expression of the different components of the GH/IGFs axis are given in Fig. 1. The expression of GHR-I linearly increased with dietary Met content (Fig. 1A). The transcription level of IGF-I was enhanced with increasing dietary Met level (Fig. 1B), being 7-fold higher when comparing the lowest (0.6% DM; Met 1) and highest (1.29% DM; Met 5) dietary Met level, while the expression of IGF-II was not affected by increasing dietary Met (Fig. 1C). The gene coding for IGFBP-1b was highly expressed in Met 2 and Met 5 (Fig. 1D).

The gene expression of GHR-I was positively correlated to that of IGF-I ($r=0.599$, $P<0.01$), but not to IGF-II ($r= 0.270$, $P =0.156$). No significant correlation was found between IGFBP-1b and IGF-I, IGF-II or GHR-I ($P=0.343$, $P=0.818$ and $P=0.539$, respectively). Interestingly, mRNA levels of GHR-I were significantly correlated to SGR ($r=0.415$, $P=0.014$) while neither the mRNA levels of IGF-I ($r=0.286$, $P=0.106$), IGF-II ($r=0.181$, $P=0.356$) nor IGFBP-1b ($r=0.059$, $P=0.753$) were correlated with SGR.

3.2.2. Protein turnover

The expression of the genes coding for TOR protein was up-regulated in fish that had been fed diets with increasing dietary crystalline Met levels, following a seemingly linear response (Fig. 2).

The expression of genes coding for enzymes involved in protein degradation pathways were affected differently by the dietary treatments (Fig. 3). The mRNA levels of Prot20D decreased in a linear manner with increasing dietary crystalline Met (Fig. 3A). The transcription of cathepsin-L was significantly affected by Met dietary level, but the trend was not linear (Fig. 3B). Hence, fish fed Met 3 had a significantly higher expression level of cathepsin-L compared to fish fed Met 5 (Fig. 3B).

The expression of Capn 1 was affected by dietary Met level responding in a weak, positive correlation to dietary increases (Fig. 3C). In contrast, Capn 2 mRNA levels were not affected by the dietary Met level. The expression of the genes coding for the long and short isoform of calpastatin (CAST-L and CAST-S, respectively), inhibitor of calpain, increased in a relatively linear manner with dietary Met levels (Fig. 3E,F). Overall, these results suggest that increasing dietary Met could lower the capacity for protein degradation, at least through the ubiquitin and calpain cascades.

The gene expression of Prot20D, and cathepsin-L were negatively correlated to the hepatic expression levels of IGF-I (r=-0.349, P=0.043; and r=-0,421, P=0.015, respectively); while CAST-L and CAST-S were up-regulated by increasing hepatic expression of IGF-I (r=0.651, P<0.001; and r=0.476, P=0.005, respectively). No correlations were found for Capn 1 and Capn 2.

4. Discussion

Increasing crystalline Met levels in the diets of rainbow trout enhanced growth, feed conversion and protein utilization in agreement with the general understanding on the effect of AA profiles on growth performance (Green and Hardy, 2008). Limited supply of dietary Met significantly reduced feed intake and, subsequently, weight gain and growth rate. Additionally, feed conversion and protein utilization for growth were significantly, negatively affected by Met deficiency as shown previously (Green and Hardy, 2008). Our results on growth performance suggest that the requirement levels of dietary crystalline Met was reached at 0.96 % DM inclusion (corresponding to Met 3), not further improved by additional Met supplementation. These results are in line with previous results by Cowey et al. (1992) but also the NRC (2011).

The GH/IGFs axis has a major function in modulating growth through complex interactions of the different components. Plasma GH levels induces increased IGF-I hepatic expression and subsequent release (Funkenstein et al., 1989; Niu et al., 1993; Duan et al., 1993), which correlates positively with growth rates (Reinecke and Collet, 1998). The signal from circulating GH is transduced into the cell by membrane receptors, GHR-I and GHR-II (Gabillard et al., 2006a). In the present study, only the expression of GHR-I was measured as this is considered the main factor involved in the hepatic transduction of the GH signal in response to nutritional changes in carnivorous fish species (Hevrøy et al., 2007; Saera-Villa et al., 2007). The

hepatic expressions of GHR-I and IGF-I were positively correlated, as previously shown for rainbow trout by Gabillard et al. (2006a). Moreover, in the present experiment the mRNA levels of GHR-I, but not IGF-I, were significantly correlated to growth rates supporting the notion that GHR-I plays a key role in the regulation of IGFs (Saera-Vila et al., 2007) and therefore in growth (Gabillard et al., 2006a). Also, the expression levels of IGFBP-1b increased together with those of IGF-I (except for diet Met 2), supporting the belief that circulating IGFs are largely bound to IGFBPs in plasma (Reinecke, 2006a; MacQueen et al., 2013), modulating their activities (Gómez-Requeni et al., 2005; Hevrøy et al., 2007).

The genetic expression and plasma levels of components of the GH/IGF axis respond to fasting/re-feeding episodes in fish (Gabillard et al., 2003; Pedroso et al., 2006), as well as to the ration level, dietary protein content (Pérez-Sánchez et al., 1995), AA profile (Gómez-Requeni et al., 2003), and protein source (Gómez-Requeni et al., 2004, 2005). In the present study, the hepatic expression of rainbow trout GHR-I increased linearly in parallel to the dietary Met content. A similar trend was found in the liver of Atlantic salmon (*Salmo salar*) in response to increasing dietary lysine (Hevrøy et al., 2007), another EAA, suggesting that the regulation of GHR-I transcripts is sensitive to the dietary AA profile in fish species including salmonids. Similarly, the hepatic expression of IGF-I, but not IGF-II, differed with dietary lysine content in salmon (Hevrøy et al., 2007), and the present results on dietary Met further suggest that the relation is of a linear form. Thus, the mRNA content of IGF-I was up-regulated in parallel to the increase of dietary Met whereas no effect was found on the transcript levels of IGF-II. In this sense, it is known that IGF-I and IGF-II respond differently according to the nutritional status in rainbow trout (Gabillard et al., 2006b). More specifically, Bower and Johnston (2010) demonstrated that Atlantic salmon myogenic cells IGF-II expression responded less to the presence of AA then did the expression of IGF-I. The mechanisms regulating IGF-I and -II at the transcriptional level seem unequally triggered by nutrients, likely reflecting the different roles of these hormones but also the tissue in which these genes are expressed (Gabillard et al., 2006b; Hevrøy et al., 2007). The expression of IGBP-1b was affected by increasing dietary Met content suggesting that, in fish as in mammals (Jousse et al., 1998), this binding protein is triggered by dietary AA availability at the transcriptional level. In this context, previous studies have shown that the expression of binding proteins are affected differently by the nutritional status (fasting and re-feeding; Gabillard et al., 2006b) but also by the protein source (Gómez-Requeni et al., 2005), and AA and hormonal signals (Bower and Johnston, 2010). IGFBP-1b is believed to be linked to the catabolic status in fish as well as in mammals, by limiting the activity of IGF-I, factor of energy-expensive growth (Kelley et al. 2001; Hevrøy et al., 2011) in response to nutritional status (Hevrøy et al., 2007). Therefore, the present results suggest that the fish fed Met 2 and Met 5 (deficient and in excess of Met, respectively) might reflect decreased active IGF-I, because bound to IGFBP-1b, leading to a decrease anabolism compared to fish fed the others diets. Taken together, the changes in the hepatic expression of IGF-I and IGFPB-1b reflect the results on growth performance and feed utilization. Indeed, while hepatic expression of IGF-I increased linearly with dietary Met content,

growth performance did not further improve with supplementation level above 0.96 %DM (Met 3) possibly due to the simultaneous increase in IGFPB-1b expression. Similarly, the hepatic expression of IGF-I for fish fed Met 2 and Met 3 were similar despite differences in growth and feed utilization. The higher IGFBP-1b expression measured for fish fed Met 2 compared to Met 3 could lead to less biologically active IGF-I in the plasma, resulting in an increase in protein catabolism in the muscle. Further studies on the relationship between IGFBPs and dietary AA levels could clarify the differential role binding proteins play in the regulation of IGF functions. However, the results suggest that both IGFs and IGFBPs should be considered together in order to determine the effect of dietary AAs on the response of the GH/IGF system, and the consequences on fish growth.

Hepatic-derived IGFs not only stimulate myogenic cells towards proliferation and differentiation, but also activate protein synthesis concomitantly with inhibiting protein degradation (Fuentes et al., 2013). Growth is achieved by protein deposition, in the case where synthesis rates exceed rates of protein degradation. The latter is a predominant factor in the efficient utilization of dietary protein for protein synthesis (Fereday et al., 1998). Previous studies have indicated that in rainbow trout muscle tissue, the three proteolytic pathways (ubiquitin, lysosomal and calpain cascade) are regulated by the nutritional status and dietary composition ,and inhibited by IGF-I through the activation of the protein kinase signalling pathway (Salem et al., 2005; Cleveland et al., 2009; Cleveland and Weber, 2010; Cleveland and Burr, 2011). The present study suggests that Met intake affects the proteolytic pathways at the transcription level in rainbow trout liver cells. The overall protein degradation activity seems to be reduced in fish fed the diet fulfilling the amino acid requirements (Met 3) compared to that of fish receiving an unbalanced amino acid supply (Met 1). The enzymes of the ubiquitin pathway (Prot 20D) and calpain cascade (Capn 1, CAST-L and CAST-S) responded linearly to dietary Met levels. That mRNA levels for IGF-I correlated with the expression levels of several enzymes in protein catabolism (negatively with Prot 20D; positively with CAST-L and CAST-S) supports the notion that both proteolytic pathways are involved in the regulation of protein turnover in response to Met availability, possibly inhibited by IGF-I (Cleveland et al., 2009; Cleveland and Weber, 2010). Furthermore, the expression of cathepsin-L was affected by the dietary Met level although not in a linear fashion. These might be in line with previous studies in rainbow trout showing that the expression of cathepsin-L in white muscle was also affected by ration size but, as in our case, following an irregular pattern (Cleveland and Burr, 2011). The present results also suggest that the control of lysosomal proteolysis is probably not achieved through the same pathways as ubiquitin-proteasome and calpain cascade, or at least it is triggered differently by the nutritional factors. This could be explained by the particularities of the different proteolysis pathways, where lysosomal protein degradation is a nonspecific bulk proteolysis process conversely to the ubiquitin pathway, which is responsible for the degradation of specific proteins (Kadowaki and Tanazawa, 2003). However, it should be mentioned that the protein turnover measured in the liver might

not reflect the growth status of the fish, but reveals the hepatic activity in response to the dietary changes designed in the present study.

The TOR pathway is a major nutrient-sensitive pathway which integrates input from several pathways to regulate gene expression. Although Met alone is not able to regulate the TOR pathway directly (Lansard et al., 2011), fish fed diets with increasing levels of Met showed an increase in hepatic expression of the TOR protein. TOR is also under the control of the GH/IGF axis and able to regulate protein turnover (Bower and Johnston, 2010; Fuentes et al., 2013). Thus, our results suggest that the capacity for regulating protein synthesis and degradation through the TOR pathway might be affected by dietary Met content. However, in order to properly reflect the effects of dietary Met changes on the TOR pathway activity, analysis should be performed at the protein level, considering the short response of the phosphorylative cascade (within few hours after meal ingestion) and the importance of this cascade in the regulation of the mTOR pathway.

In conclusion, changes in the dietary levels of Met, one of the ten EAA, affect the expression of genes involved in the GH/IGF axis, likely influencing the growth performance of the fish. The overall hepatic expression of genes coding for the proteolytic pathways also responded differentially to changes in the dietary Met levels. However, to elude the role of the TOR pathway in response to increasing dietary Met level, further studies on the activity of proteins of the cascade are needed. Finally, to confirm the effect of Met supplementation on growth through the regulation of protein degradation, further analysis should be performed on the gene expression patterns in muscle cells. The response of GH/IGF axis and protein catabolism to dietary level Met indicates that the signalling role of AAs represent an important factor regulating growth performance, giving a very valuable information when evaluating the suitability of new formulations for aquafeeds.

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Table 1

Ingredients (g $100g^{-1}$), proximate composition (g $100g^{-1}$ feed) and essential amino acid (% Dry Matter) content of the seven experimental diets.

	Met 1	Met 2	Met 3	Met 4	Met 5
Ingredients (g $100g^{-1}$)					
Fish Meal	8.0	8.0	8.0	8.0	8.0
Krill Meal	2.0	2.0	2.0	2.0	2.0
Soya Protein Concentrate	10.0	10.0	10.0	10.0	10.0
Pea Protein concentrate	36.5	36.5	36.5	36.5	36.5
Wheat	18.9	18.9	18.9	18.9	18.9
Fish Oil	11.0	11.0	11.0	11.0	11.0
Rapeseed Oil	11.0	11.0	11.0	11.0	11.0
Monocalcium phosphate	1.1	1.1	1.1	1.1	1.1
L-Histidine	0.1	0.1	0.1	0.1	0.1
Vitamin and mineral Premix[a]	0.9	0.9	0.9	0.9	0.9
D-L Methionine	-	0.1	0.3	0.4	0.7
Proximate composition (g $100g^{-1}$ feed)					
Dry Matter	92.2	91.7	91.1	91.7	91.7
Moisture	7.8	8.3	8.9	8.3	8.3
Protein	42.1	42.0	41.8	41.9	41.8
Fat	27.9	27.2	27.6	28.4	28.0
Ash	4.8	4.8	4.6	4.6	4.6
Phosphorous	1.0	1.0	1.0	1.0	1.0
Energy (MJ kg^{-1} feed)	23.3	23.1	23.0	23.2	23.2
Essential amino acid composition (% DM[b])					
Arginine	3.39	3.42	3.55	3.71	3.47
Histidine	1.24	1.23	1.25	1.38	1.22
Isoleucine	1.80	1.90	1.98	2.03	2.07
Leucine	3.44	3.50	3.64	3.77	3.65
Lysine	2.91	3.09	3.06	3.08	3.23
Methionine	0.60	0.68	0.96	1.09	1.29
Phenylalanine	2.18	2.21	2.46	2.56	2.42
Threonine	1.66	1.70	1.73	1.79	1.72
Valine	1.87	1.96	2.01	2.09	2.14
Sum essential AAs	19.10	19.69	20.64	21.49	21.21

[a]Adjusted to meet the level recommenced by the NRC (2011)
[b] Dry Matter

Table 2

Summary of forward and reverse primers used for RT-qPCR analysis and accession numbers of genes sequences (GenBank).

Gene name	Abbreviation	Forward Primer	Reverse Primer	Acc. Number
Reference gene				
Elongation factor 1 alpha	*EF1α*	CGGAGGCATTGACAAGAGAAC	AGCACCCAGGCATACTTGAA	AF498320
Protein turnover				
Target of rapamycin	*TOR*	ATGGTTCGATCACTGGTCATCA	TCCACTCTTGCCACAGAGAC	EU179853
Proteasome 20 delta	*Prot 20D*	GGTTCTGCTGCCGATACTCA	GGGGCTTCATCCAGCTCAATA	AF115539
Cathepsin L	*Cathepsin-L*	CAACTACCTGCAGGCACCTA	ACATGATCCCTGGTCCTTGAC	AF358668
Calpain 1	*Capn 1*	CAGAGCTGCAGACCATACTCA	AAGCTTCCGGTCCAAATCCA	AY573919
Calpain 2	*Capn 2*	AGCCTGAACAACACTCTCCA	CGCAAGCCACGAAGTTATCA	AY573920
Calpastatin L	*CAST-L*	ACATAGTCACGGAGGGCAAA	ACTTTGGTGGGAGTGTGTCA	AY937407
Calpastatin S	*CAST-S*	ATAGAGCCCTCCATGGACTCA	CAGTTGGAGCCACAGAGGAA	AY937408
Somatotropic axis				
Growth hormone receptor-I	*GHR-1*	TGGAAGACATCGTGGAACCA	ATCAAAGTGGCTCCCGGTTA	AF403539
Insulin-like growth factor-I	*IGF-I*	TGCGGAGAGAGGCTTTTA	AGCACTCGTCCACAATACCA	M81904
Insulin-like growth factor-II	*IGF-II*	CATGGTGCCCACACTCAAAC	CTTCCTCTGCCACACCTCATA	AY049955
Insulin-like growth factor binding protein-1b	*IGFBP-1b*	TCCGCAAGAAACTGGTGGAA	GTTCCTGCTGAGAGCTGGTTA	AY662657

Table 3

Effect of increasing dietary methionine level on growth performance of fish fed the experimental diets for 6 weeks. Each value represents mean ± SD of data from triplicate groups. Results within rows differing significantly from each other are followed by different superscript letter.

		Met 1	Met 2	Met 3	Met 4	Met 5	p-value
Initial Weight (kg)	Wi	8.29±0.04	8.42±0.09	8.34±0.36	8.21±0.14	8.12±0.43	0.732
Final Weight (kg)	Wf	14.74±0.79^{a}	16.63±0.98^{ab}	17.52±0.44^{ab}	17.60±0.46^{b}	15.81±1.26^{ab}	0.022
Feed Intake (kg)	FI	6.05±0.76	7.42±0.92	7.89±0.54	7.92±0.43	6.56±0.67	0.044
SGR[1]	SGR	1.55±0.13^{a}	1.84±0.17^{ab}	2.01±0.15^{b}	2.06±0.12^{b}	1.80±0.09^{ab}	0.005
FCR[2]	FCR	0.94±0.01^{a}	0.90±0.00^{b}	0.86±0.01^{c}	0.85±0.01^{c}	0.85±0.01^{c}	<0.001
PER[3]	PER	2.75±0.25^{a}	2.87±0.01^{b}	3.06±0.03^{c}	3.08±0.03^{c}	3.06±0.03^{c}	<0.001
EER[4]	EER	49.66±0.45^{a}	52.20±0.16^{b}	55.55±0.47^{c}	55.66±0.55^{c}	55.10±0.55^{c}	<0.001

[1] Specific growth rate (%) = 100 × (ln Wf. - ln Wi.)/days

[2] Feed conversion ratio (g/g) = feed intake/wet weight gain

[3] Protein efficiency ratio (g/g) = wet weight gain/crude protein intake

[4] Energy efficiency ratio (g/J) = wet weight gain/energy intake

Fig. 1. Hepatic relative gene expression of (A) GHR-I, (B) IGF-I, (C) IGF-II, and (D) IGFBP-1b in response to increasing dietary level of crystalline methionine. Results differing significantly from each other are indicated by different lower case letters. The correlation coefficient (r) and *P*-value for Pearson's correlation test are given for each gene. All values are reported as mean ± SE (n=7).

Fig. 2. Hepatic relative gene expression of target of rapamycin protein (TOR) in response to increasing dietary level of crystalline methionine. Results differing significantly from each other are indicated by different lower case letters. The results for Pearson's correlation test are given as correlation coefficient (r) and *P*-value. All values are reported as mean ± SE (n=7).

Fig. 3. Hepatic relative gene expression of (A) Prot 20D, (B) Cathepsin-L, (C) Capn 1, (D) Capn 2 ,(E) CAST-1, and (F) CAST-S, respectively) in response to increasing dietary level of crystalline methionine. Results differing significantly from each other are indicated by different, lower case letters. The correlation coefficient (r) and p-value for Pearson's correlation test are given for each gene. All values are reported as mean ± SE (n=7).

Fig. 1.

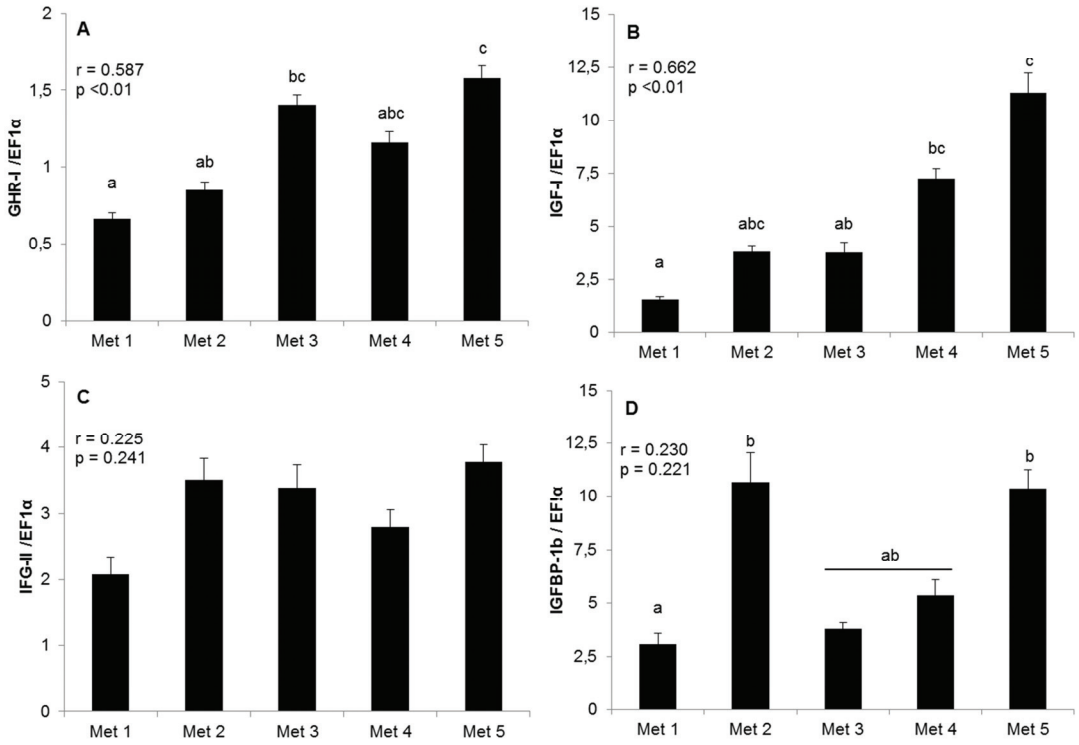

A
r = 0.587
p <0.01
GHR-I /EF1α
a
ab
bc
abc
c
Met 1
Met 2
Met 3
Met 4
Met 5
B
r = 0.662
p <0.01
IGF-I /EF1α
a
abc
ab
bc
c
C
r = 0.225
p = 0.241
IFG-II /EF1α
D
r = 0.230
p = 0.221
IGFBP-1b / EF1α
a
b
ab
b

Fig. 2.

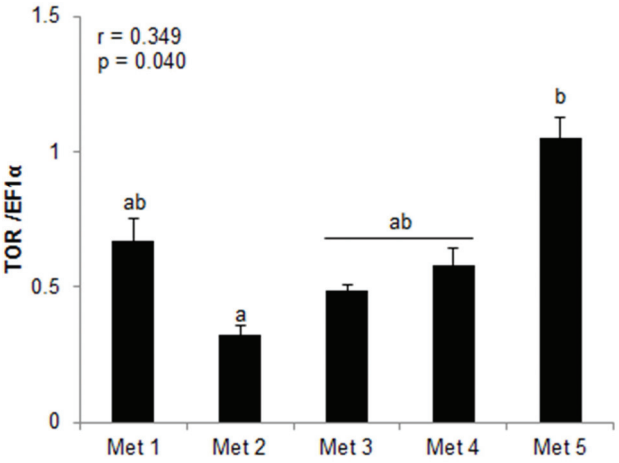
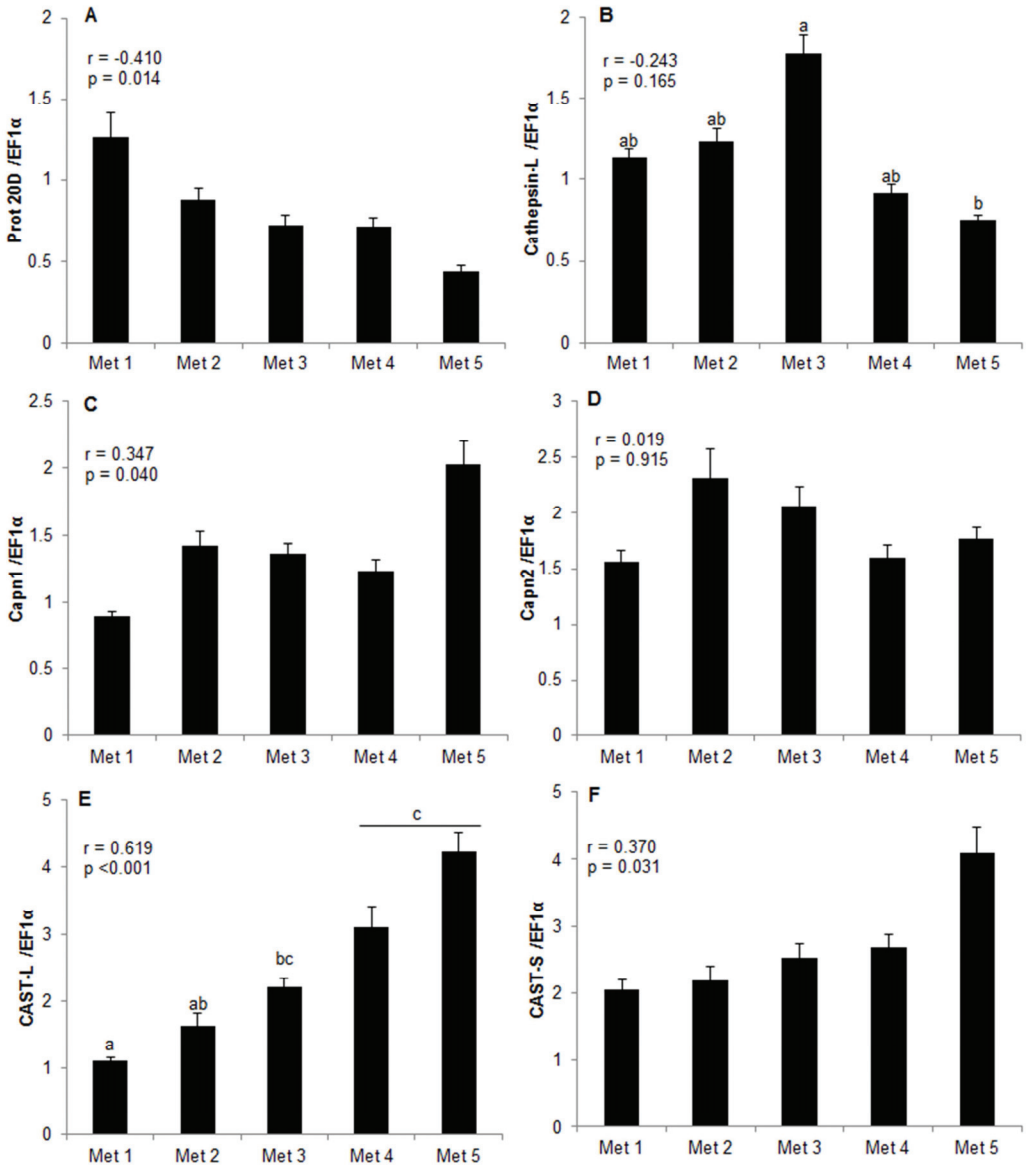


Fig. 3.


A
r = -0.410
p = 0.014
Prot 20D /EF1α
B
r = -0.243
p = 0.165
Cathepsin-L /EF1α
a
ab
ab
ab
b
C
r = 0.347
p = 0.040
Capn1 /EF1α
D
r = 0.019
p = 0.915
Capn2 /EF1α
E
r = 0.619
p <0.001
CAST-L /EF1α
a
ab
bc
c
F
r = 0.370
p = 0.031
CAST-S /EF1α
Met 1
Met 2
Met 3
Met 4
Met 5

Paper IV: Dietary methionine level affect plasma methionine profile, ammonia excretion and the expression of genes related to the hepatic intermediary metabolism in rainbow trout (Oncorhynchus mykiss) fed plant based diets.

Dietary methionine level affect plasma methionine profile, ammonia excretion and the expression of genes related to the hepatic intermediary metabolism in rainbow trout (*Oncorhynchus mykiss*) fed plant based diets.

Running title: Methionine intake affects hepatic gene expression

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Abstract

In fish nutrition, a growing interest has been given to the role of amino acids as metabolic regulators at the transcriptional level. The present study investigated the effects of dietary methionine level on the hepatic intermediary metabolism in rainbow trout (*Oncorhynchus mykiss*) after ingestion of a single meal. For this purpose, five plant-based diets were formulated with methionine content ranging from 0.60 to 1.29 % dry matter. The diets were fed in excess to juvenile rainbow trout during a six weeks period. Subsequently, sample collection was performed for i) hepatic gene expressions, ii) hepatosomatic index (HSI), iii) postprandial ammonia excretion, and iv) plasma methionine analyses. Postprandial plasma methionine concentrations correlated significantly with the dietary level ($P<0.001$) at the different sampling time. The expression of genes coding for enzymes involved in lipid metabolism (Fatty acid synthase, Glucose 6 phosphate dehydrogenase and Carnitine palmitoyl transferase 1 a), gluconeogenesis (Frutose-1,6-biphosphatase) and amino acid catabolism (Alanine transferase and Glutamate dehydrogenase) responded in a linear manner ($P<0.05$) to gradual increases in dietary crystalline methionine; which was accompanied by a concomitant, linear decrease in ammonia excretion and HSI ($P=0.22$ and $P<0.001$, respectively). This study is the first to demonstrate that the expression of several genes related to hepatic intermediary metabolism respond in a dose-response manner to increasing levels of dietary methionine and reflect the metabolic response.

Keywords

Amino acid catabolism; Lipid metabolism; Gluconeogenesis; Crystalline amino acid; Hepatosomatic index

Introduction

An increased understanding on the role of amino acids in the regulation of genes involved in growth and metabolism has been obtained from studies on mammalian species (Fafournoux et al. 2000; Kadowaki and Kanazawa 2003; Jousse et al. 2004; Avruch et al. 2009), and is rapidly emerging as an independent field of research in fish nutrition (Panserat and Kaushik 2010). Amino acids were until recently considered merely as substrates for metabolic reactions, with little or no attention given to their role as signalling factors. Carnivorous fish, such as salmonids, have a high requirement for dietary protein compared to other vertebrates (Bowen 1987). Moreover, they are able to utilize dietary protein more efficiently for growth despite the utilization of amino acids as energy source or as precursors in the synthesis of metabolic intermediates. These peculiarities of carnivorous fish call for further studies on the role of amino acids as signalling molecules in the regulation of genes related to intermediary metabolism.

At present, aquafeeds are typically formulated to desired energy and protein levels using a blend of raw materials, and are often supplemented with crystalline amino acids to balance profile to the species requirements. To support a sustainable aquaculture production, utilization of raw materials from various origins is necessary (Bendiksen et al. 2011). However, protein sources differ, among other things, in their amino acid profile (Médale and Kaushik 2009), and in plant-based protein sources methionine and lysine are often limiting essential amino acids. Supplementation with crystalline amino acids of the deficient diets improves nitrogen utilization (Green and Hardy 2008), but the utilization is most often still inferior to that of a diet containing only protein-bound amino acids such as a fish meal-based diet (Bodin et al. 2012). The plasma pool of free amino acids is an important component of the homeostasis of proteins and amino acids (Fafournoux et al. 2000; Panserat and Kaushik 2010), and the diet has been shown to affect the postprandial amino acid plasma profiles (Rolland et al. submitted).

Amino acids are versatile molecules used as building blocks in protein synthesis, as energy source to fuel metabolism, and as precursors for the synthesis of multiple biomolecules (e.g. hormones, pyrimidine, non-essential amino acids, etc.). In addition, amino acids operate as signalling molecules, and there is a growing interest in their ability to regulate metabolic pathways at the gene expression level. In a study on isolated rainbow trout hepatocytes, Lansard et al. (2010) demonstrated that the mRNA levels of genes involved in glucose metabolism (glucose-6-phosphatase, phospohoenolpyruvate carboxylase, and 6-phospho-fructo-1-kinase) increased in a dose-response manner to the concentration of amino acids in the medium. In mammals, some amino acids have been shown to have a predominant signalling role. Leucine is, for example, believed to have a greater regulatory capacity on protein synthesis through the target of rapamycin pathway (TOR) than valine and isoleucine (Kimball and Jefferson 2006). In the *in vitro* study on rainbow trout hepatocytes mentioned above, Lansard et al. (2010) showed that the presence of single amino acids (L-leucine, L-methionine and L-lysine) affected the expression of genes related to glucose and lipid metabolism in different ways. Hence, leucine in association with insulin was shown to regulate lipogenesis and

gluconeogenesis to a similar extent as did a mixture of amino acids. Methionine was shown to be involved in the regulation of particular genes related to the intermediary metabolism, while lysine only had a marginal regulatory effect. *In vitro* studies suggest that the amino acid regulation of gene transcription related to the hepatic metabolism is complex and remains to be fully elucidated. In fish, the transcription of hepatic metabolism-related genes is largely under the control of dietary proteins (Skiba-Cassy et al. 2013a), and affected by changes in the dietary amino acid profile (Wacyk et al. 2012), the content of a single amino acid (Hevrøy et al. 2007; Espe et al. 2008) and the ratio between indispensable to dispensable amino acids (Gómez-Requeni et al. 2003). However, the response to an increasing dietary dose of a single amino acid on the hepatic intermediary metabolism *in vivo*, using gene expression and metabolic indicators has, to our knowledge, not been investigated previously.

The current study focusing on the hepatic intermediary metabolism is a follow-up of a previous study investigating the expression of several genes involved in the growth somatotropic axis and protein turnover in response to increase of dietary methionine (Rolland et al. 2015). Therefore, the aim of the present study was to determine the hepatic metabolic response at the transcriptional level to dietary methionine content, as well as associated changes in ammonia excretion and hepatosomatic index (HSI) in rainbow trout (*Oncorhynchus mykiss*) juveniles after a single meal. Additionally, postprandial plasma methionine concentrations were measured to estimate the concomitant effect of different dietary methionine levels on the plasma free methionine pool. As the liver is the main site for intermediary metabolism, focus was directed at the hepatic expression of specific genes involved in lipid metabolism, glucose synthesis and amino acid catabolism. The expression of fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PD), and carnitine palmitoyltransferase 1 isoform a (CPT1a) were measured in the present study as indicators of lipid synthesis and oxidation. FAS is a multi-enzyme complex catalysing lipogenesis in a process requiring reductive power produced in a reaction catalysed by G6PD. CPT1 is a rate-limiting enzyme responsible for lipid oxidation in the liver, and the expression of the examined genes has been shown to be regulated by dietary ingredients (Espe et al. 2010; Seiliez et al. 2011). Some amino acids, like methionine, are carbon donors for *de novo* synthesis of glucose and are able to regulate the expression of genes related to enzymes catalysing different reactions of the gluconeogenic pathway (Lansard et al. 2010, 2011). The dietary profile of methionine was consequently anticipated to affect glucose synthesis through modulating the transcription of the main enzymes in the pathway, i.e. phophoenolpyruvate carboxykinase (PEPCK), fructose-1,6-biphosphate (FBPase) and glucose-6-phosphatase (G6Pase). In addition, the expression of five genes coding for enzymes involved in amino acid catabolism, and which have previously been shown to be affected by the dietary composition and/or amino acid profile (Wacyk et al. 2012), was measured. Hence, alanine aminotransferase (ALT1) is a transaminase catalysing pyruvate production from alanine in a reversible reaction, while glutamate dehydrogenase (GDH) converts glutamate into α-ketoglutarate (intermediate of the Krebs cycle) by releasing ammonia (Ballantyne 2001). Aspartate aminotransferase (GOT2) is another

transaminase using aspartate and α-ketoglutarate to produce oxaloacetate (another intermediate of the Krebs cycle). Finally, glutamine synthetase 1 and 2 (GLS01 and GLS02, respectively) eliminate free ammonia by producing glutamine from glutamate.

Materials and Methods

Fish husbandry and experimental diets

Juveniles of rainbow trout (116.8±0.6 g; mean ± SD) were obtained from a commercial fish farm (Funderholme Dambrug A/S, Denmark). Fish were kept for three weeks in quarantine and hand-fed a commercial diet (BioMar A/S, Brande, Denmark).

The proximate composition of the five experimental diets was reported by Rolland et al. (2015) and is provided in Table 1. In brief, a plant-based diet, containing mainly wheat gluten (18.9 g $100g^{-1}$ feed), soya protein concentrate (10.0 g $100g^{-1}$ .feed) and pea protein concentrate (36.5 g $100g^{-1}$ feed) largely deficient in methionine (0.6 % dry matter, DM) was used as reference recipe (Met 1). Graduating levels of crystalline D-L methionine were added at 0.11, 0.32, 0.44 and 0.65 g $100g^{-1}$ feed, to obtain five diets differing only in the methionine content (Met 1 to Met 5). As per design, the diets differed mainly in methionine content, increasing from 0.60 to 1.29 % DM. The proximate compositions of the plant-based diets (Met 1 to Met 5) were very similar.

Experimental design

A total of 15 fiberglass tanks were each stocked with 70 fish (8.2±0.4 kg $tank^{-1}$; mean ± SD). The freshwater in the recirculation unit was replaced at a rate of 8 % of the total volume per day and the temperature was maintained at 15.1±0.1 ºC. Fish were fed in excess for six weeks with one of the five experimental diets (triplicate per diets) each day, from 8:00 to 12:00 h, using automatic belt feeders.

Tissue sampling. After a six weeks feeding period, fish were fasted for 24 h and seven individuals per treatment were randomly selected and scarified in a lethal dose of phenoxyethanol (1 ml l^{-1}). Liver were dissected and sampled as detailed by Rolland et al. (2015). Another seven individuals per treatment were subsequently sacrificed following the same protocol and body and liver masses were recorded. Hepato-somatic index was calculated as: HSI = liver wet weight (g) / body wet weight (g) × 100. Different individuals were used for tissue sampling and HSI calculation in order to minimize the time before flash freezing of the livers.

Then 45 individuals per tank were randomly selected and pooled by dietary treatment in five tanks. The fish were acclimatized to a new feeding strategy over a period of one week being hand fed in excess over a 20

min period with their respective diets for the purpose of measuring ammonia excretion and methionine plasma profile during digestion.

Ammonia excretion. To measure ammonia excretion, 12 fiberglass tanks with a volume of 700 L were meticulously cleaned to remove any biofilm. Diet Met 2 was omitted from this part of the study due to space limitations. Met 2 was chosen for exclusion because it was below methionine requirements but less deficient than Met 1. From each of the four remaining dietary treatment groups (Met 1, Met 3, Met 4 and Met 5), triplicate groups of 20 fish were distributed into the 12 tanks (6.1±0.6 kg per tank) and fasted for 48 hours. The experiment was initiated by hand feeding 50 g of the respective diet to each tank over a 20 min period. The tanks were then temporarily modified into single recirculation units without biofiltration and direct oxygen supply to allow for the measurement of ammonia accumulation. Any uneaten pellets were collected in swirl separators located in each tank, enumerated and deducted from the ration to calculate the feed intake. A 50 ml water sample was obtained directly from the outlet of each tank 48 h after meal ingestion and the system was closed (T0). Water sample was filtered through a 0.20µm sterile filter (Sarstedt AG & Co, Nüremberg, Germany), and stored at -20 º C until analysis for total ammonia nitrogen (TAN). To check for internal nitrogen turnover in the tanks the fish were carefully removed from the tanks and water was re-sampled (T48). The systems were kept closed for another 24 h before a final water sample was obtained (T72). Finally, the water volume in each system was verified.

Plasma methionine concentrations. The final part of the study was carried out to investigate the effects of dietary methionine level and form on plasma free methionine pool, measured as postprandial plasma methionine concentrations. From each of five original dietary treatment groups (Met 1 to Met 5), triplicate groups of 20 fish were distributed in 15 700 L tanks and fasted for 48 hours. On the sampling day, the 15 tanks were hand fed 120 g feed within 20 min, and blood samples were obtained at time 0, 3, 6, 12, 20 and 36 h after feeding. In order to minimize disturbance during digestion, each tank was sampled only twice, with a minimum interval of 12 h. All blood samples were obtained as follows: seven fish were caught and euthanized in phenoxyethanol (1 ml l^{-1}) for three min. Upon removal from the anaesthetic solution each fish was killed by a blow to the head, and blood was sampled directly from the ventricle. The spinal cord was severed and fish were dissected to verify the presence of food in the gastrointestinal tract. Blood samples from fish with no signs of food in the stomach or intestine were discarded. The samples were immediately centrifuged at 3000 rpm for 5 min, the plasma fraction frozen on dry ice, and subsequently stored at -80 ºC until analysis.

Chemical analysis

Feed samples were homogenized using a Krups Speedy Pro homogenizer and analysed for dry matter and ash (Kolar 1992), crude protein (ISO 2005; assuming crude protein = 6.25×Kjeldahl nitrogen) and lipid

(Bligh and Dyer 1959). Dietary gross energy was measured using a bomb calorimeter (IKA-Calorimeter C7000, IKA Analysentechnik, Heitersheim, Germany) after drying of the diet samples for 48 h at 60 ºC.
The methionine content of each diet (% DM) and plasma concentrations (nmol ml^{-1}) were measured following the methods described by Rolland et al. (2014).
Water samples were analysed for ammonia using a colorimetrical method (DS 1975). The turnover of ammonia by the system was calculated as the difference between the ammonia concentration at T48 and T72. Total ammonia excretion values after 48 h were expressed as mg N per g protein intake (mg N g^{-1}PI).

Gene expression analysis

Gene expression levels were determined using a real-time, quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the targeted genes involved in the hepatic intermediary metabolism. The primer sequences as well as the accession number in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank) are given in Table 2. The analyses were carried out using the method described by Rolland et al. (2015).
The relative quantification of target gene was performed using the mathematical model described by Pfaffl (2001). The relative expression ratio (R) of a target gene was calculated on the basis of real-time PCR efficiency (E) and CT deviation (ΔCT) of the average control sample versus average control sample, and expressed in comparison to the EF1α reference gene as the PCR efficiency was slightly better than for β-actin. PCR efficiency (E) was measured by the slope of a standard curve using serial dilutions of cDNA of a pool sample. PCR efficiency values ranged between 1.9 and 2.1.

Statistical analysis

All statistical analyses were performed using SigmaPlot v. 11 (Systat Systems Inc., USA), considering a probability of $P<0.05$ to be significant. The data were tested for normal distribution using a Shapiro test and if required, simple transformations (logarithm, reverse or square root) were performed. When all data had passed the normality test, one-way ANOVA followed by the Holm–Sidak multi comparisons procedure, was carried out. Correlations between data were examined using Pearson's Correlation test. Values are throughout presented as mean ± SD (SE for gene expression results presented in figures).

Results

Hepatosomatic index and ammonia excretion

Fish fed diets Met 1 and 2 had significantly higher HSI values than fish fed the highest level of crystalline methionine (Met 5; Table 3). The ammonia excretion for the five dietary treatment did not differ

significantly from each other (P=0.065; Table 3). However, both ammonia excretion and HSI decreased linearly as the level of dietary methionine increased (P<0.05; Table 4).

Plasma methionine concentrations

Postprandial plasma methionine concentrations were significantly affected by dietary level (Fig. 1). Plasma concentrations of methionine correlated positively with increasing levels of methionine (Table 4) at all postprandial sampling points Following feeding, plasma methionine levels rose rapidly and peaked after 12 h in fish fed Met 3 and Met 5, with peak plasma concentration values that were 2.7 and 4-fold higher, respectively, than at T0. At 12 h, plasma methionine concentration for Met 5 was 11.5-fold higher than for Met 1 (789.1±129.6 and 66.9±16.3 μmol ml^{-1}, respectively).

Gene expression

Lipid metabolism. Expression of genes related to lipid metabolism (FAS, G6PD, and CPT1a) responded in a linear manner to increasing dietary methionine (Fig. 2). The relative expression of FAS decreased negatively with increasing dietary methionine (Fig. 2a). Fish fed the methionine deficient diets (Met 1 and 2) had significant higher levels of FAS mRNA than that of fish fed a diet with methionine largely in excess (Met 5), possibly reflecting a lower lipogenic capacity. Conversely, the expressions of CPT1a and G6PD were enhanced by crystalline methionine supplementation (Fig. 2b,c). Based on the expression of CPT1a, the apparent capacity for fatty acid oxidation was significantly higher in fish fed Met 5 than in fish fed the deficient diet Met 2 (Fig. 2b). The transcript levels of the gene coding for G6PD were higher in fish fed diets supplemented to or above requirement (Met 3, Met 4 and Met 5) compared to those fed the deficient diets (Met 1 and Met 2).

Gluconeogenesis. Among the enzymes catalysing gluconeogenesis (Fig 3), only the expression of FBPase was significantly affected by dietary methionine levels (Fig. 3b). The gene expression of PEPCK and G6Pase (Fig. 3a and c, respectively) do not show any specific response to increasing dietary methionine levels while the mRNA levels of FBPase responded in a linear manner (r=0.595, P<0.001). The expression for Met 5 was 7-fold higher than for Met 1.

Amino acid catabolism. The expression of hepatic genes involved in amino acid catabolism (ALT1, GDH, GOT2, GLS01 and GLS02) was differently affected by the dietary levels of crystalline methionine (Fig. 4). ALT1 and GDH mRNA expressions were strongly up-regulated by increasing the concentration of crystalline dietary methionine (Fig. 4a,b), whereas the transcript levels of GOT2 were not significantly affected (Fig. 4c). Hepatic GLS01 and GLS02 showed similar expression patterns, being the highest in fish fed Met 3, containing methionine close to requirements according to the growth performance results, and significantly higher than in fish fed diet Met 1 (Fig. 4d,e).

Discussion

The present study, to the best of our knowledge, showed for the first time that the hepatic expression levels of several genes involved in lipid, glucose and amino acid metabolism vary with dietary methionine level, sometime in a linear manner. Growth performances, postprandial ammonia excretion following a single meal and the HSI were used as metabolic indicators to verify whether or not these relationships reflected hepatic metabolic changes.

The growth performance results previously published for these diets indicated that dietary methionine requirement was met in Met 3 (Rolland et al. 2015). For methionine content above 0.96 % DM, growth, feed and protein utilization were not further improved. Interestingly these results were not reflected by the linear decrease of ammonia excretion measured in the current study.

The liver is an important site for lipid synthesis, storage and export in fish (Tocher 2003). Lipid is an important energy source for fuel metabolism in fish (Wood 2001), and amino acids, through the synthesis of metabolic intermediates, serve as carbons donor for *de novo* lipid synthesis. In addition to their role as substrates, amino acids are able to modulate lipogenic pathways at the transcriptional level. In a controlled environment, methionine in association with insulin up-regulate the hepatic expression of FAS in rainbow trout; however to a much lower extent than a mixture of amino acids (Lansard et al. 2011). In the present study, the mRNA levels of FAS showed a linear decrease with increasing dietary methionine content. The FAS expression in the hepatic tissue of rainbow trout was previously shown to be exclusively controlled though the target of rapamycin (TOR) pathway (Dai et al. 2013); a nutrient-sensitive pathway that methionine alone is not able to activate (Lansard et al. 2011). The decrease in FAS mRNA expression, coinciding with decreased ammonia excretion, suggests a reduced amino acid deamination when dietary methionine levels increase. This would indicate that the expression of FAS is possibly modulated by the amount of amino acid available for catabolism. The expression of G6PD, catalysing a reaction resulting in the production of reductive power necessary for lipid synthesis, was up-regulated with the increase of dietary methionine. The expression pattern differs to that of FAS suggesting that the transcriptional regulation of these two enzymes occurs through different pathway(s) and/or is modulated by different factor(s). However, the decrease in HSI with increasing dietary methionine level supports the notion that the hepatic lipid deposition is reduced, probably due to the decrease of *de novo* synthesis. Feeding Atlantic salmon with a methionine-deficient diet resulted in a higher hepatic FAS activity, triacylglycerol (TAG) concentration, and higher HSI compared to individuals fed with adequate dietary amino acid profile (Espe et al. 2008, 2010). Additionally Espe et al. (2010) showed that the hepatic lipid oxidation capacity was unaffected by methionine limitation, as also suggested by the results on CPT1a expression in the present experiment on rainbow trout. Conversely, the excess of methionine induces an increase in CPT1a mRNA levels possibly reflecting a higher mobilization of endogenous lipid stores as previously suggested by Skiba-Cassy et al. (2013b). The increased protein deposition, suggested by the reduced ammonia excretion, could result in a

lower share of amino acid available for energy production. In response, the mobilization of endogenous lipid to fuel metabolism would increase, as reflected in the higher CPT1a gene expression levels and lower HSI measured in fish fed Met 5. Hence, the lipid metabolism in fish appears to be closely regulated by the dietary amino acid profile, both at transcriptional and post-transcriptional levels.

The natural diet of carnivorous species such as the rainbow trout is typically devoid in carbohydrate, and the supply of glucose (an indispensable energy source for certain tissues including the central nervous system) is ensured by endogenous *de novo* synthesis from metabolic intermediates. Previous findings have suggested that the regulation of gluconeogenesis at the transcriptional level in carnivorous fish is under the control of dietary protein content rather than carbohydrate content (Panserat et al. 2001a,b,c; Enes et al. 2009). PEPCK, FBPase and G6Pase are the three main enzymes catalysing reactions of the gluconeogenic pathway and their regulation at the transcriptional level responds differently to nutritional factors. In rainbow trout, the expression of G6Pase increases with dietary lipid (Panserat et al. 2002), and protein content (Kirchner et al. 2003). Similarly, increasing dietary protein level induced the hepatic transcription of FBPase but not PEPCK (Kirchner et al. 2003). In addition to being important carbon donors for gluconeogenesis, amino acids are able to regulate the expression of PEPCK and G6Pase coding for two enzymes catalysing major steps of *de novo* glucose synthesis (Lansard et al. 2010). Methionine, in association with insulin, has been shown to down-regulate the expression of PEPCK and G6Pase in cultured rainbow trout hepatocytes (Lansard et al. 2011). In the present experiment we did not observe any significant effect of dietary methionine level. However, gradual increases of crystalline methionine induced a linear up-regulation of FBPase expression. This is in contrast to the observations of Lansard et al. (2010), who found that increasing doses of amino acids in rainbow trout hepatic cells did not affect FBPase mRNA levels. Additional factors (e.g. other nutrients and hormonal signals) might interact with methionine *in vivo*, possibly explaining the discrepancy between results when compared to *in vitro* conditions. Methionine is a glucogenic amino acid and as such the increased intake seems to regulate gluconeogenesis through the up-regulation of FBPase expression. In addition, the increasing mRNA levels of ALT1, a transaminase responsible for the production of pyruvate from alanine, could indicate an enhanced *de novo* glucose synthesis. Through this pathway and the synthesis of pyruvate, alanine is an effective precursor for gluconeogenesis (González et al. 2012). However, the effects of increasing dietary methionine on the glucose synthesis in the present study are uncertain, considering that the mRNA levels of PEPCK and G6Pase were unaffected by the dietary treatment.

Similarly to those of ALT1, the expression levels of GDH were enhanced by dietary methionine levels at or above requirements compared to deficient diets. The main catabolic pathway involves the transmission of the amine group of amino acids to glutamate, further deaminated by GDH to form α-ketoglutarate (Ballantyne 2001). The gene expression levels of these two catabolic enzymes were not significantly affected by increasing the dietary protein content (Kirchner et al. 2003), indicating that amino acid catabolism might be induced by an excess of specific dietary amino acids, including methionine (Skiba-Cassy et al. 2013b). In the

present study, increasing dietary methionine levels induced a positive response on the plasma methionine concentrations. The hepatic expression of ALT1 and GDH could be driven by an increased availability of methionine and not reflect the amino acid utilization *per se*. A previous study suggested that the hepatic activities of GDH and ALT1 do not reflect the cumulative ammonia excretion induced by feeding gilthead sea bream (*Sparus aurata*) with diets differing in their indispensable to dispensable amino acid ratio (Gómez-Requeni et al. 2003). Similarly, in the present experiment the expression profiles of these two enzymes were not in agreement with the ammonia excretion results.

Similarly, the expression levels of GLS01 and GLS02 did not reflect ammonia excretion results and were the highest for fish fed a diet with adequate methionine supplementation with no further increase when dietary methionine was added above requirements. GLSs catalyse glutamine synthesis from glutamate and ammonia. At first GLS01 and GLS02 seem to be up-regulated by increasing methionine and then reached a plateau, possibly due to a negative feed-back from other factors or a limited turnover. Similarly to GLSs, GOT2 catalyse a reaction that removes ammonia by synthetizing glutamate from α-ketoglutarate and the expression pattern did not reflect ammonia excretion. The results put forward a complex control of amino acid catabolism at transcriptional and/or post-transcriptional levels, likely involving a large enzymatic machinery.

Thus from the present experiment we suggest that, in the case of a methionine-deficient diet, the amine group is removed from the amino acids and the resulting α-keto acid is preferentially orientated towards *de novo* lipid synthesis rather than being fully oxidized in the Krebs cycle. This is supported by the higher ammonia excretion and HSI of fish fed deficient diets compared to that of fish fed excess amounts of methionine. Conversely, rainbow trout fed excess methionine tended to improve the apparent nitrogen retention as deduced from the lower ammonia excretion and HIS. This might indicate a reduced utilization of amino acids for fatty acid synthesis and improved mobilization of endogenous lipid reserves to fuel metabolism. The regulation of gene expression related to amino acid catabolism could be a response to high methionine availability and contributing to preserve the free pool and ensure that plasma levels do not exceed possible critical levels (Katz and Baker 1975; Garlick 2006), if any in rainbow trout. As methionine is a glucogenic amino acid, increasing plasma levels could up-regulate the transcription of FBPase.

In summary, our results showed that the expression of several genes related to the hepatic intermediary metabolism responded in a linear manner to increasing dietary methionine level, reflecting a wide range of metabolic changes. Therefore it seems evident that the modulation of the hepatic metabolism is closely related to the methionine content in the diet, although we cannot discard an effect of changes in the balance of total amino acids and/or in the ratio between essential and non-essential amino acids. Even though more research is needed on other amino acids, these results provide valuable information to better comprehend the consequences of changing amino acid profile in current formulations for the aquaculture industry.

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Table 1: Ingredients (g 100 g^{-1}), proximate composition (g 100 g^{-1}) and methionine content (% dry matter; DM) of the five experimental diets.

	Met 1	Met 2	Met 3	Met 4	Met 5
Recipies (g 100g^{-1} feed)[a]					
Fish Meal	8.0	8.0	8.0	8.0	8.0
Krill Meal	2.0	2.0	2.0	2.0	2.0
Soya Protein Concentrate	10.0	10.0	10.0	10.0	10.0
Pea Protein concentrate	36.5	36.5	36.5	36.5	36.5
Wheat	18.9	18.9	18.9	18.9	18.9
Fish Oil	11.0	11.0	11.0	11.0	11.0
Rapeseed Oil	11.0	11.0	11.0	11.0	11.0
Monocalcium phosphate	1.10	1.10	1.10	1.10	1.10
L-Histidin	0.10	0.10	0.10	0.10	0.10
Vitamin and mineral Premix[b]	0.94	0.94	0.94	0.94	0.94
D-L Methionine	-	0.11	0.32	0.43	0.65
Proximate composition (g 100g^{-1} feed)[a]					
Moisture	7.8	8.3	8.9	8.3	8.3
Protein	42.1	42.0	41.8	41.9	41.8
Fat	27.9	27.2	27.6	28.4	28.0
Ash	4.8	4.8	4.6	4.6	4.6
Energy (MJ kg^{-1} feed)	23.3	23.1	23.0	23.2	23.2
Essential amino acids (% DM)[a]					
Arginine	3.39	3.42	3.55	3.71	3.47
Histidine	1.24	1.23	1.25	1.38	1.22
Isoleucine	1.80	1.90	1.98	2.03	2.07
Leucine	3.44	3.50	3.64	3.77	3.65
Lysine	2.91	3.09	3.06	3.08	3.23
Methionine	0.60	0.68	0.96	1.09	1.29
Phenylalanine	2.18	2.21	2.46	2.56	2.42
Threonine	1.66	1.70	1.73	1.79	1.72
Valine	1.87	1.96	2.01	2.09	2.14
Sum essential AAs measured	19.10	19.69	20.64	21.49	21.21

[a]Rolland et al. (2015); [b]Adjusted to meet the level recommenced by the NRC (2011)

Table 2 Summary of forward and reverse primers used for RT-qPCR analysis and accession numbers of genes sequences (GenBank).

ne name	Abbreviation	Forward Primer	Reverse Primer	Acc. Number
ference gene				
Elongation factor 1 alpha	*EF1α*	CGGAGGCATTGACAAGAGAAC	AGCACCCAGGCATACTTGAA	AF498320
vid metabolism				
Fatty acid synthase	*FAS*	ATGCGTATCCAAGCCCCAAA	CCCACCAATCCTGGTCATCC	BT073300
Carnitine palmitoyl transferase 1 a	*CPT1a*	TGGCTTCCAGTTCACCGTAA	GGTAGACCCCTGTCAATATCCC	AF327058
Glucose 6 phosphate dehydrogenase	*G6PD*	TCTCTGGTTGCCATGGAGAA	ATGCACTTCAGCACCTTCAC	EF551311
uconeogenesis				
Phosphoenolpyruvate carboxylase	*PEPCK*	GGTGTGTTTGTAGGAGCCTCA	AGGGGTCGTGCATGATAACC	AF246149
Frutose-1,6-biphosphatase	*FBPase*	TCCATCGGGGAGTTCATCCTA	TGGGTAGAAGTGCTGAGCAAA	AF333188
Glucose-6-phosphatase	*G6Pase*	CTTCCCACACCAGGTCATCA	GATCCACTGCACTCTGGAGAA	AF120150
nino acid metabolism				
Alanine transferase	*ALT1*	GGGAGTAGTTTGGGTGCGTACA	CGTCCTGCCGCACACA	CA373015
Glutamate dehydrogenase	*GDH*	TGTCGGTCGATGAGGTGAAA	TGGCTCCTCCAAATGGAACA	AJ556997
Aspartate aminotransferase 2	*GOT2*	CGCAGTCGTTTGCTAAGAACA	TTCCTCGGCATCGTTACACA	BT045760
Glutamine synthase 1	*GLS01*	TGGGCCATGTGAAGGTATCA	ACGCCAAAGTCTTCACACAC	AF390021
Glutamine synthase 2	*GLS02*	TTCAGAGATCCCTTCCGCAAA	TCTGCAGGCTTGTGGTTGTA	AF390022

Table 3 Results of the hepatosomatic index (% body weight) and ammonia excretion (mg nitrogen per g protein intake) for the diets Met 1 to Met 5. Results within rows differing significantly from each other are followed by different subscript letters.

		Met 1	Met 2	Met 3	Met 4	Met 5	p-value
Hepatosomatic Index (%)	HSI	1.55±0.26^{a}	1.47±0.24^{a}	1.33±0.37^{ab}	1.19±0.16^{ab}	0.99±0.18^{b}	0.002
Ammonia excretion (mg N g PI-1)		89.20±2.04	nd*	77.38±6.63	72.97±0.48	66.09±10.63	0.065

*not determined

Table 4 Correlation between dietary methionine levels in diets Met 1 to Met 5 and (A) the plasma methionine concentrations at 0, 3, 6, 12, 20 and 36 h after feeding; (B) ammonia excretion; and (C) hepato-somatic index (HSI). A value of $P<0.05$ was considered significant.

	Correlation of dietary methionine level (% DM) with							
	Ammonia excretion (mg N g PI^{-1})	HSI (% body weight)	Plasma methionine concentrations (nmol ml^{-1})					
			T0	T3	T6	T12	T20	T36
r	-0.707	-0.642	0.839	0.928	0.941	0.943	0.844	0.900
p-value	0.022	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Fig. 1 Postprandial plasma methionine concentration (nmol ml^{-1}) profile for the diets Met 1 to Met 5 after a single meal ingestion. Each value is the mean±SD of 7 individuals

Fig. 2 Expression of genes involved in lipid metabolism (a) FAS, (b) G6PD and (c) CPT1 in the liver of fish fed diets Met 1 to Met 5. The results of Pearson's correlation test with dietary methionine content (% DM) are given as r and *P*-values. Each value is the mean±SE of 7 individuals. Results followed by a similar letter do not differ significantly (One-way ANOVA test, $P<0.05$)

Fig. 3 Hepatic expression of genes involved in gluconeogenesis (a) PEPCK, (b) FBPase, and (c) G6Pase CPT1for fish fed diets Met 1 to Met 5. The results of Pearson's correlation test with dietary methionine content (% DM) are given as r and *P*-values. Each value is the mean±SE of individuals. Results followed by a similar letter do not differ significantly (One-way ANOVA test, $P<0.05$)

Fig. 4 Gene expression of selected enzymes involved in amino acid catabolism (a) ALT1, (b) GDH, (c) GOT2, (d) GLS01, and (e) GLS02 in the liver of fish fed diets Met 1 to Met 5. The results of Pearson's correlation test with dietary methionine content (% DM) are given as r and *P*-values. Each value is the mean±SE of 7 individuals. Results followed by a similar letter do not differ significantly (One-way ANOVA test, $P<0.05$)

Fig. 1

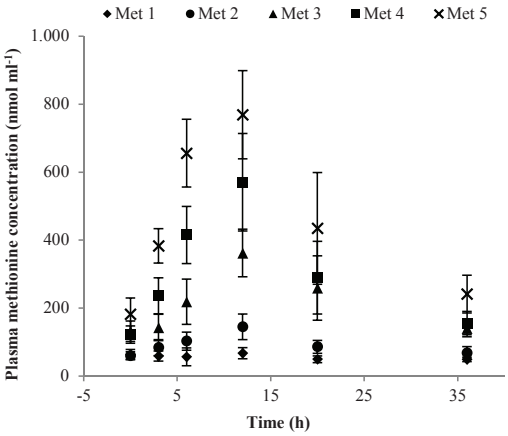

Met 1
Met 2
Met 3
Met 4
Met 5
Plasma methionine concentration (nmol ml-1)
1.000
800
600
400
200
0
-5
5
15
25
35
Time (h)

Fig. 2

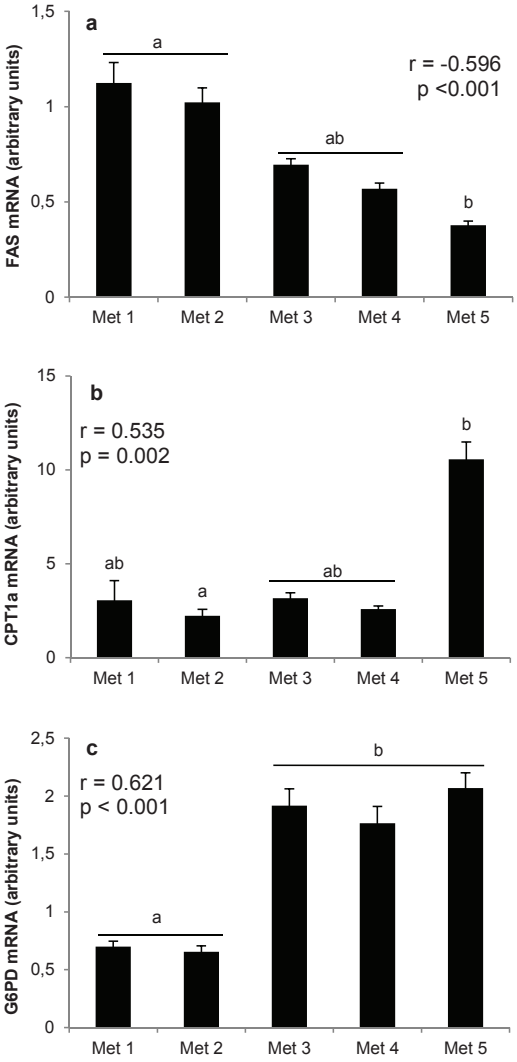

a
FAS mRNA (arbitrary units)
1,5
1
0,5
0
a
ab
b
r = -0.596
p <0.001
Met 1
Met 2
Met 3
Met 4
Met 5
b
CPT1a mRNA (arbitrary units)
15
10
5
0
ab
a
ab
b
r = 0.535
p = 0.002
Met 1
Met 2
Met 3
Met 4
Met 5
c
G6PD mRNA (arbitrary units)
2,5
2
1,5
1
0,5
0
a
b
r = 0.621
p < 0.001
Met 1
Met 2
Met 3
Met 4
Met 5

Fig. 3

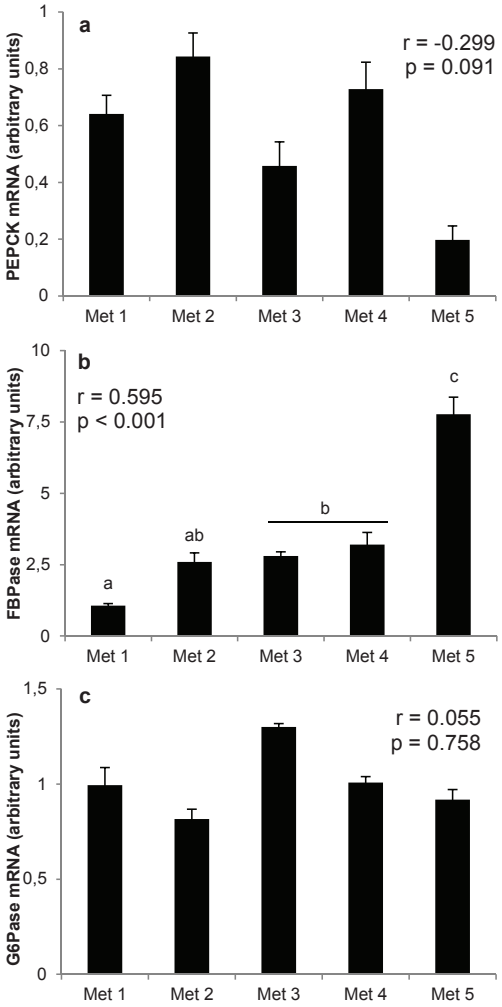
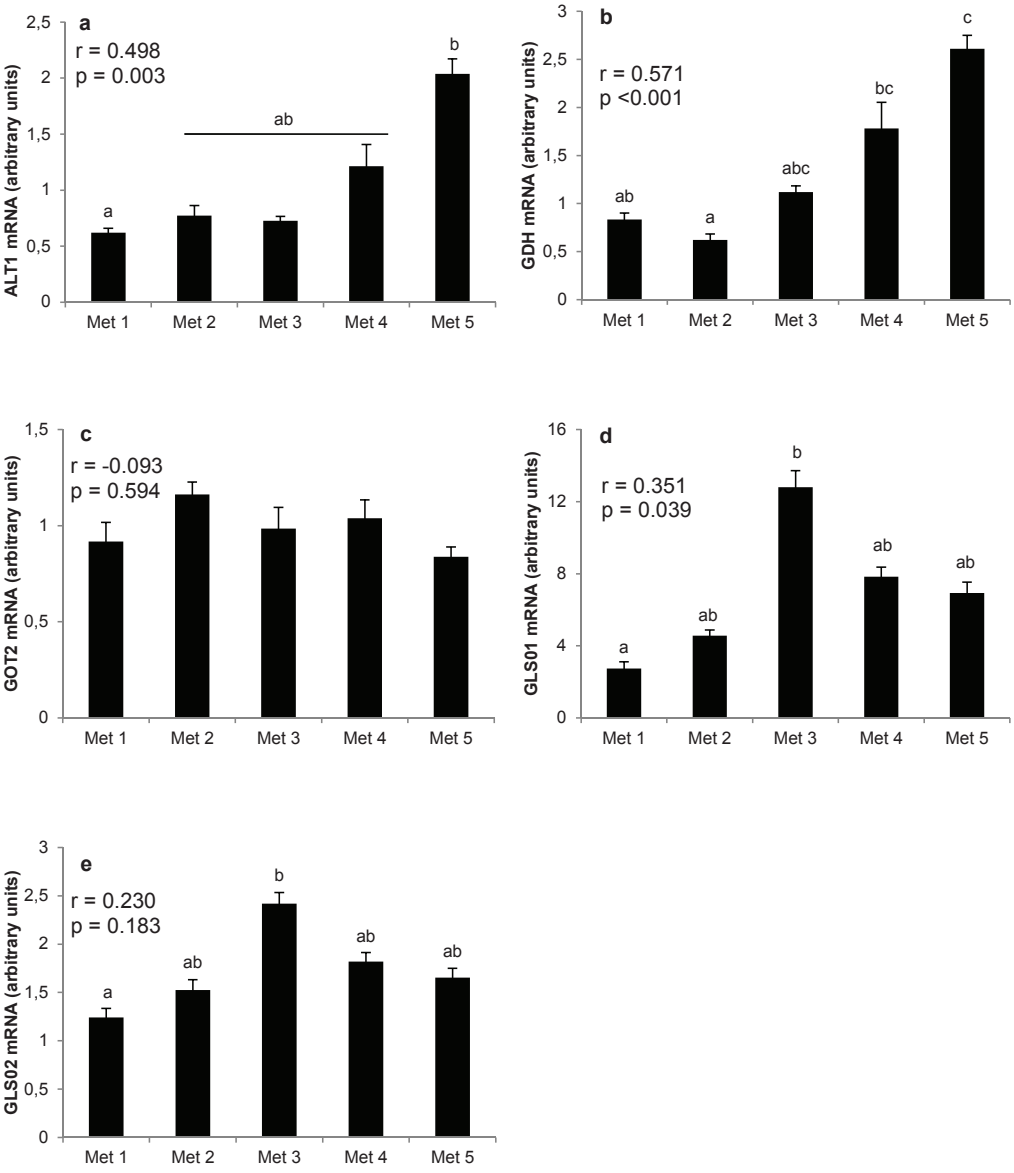

a
r = -0.299
p = 0.091
PEPCK mRNA (arbitrary units)
1
0,8
0,6
0,4
0,2
0
Met 1
Met 2
Met 3
Met 4
Met 5
b
r = 0.595
p < 0.001
FBPase mRNA (arbitrary units)
10
7,5
5
2,5
0
a
ab
b
c
c
r = 0.055
p = 0.758
G6Pase mRNA (arbitrary units)
1,5
1
0,5
0

Fig. 4


a
r = 0.498
p = 0.003
ALT1 mRNA (arbitrary units)
2,5
2
1,5
1
0,5
0
a
ab
b
Met 1
Met 2
Met 3
Met 4
Met 5
b
r = 0.571
p <0.001
GDH mRNA (arbitrary units)
3
2,5
2
1,5
1
0,5
0
ab
a
abc
bc
c
c
r = -0.093
p = 0.594
GOT2 mRNA (arbitrary units)
1,5
1
0,5
0
d
r = 0.351
p = 0.039
GLS01 mRNA (arbitrary units)
16
12
8
4
0
a
ab
b
ab
ab
e
r = 0.230
p = 0.183
GLS02 mRNA (arbitrary units)
3
2,5
2
1,5
1
0,5
0
a
ab
b
ab
ab

DTU Aqua – National Institute of Aquatic Resources – is an institute at the Technical University of Denmark. DTU Aqua's mission is to conduct research, provide advice, educate at university level and contribute to innovation in sustainable exploitation and management of aquatic resources. We investigate the biology and population ecology of aquatic organism, aquatic physics and chemical processes, ecosystem structure and dynamics, taking account of all relevant natural and anthropogenic drivers.

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