Increased levels of dietary crystalline methionine affect plasma methionine profiles, ammonia excretion, and the expression of genes related to the hepatic intermediary metabolism in rainbow trout (Oncorhynchus mykiss)

Strictly carnivorous fish with high requirements for dietary protein, such as rainbow trout (Oncorhynchus mykiss) are interesting models for studying the role of amino acids as key regulators of intermediary metabolism. Methionine is an essential amino acid for rainbow trout, and works as a signalling factor in different metabolic pathways. The study investigated the effect of increasing dietary methionine intake on the intermediary metabolism in the liver of juvenile rainbow trout. For this purpose, five diets were formulated with increasing methionine levels from 0.60 to 1.29% dry matter. The diets were fed in excess for six weeks before three sampling campaigns carried out successively to elucidate (i) the hepatic expression of selected genes involved in lipid, glucose and amino acid metabolism; (ii) the postprandial ammonia excretion; and (iii) the postprandial plasma methionine concentrations. The transcript levels of enzymes involved in lipid metabolism (fatty acid synthase, glucose 6 phosphate dehydrogenase and carnitine palmitoyl transferase 1 a), gluconeogenesis (fructose-1,6-biphosphatase) and amino acid catabolism (alanine amino transferase and glutamate dehydrogenase) were significantly affected by the increase in dietary methionine. Changes in gene expression reflected to some extent the decrease in ammonia excretion (P=0.022) and in the hepatosomatic index (HSI; P
Modelling the effects of dietary methionine level and form on postprandial plasma essential amino acid profiles in rainbow trout (Oncorhynchus mykiss)

Aquafeed 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 6.01 to 16.17 g kg⁻¹ 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 (six sampling points up to 36 h, seven fish each time), further analysed for EAA content. Using generalized additive models, we show that (i) dietary methionine level and form explained 74% postprandial methionine plasma variations and that (ii) the methionine dietary form and plasma concentrations significantly affected the plasma concentrations of the other EAAs. Finally, linear model revealed a positive relationship (R² > 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.
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

The effects of dietary level of methionine were investigated in juvenile rainbow trout (Oncorhynchus mykiss) fed five plant-based diets containing increasing content of crystalline methionine (Met), in a six week 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 hormones land 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,
Calpains 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. (C) 2014 Elsevier Inc. All rights reserved.
Effect of plant proteins and crystalline amino acid supplementation on postprandial plasma amino acid profiles and metabolic response in rainbow trout (Oncorhynchus mykiss)

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 (1) fish meal (FM), (2) pea protein concentrate (PPC), or (3) 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 compared 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 compared to FM. The strongest effect was observed for methionine, presenting threefold 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.
Effects of dietary methionine on feed utilization, plasma amino acid profiles and gene expression in rainbow trout (Oncorhynchus mykiss)

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, benefitting from a high availability, low cost and similar nutritive properties. The major limitation in using plant 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 form (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 (R²=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 and form (crystalline or protein-bound) of methionine affected the expression of hepatic genes related to i) the somatotropic axis and ii) protein turnover. For this purpose seven 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 either in crystalline or protein-bound form. 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. In comparison, the methionine source appeared to have limited effect on the expression pattern of protein degradation enzymes. The results suggest that methionine availability, influenced by dietary content or form, modulates the expression of genes involved in the GH/IGF response and protein turnover, further affecting growth performances.

Paper IV presents the results of the effects of dietary methionine level and form (free or bound) 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. Additionally, the dietary form of methionine significantly affected postprandial plasma methionine concentrations as well as the expression of specific hepatic genes (G6PD, PEPCK, FBPase, G6Pase, ALT1, GHD and GLS02; P <0.05). Nitrogen excretion was found to be consistently higher for fish fed diets supplied with crystalline methionine than fish fed protein-bound 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, and that the dietary source of methionine affect hepatic metabolism at the transcriptional level.

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 seem to be significantly affected by crystalline methionine supplementation, as a possible explanation to the different utilization of CAA supplemented plant based diets, compared to conventional fish meal based diets.

**General information**

State: Published
Organisations: National Institute of Aquatic Resources, Section for Aquaculture
Authors: Rolland, M. (Intern), Skov, P. V. (Intern), Dalsgaard, A. J. T. (Intern)
Number of pages: 173
Publication date: 2014

**Publication information**
Place of publication: Charlottenlund
Publisher: Technical University of Denmark. National Institute of Aquatic Resources
Original language: English
Note re. dissertation: The thesis was founded by BioMar A/S and Danish Ministry of Higher Education and Science
Main Research Area: Technical/natural sciences
Electronic versions:
Publishers version
Publication: Research › Ph.D. thesis – Annual report year: 2014

Influence of inclusion level and form of dietary methionine in plant protein based diets on growth performances, ammonium excretion and postprandial methionine plasma levels in rainbow trout (Oncorhynchus mykiss).
**Influence of protein source on amino acid uptake patterns and protein utilization in rainbow trout Oncorhynchus mykiss**

Matrixes of different protein sources (fish and plant products) combined with the use of crystalline amino acids allow for formulation of diets that meet fish requirements with little or no effect on protein digestibility and/or feed intake. Despite this, a total or partial replacement of fish meal induces reduced growth performances that remain partly unexplained. The aim of the current study was to investigate the effect of exchanging the protein source on protein utilization. Marine (fish meal) and vegetable (pea protein) sources were used with or without supplementation of crystalline amino acids to the fishmeal diet level (see Table 1). Amino acid uptake patterns were assessed by the appearance of amino acids in the blood stream following the ingestion of a meal, while dietary protein utilization was evaluated by examining the metabolic response to digestion and ammonium and urea excretion rates during digestion. Four treatments, 3 diets and 1 control (no feeding), were applied to rainbow trout with an average body mass of 500 grams. Fish were either force fed one of the 3 diets at a ration corresponding to 0,75% of the body mass, or no force feeding. Four fish at a time (one per treatment) were placed in individual chambers for 48h. Blood and water samples were collected at time 0 and then at 2, 4, 6, 8, 12, 20, 32 and 48 hours post feeding. The protocol was repeated until 8 replicates per treatment were obtained.

The results were obtained through 2 separate experiments.

In the first part, oxygen consumption was recorded continuously, while water was sampled as detailed above and analyzed for ammonium and urea content.

The second part of the experiment was designed to collect blood samples. After the feeding treatment fish were held in separate containers for the above described time sampling. Fish were killed by a blow in the head and blood was collected from the caudal vein with heparinized syringes. Plasma and red blood cells content were stored separately at -80 for amino acid content analysis. The ammonium excretion profiles (Figure 1) will be correlated with the amino acid profile in the blood and oxygen consumption during digestion to investigate the effect on protein utilization for each treatment.

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**Projects:**

*Amino acid uptake patterns in fish fed plant based protein and the effects on protein utilization.*

National Institute of Aquatic Resources

Period: 01/04/2011 → 27/08/2014

Number of participants: 7

PhD Student:

Rolland, Marine (Intern)

Supervisor:

Dalsgaard, Anne Johanne Tang (Intern)

Holm, Jørgen (Ekstern)
HPLC and amino acids uptake patterns in fish fed plant-based protein (38803)

One of the issues of the rapidly growing aquaculture sector is to find fish meal substitutes. The main focus has been on plant proteins as a substitute for fish meal in the diet formulation. However, significant incorporation of plant proteins in the fish diet often results in reduced growth and/or impaired feed efficiency. Recent trials performed at our lab have shown that the profile of amino acid uptake (timeline) varies between rainbow trout fed plant based diet and fish meal diet. This difference in amino acid availability might well influence the protein synthesis and could add to the explanation of reduced performance of fish fed plant based diets and also the observed increased ammonia excretion.

Following these initial observations made in 2011 the project will perform a series of experiments to further examine how and why amino acid uptake patterns differ. Correlations between amino acid profile in the diet and amino acid in the blood following feeding will be made for different plant protein sources and added crystalline amino acids.

The concomitant effects on liver enzyme activity and protein synthesis will be examined and relevant indicators for protein synthesis (i.e. growth) hopefully determined. Specific digestibility and nitrogen excretion studies as well as traditional growth studies will be performed to support the findings.

The project is coordinated by DTU Aqua.

National Institute of Aquatic Resources
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