Norwegian University of Life Sciences
Faculty of Veterinary Medicine and Biosciences
Department of Animal and Aquacultural Sciences

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Hepatic Enzymes` Transcription Controls Utilization of Amino Acids in Nile Tilapia

Elena Gusakova

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Abstract

Nile tilapia is an attractive fish species for farming, due to its resistance to diseases and poor environmental quality. Tilapia can efficiently utilize low quality feed. Fish feed occurs approximately 40% of total farming costs. Animal nutritionists try to deal with feed costs reduction by using alternative less expensive ingredients, which could compose valuable feed, well-balanced with all nutrients. One of the major nutrient is protein, which takes 30% of the feed contents. Protein utilization and nitrogen excretion are the stumblingstones in aquaculture.

In our experiment, we prepared plant ingredient based diet with 31% of crude protein, 37% of starch and 6 % of fat in the dry matter, and supplemented with crystalline amino acids. The task was to see the physiological responses of the fish during protein turnover, as well as perform transcriptome analysis, based on qRT-PCR. The hypothesis was that enzymes within the organism control amino acid utilization, and their transcription is linked to fish physiological responses.

RNA were isolated from fish liver from each of 8 tanks at 2 hours after last feeding and 10 hours after last feeding. Additionally, we defined free amino acid blood plasma at 2, 6 and 10 hours after last feeding. At 45th day of experiment, ammonia was measured in closed stagnant system.

In our experiment we performed that enzymes' transcription controls utilization f amino acids in Nile tilapia. For the analysis of correlations between blood clearance rate of individual amino acids and gene expression levels, we divided all correlations on groups due to metabolic intermediates, which could participate in gluconeogenesis or citric acid cycle. ALAT correlates to alanine, cysteine, glycine, serine, threonine, which share pyruvate as metabolic intermediate. Asparagine and aspartate, phenylalanine, tyrosine, arginine, glutamine, histidine, proline ends up with fumarate, α-ketoglutarate, oxaloacetate. These amino acids correlates to AMPD, which is the limit enzymes in purine nucleotide cycle.

MAB has the majority correlations with clearance pattern of branch-chained amino acids (leucine, valine, isoleucine), some non-polar amino acids (methionine, leucine, valine) and aromatic amino acids (phenylalanine, tyrosine), those which have acetoacetyl-CoA intermediate (tryptophan, lysine) and pyruvate intermediate (cysteine and glycine). Frequent correlations of MAB with the majority of amino acids and especially leucine, has a direct influence in proteolysis and amino acid catabolism. This hypothesis is also proved by MAB correlations with

digested protein (R^2=0.792, P=0.003) and excreted nitrogen. Moreover, correlations between MAB and nitrogen excreted and correlations between digested protein and nitrogen excreted share common pattern. We suppose MAB encodes enzyme, participating directly in one of reactions in protein turnover, or serving as indicator of protein catabolic reaction.

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List of abbreviations

%	per cent	M	Molar
ASAT	Aspartate aminotransferase	BW	Body weight
ALAT	Alanine aminotrasferase	qRT-PCR	Quantitative real time polymerase chain reaction
GDH	Glutamate dehydrogenase	cDNA	Complementary Deoxyribonucleic acid
AMPD	AMP-deaminase	RNA	Ribonucleic acid
AASS	Alpha-aminoadipic semialdehyde synthase	U	Enzyme unit
MAB	MB21 domain 2 transcript	EAA	Essential amino acids
GIFT	Genetically improved farmed tilapias	NEAA	Non-essential amino acids
g	Gram	2MF	Two times fully fed meal
l	Litre	2M	Two times restricted meal
^{o}C	Degree Celsius	4M	Four times restricted meal
m	Meter		

Table of contents

1 INTRODUCTION

1.1 Nile tilapia farming history and worldwide production

The first traces of aquaculture of Nile tilapia (*Oreochromis niloticus*) comes from Northern Africa, around 4000 years ago. The real popularity of this species, as cultured fish, came in 1960s. During 1960s to 1980s, Nile tilapia was introduced to Thailand and Philippines from Japan. It was introduced to Brazil and later to United States from the Ivory Coast. In 1978, Nile tilapia was expanded to China, which has become the major producer of this species since 1992 (El-Sayed 2006).

Figure 1. Main producer countries of Oreochromis niloticus (Adopted from FAO Fishery Statistics, 2006)

The map, obtained from Food and Agriculture Organization of the United Nations, shows the main producing countries of Nile tilapia marked in orange color. The majority is in Africa and Asia.

The total production of farmed Nile tilapia has the increased significantly in last 30 years. In 2012 global production reached 3.2 million tons. China was the leading producer with 806 thousand tons in 2003. While totally in 2003 it was 1272 thousand tons produced worldwide. It means, ten years ago, China produced more than 60% of farmed Nile tilapia (FAO Fishery Statistics).

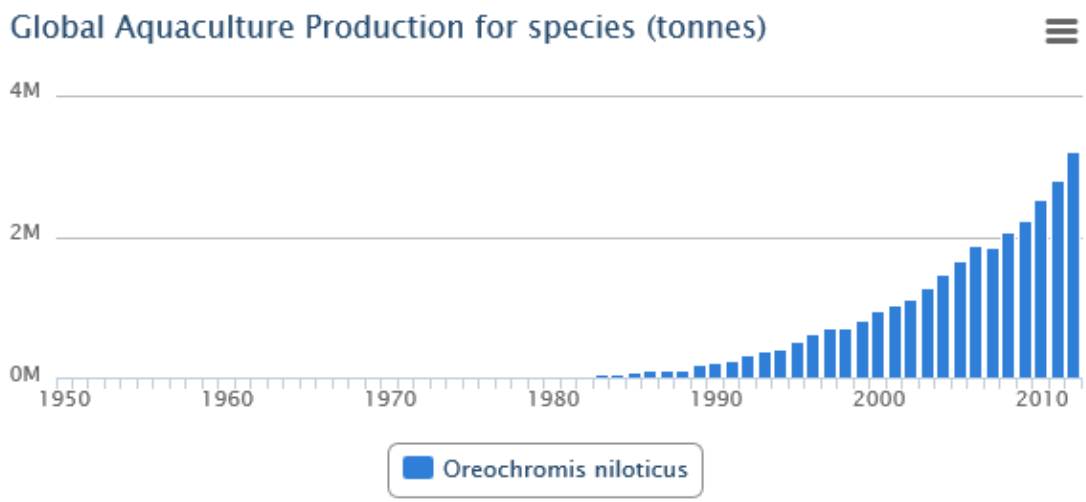


Figure 2. Global production for Nile tilapia (Food and Agriculture Organization of the United 2014)

1.2 Why is Nile tilapia attractive for farming?

Tilapia has became one of the most popular farmed fish species in the world (Figures 1 and 2). Due to its high adaptability, it has been commercialized in over 100 countries. Tilapia is resistant to diseases and poor environmental quality, and it has been proved that tilapia can efficiently utilize low quality feed.

Fish food could be divided into several ways: by taxonomic group, quality, and physiological characteristics of the fish (Evans and Claiborne 2005). Types of food are typically classified in trophic categories. These are carnivore, herbivore, detritivore and omnivore (Evans and Claiborne 2005). Carnivore fishes consume animal protein. Herbivores eat plant protein foods (ex. algae). Detritivoures utilize detritus. Omnivores, like Nile tilapia, feed on both plant and animal protein foods, thus, complementing protein sources. For tilapia fish meal can readily be taken out of the diet without influencing on growth parameters. However, in other species such as salmon and rainbow trout utilization of plant protein may reduce growth performance or negatively affects body composition or fish health (Refstie, Korsøen et al. 2000).

Since 40% of fish farming costs goes to provide feed, replacing fish meal with plant protein and utilizing higher level of carbohydrates in fish feed reduces feeding costs and makes tilapia as an economically good option for fish farming in warm waters (El-Sayed 2006). One of the main advantages of tilapia over the carnivorous farmed fish is acceptance of feeds with lower protein and higher carbohydrate levels (El-Sayed 2006). Moreover, utilizing some

industrial secondary products such as rapeseed meal as a source of protein instead of fish meal makes fish feed production more sustainable and environmentally friendly (Davies, McConnell et al. 1990). There have been experiments with cassava leaf meal (Ng and Wee 1989), barley and alfalfa (Belal 1999), soybeans (Nyirenda, Mwabumba et al. 2000, Koumi, Atse et al. 2009), ipil ipil (Zamal, Barua et al. 2008). Those experiments concluded about possibility of using feeds for tilapia, based on plant protein. Thus, potential manipulations with protein sources within Tilapia's feed and its high adaptability make it attractive for farmers (El-Sayed 2006).

1.3 Protein nutritional value

Fish needs around 40 nutrients at the cellular level (Rust 2002). Proteins, lipids and carbohydrates are major macronutrients of the food, participating in beneficial metabolic pathways. Proteins are the main structural material of fish body, and make 65-75% of dry weight tissue (Wilson 2002), and are highly related to fish growth performance (Bowen, Lutz et al. 1995). Proteins also contribute to energy metabolism. Efficiency of conversion dietary protein into body protein is higher for fish then for cattle, pigs and chicken, but dietary requirements in protein are up to 4 times higher comparing to the other vertebrates (Tacon and Cowey 1985).

For tilapia, the average percentage of required protein in feed is around 30% of the diet for adult fish (>25 grams). Dietary protein requirements are varying on fish size and age, energy contents of the feed and protein source and quality. Thus, tilapia needs higher protein level at stage of first feeding, up to 50%. Then, when it becomes fry, protein requirement decreases up to 40%, 35-40% for fingerlings, and 30-35% for juveniles (Food and Agriculture Organization of the United 2014).

Well-composed diet is a balance between ingredients and their nutritional value. For the best nutritional value it is important to know the content and ratio between nutrients such as content of vitamins, minerals and essential amino acids and the ratio of lipid to protein in diet (Halver and Shanks 1960). All compounds will be digested and utilized for the better growth performance. Thus, exchange or replacement of one of the nutrients in feed or its source, will lead to changes in its nutritional value and affect growth performance for fish (Montgomery and Gerking 1980).

Amino acids are of special interest, because of their tight relationship to protein anabolism. For each amino acid to be utilized with high efficiency, all essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine,

tryptophan, valine; cysteine and tyrosine as semi essential) are supposed to be in optimum ratio to each other (Hoar, Randall et al. 2001). One of the disadvantages of plant proteins is lack of one or several essential amino acids. To avoid deficiency of essential amino acids, synthetic amino acids are added to feed. Nevertheless, scientists made a research within synthetic amino acids additives and reported that dietary non-essential amino acids may affect amino acid metabolism in fish body and its growth performance (Gaye-Siessegger, Focken et al. 2007). Feeding frequency (feeding regime) has an effect on growth performance of fish too. Feeding regime of three meals in a day gave increased feed consumption and, as a result, increased growth rate (Wang, Hayward et al. 1998).

1.4 Food digestion

Firstly, food comes into mouth with teeth. It moves through esophagus, where it is lubricated, to stomach. In stomach food breakdown occurs. Tilapia has Y-shaped stomach with cecum (Rust 2002). By the action of enzymes and gastric juices, food undergoes mechanical destruction there. Storage of the food (in cecum) in the stomach and mixing with digestive enzymes provides preliminary stage of digestion. The main gastric enzyme is pepsin. Pepsin is endopeptidase, which breaks the peptide bonds. The products of its action are short polypeptides and peptides. Pepsin and hydrochloric acid are secreted by oxynticopeptic cells from stomach mucosa. Simultaneously, gastric mucosa secretes hormones (gastrin, somatostatin) by endocrine cells (Rust 2002). At the end of stomach, there are pyloric cecae, which increase absorptive area of intestine, without effect on its thickness or length.

After the first stage of digestion in stomach, feed goes into the intestine. The intestine varies in length, and is the longest in herbivorous fish, due to necessity to process food of the lower quality. Food is spread whole along the intestine surface, maximizing digestion and absorption. Digestion and absorption both take place in the brush border of the columnar epithelial cells, where the enzymes – endoproteases and peptidases are excreted. These enzymes and their activities are highly related to food preferences. Endoproteases break peptide bonds and degrade proteins to oligopeptides. Endoprotease action is specific and affected by chemical group next to peptide bond. Trypsin and chymotrypsin are endoproteases, produced by pancreas and released in intestine. They use proteins and long polypeptides as substrates, and brake them into shorter peptides. Carboxypeptidase, aminopeptidase and di-tripeptidases converts proteins and polypeptides into amino acids. Shorter peptides and amino acids are further digested in first three gut regions. In the fourth part of intestine peptidase

activity is weak, concluding about low contribution to protein hydrolysis in this region (Tengjaroenkul, Smith et al. 2000).

Besides proteins, there are polysaccharides and lipids, which go through enzymatic hydrolysis and further absorption. Lipases are responsible for lipid catabolism, amylase and disaccharidases – for starch and polysaccharide degradation, chitinases – for chitin (Tengjaroenkul, Smith et al. 2000).

The figure below (Figure 3) represents mechanisms of absorption within intestinal enterocytes (or columnar epithelial cells).

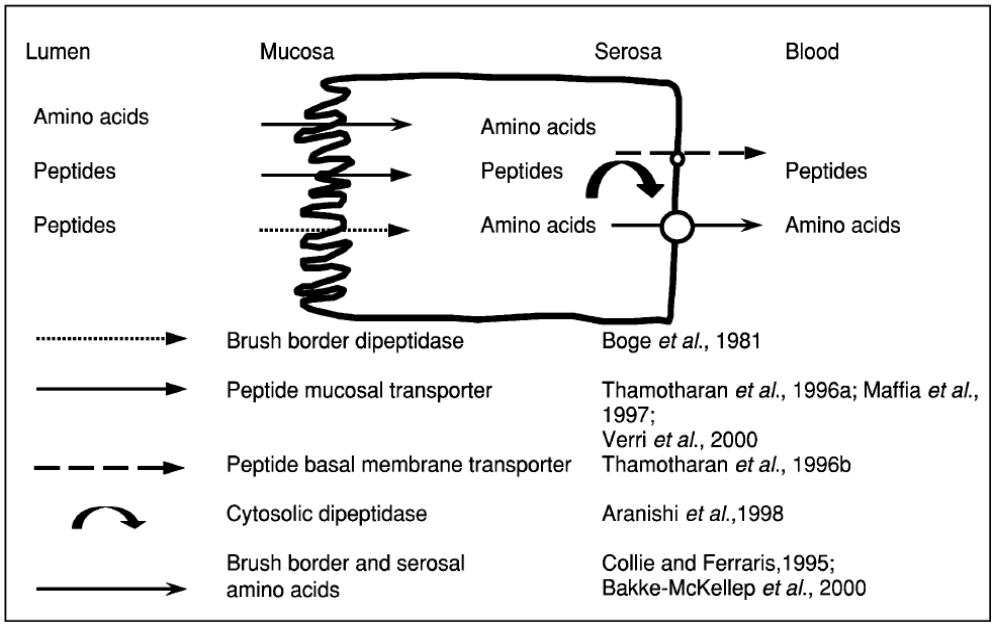


Figure 3. Nutrient absorption and hydrolysis in teleost intestinal enterocytes. Adopted from (Portella and Dabrowski 2005)

Proteins come to intestinal lumen as amino acids and polypeptides, and sometimes whole protein molecules. Amino acids are absorbed by intestine enterocytes by the help of active sodium-linked transport and carrier molecules. Polypeptides are hydrolyzed to amino acids and shorter peptides in intestinal epithelium and become absorbed through transcellular movement (through epithelial cells). Mechanism of peptides' conversion is not studied well, but there is possibility of both, active and passive transport. Remaining particles are captured by the paracellular route (between epithelial cells) or pinocytosis. Some changes, like deamination, protein synthesis could occur already in enterocytes. Amino acids are transported from serosa membrane of intestine to blood plasma, lately, to liver, where amino acid catabolism occurs. (Srivastava, Kurokawa et al. 2002, Evans and Claiborne 2005, Portella and Dabrowski 2005)

Herbivorous fishes also have microorganisms in the posterior part of the gut, where polysaccharides are fermented into short chain fatty acids (Evans and Claiborne 1997).

1.5 Amino acid catabolism

Amino acids are the main oxidative substrates in liver, and are represented in high concentrations there. Amino acids may be used as energy source (around 14-85% of teleost fish energy requirement is covered by amino acids) and participate in gluconeogenesis (Van Waarde 1983). The final excretion product of amino acid catabolism is ammonia (Van Raaij 1995). There are several pathways for ammonia production in fish from amino acids.

1.6 Removal of nitrogen

Generally, amino acid catabolism starts with removal of nitrogen from the molecule. That is trans- or deamination (Braunstein and Bychkov 1939, Braunstein 1957). Transamination is catalyzed by transaminases (or aminotransferases), which transfer α-amino groups to α-ketoglutarate to form glutamate and α-keto acid. Glutamate enters mitochondria, where it is oxidatively deaminized by glutamate dehydrogenase (deamination reaction). Combination of any aminotransferase and glutamate dehydrogenase works as amino acid oxidase system and forms glutamate in liver cytosol (Berg, Tymoczko et al. 2011).

The two most studied aminotransferases are aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT). Both are located in the liver, and their amount is about equilibrium, depending on the amount of incoming amino acids. Increase of amino acids in blood leads to proportional increase of ASAT and ALAT (Berg, Tymoczko et al. 2011).

ALAT (EC 2.6.1.2) catalyzes transamination between alanine and α-ketoglutarate to produce pyruvate and L-glutamate (Yang, Park et al. 2009):

L-alanine + α-ketoglutarate <=> pyruvate + L-glutamate.

Alanine aminotransferase is a homodimeric cytoplasmic enzyme, which plays a key role in the intermediary metabolism of glucose (liver gluconeogenesis), amino acids (protein turnover) and nitrogen metabolism (Ishiguro, Takio et al. 1991). Mainly, alanine aminotransferase is found in liver mitochondria, but some is also detected in kidney, brain and heart cells. In liver it plays major role of alanine turnover into glucose, which further participates in several metabolic pathways. Thus, level of ALAT in liver might be a marker of fish health, and spontaneous changes could affirm about disease or tissue injury (Prati, Taioli et al. 2002).

Aspartate aminotransferase (ASAT, EC 2.6.1.1) has role in the reaction (http://enzyme.expasy.org/EC/2.6.1.1):

L-aspartate + α-ketoglutarate <=> oxaloacetate + L-glutamate

ASAT is represented as two dimer isoenzymes in animal cells: one is in cytosol and the other is in mitochondria (Sonderegger, Jaussi et al. 1982). Aspartate aminotransferase isoenzymes are responsible for ping-pong conversion of L-aspartate and L-glutamate, their synthesis and degradation and participation in nitrogen metabolism (Christen and Metzler 1985).

Glutamate dehydrogenase (GDH, EC 1.4.1.2), as previously mentioned, works together with aminotransferases, forming glutamate (http://enzyme.expasy.org/EC/2.6.1.1):

L-glutamate + H2O + NAD+ <=> 2-oxoglutarate + NH3 + NADH

GDH is located in in mitochondrial matrix. It plays a central role in nitrogen and carbon metabolism, due to participation in both oxidative deamination and reductive amination. In deamination GDH converts L-glutamate to 2-oxoglutarate in the TCA cycle. In the reductive amination GDH is involved in reactions, which supply nitrogen for several biosynthetic pathways (Structures of bovine glutamate dehydrogenase complexes elucidate the mechanism of purine regulation (Smith, Peterson et al. 2001). Due to high relation to protein turnover and/or ammonia formation, GDH could be used as a marker for these processes (Liu, Zhou et al. 2012). The summarizing reaction for the amino acid oxidase system, which includes aminotransferase and glutamate dehydrogenase, is:

α-amino acid+NAD^++H2O<=> α-ketoacid+$NH4^+$+NADH+H^+ (or NADPH)

The enzymes, which were described above, are of a great interest. They are mostly expressed in liver. And liver is the central organ for protein turnover. Any deviations in levels of these enzymes indicate changes in protein turnover and liver health itself.

An alternative way to amino acid catabolism is during transamination by the purine nucleotide cycle. Due to its mechanism, amino group from α-amino acid is transferred to oxaloacetate by aminotransferases. Oxaloacetate is converted to aspartate, which subsequently is deaminated in purine nucleotide cycle by the action of three enzymes: adenylosuccinate synthetase, adenylosuccinate lyase and AMP-aminohydrolase. Mostly, reaction is described by the activity of AMP-aminohydrolase (AMP-deaminase). There are four gene isoforms, encoded AMP-deaminase. They are AMPD1, AMPD2, AMPD3 and AMPD4. They are different by their location in tissue. AMPD2 is located in liver tissues (Braunstein 1957, Lowenstein 1972).

Amino acid catabolism might start without transamination. The example is flavoprotein L-amino acid oxidase, which converts L-amino acids to corresponding α-keto acids and ammonia. But scientists found that L-amino acid oxidases less contribute to amino acid catabolism, and has less significance in it (Campbell 1973).

Some of the amino acids could be catabolized with a specific enzyme. For example, proline is converted to Δ-pyrroline-5-carboxylate by the help of proline oxidase. While serine dehydratase and threonine dehydratase could deaminate serine and threonine (Campbell 1973).

Alpha-aminoadipic semialdehyde synthase (AASS) is common in lysine catabolism. Lysine catabolism in plant and animal cells could go through two pathways: via saccharopine and via pipecolic acid. These reactions are catalyzed by a bifunctional enzyme, which is encoded by AASS gene. Bifunctional enzymes contains lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH). LKR (EC 1.5.1.8) catalyses the reaction between lysine and oxoglutarate, using a molecule of NADPH (http://enzyme.expasy.org/EC/1.5.1.8):

N(6)-(L-1,3-dicarboxypropyl)-L-lysine + NADP(+) + H(2)O <=> L-lysine + 2-oxoglutarate + +NADPH

While saccharopine dehydrogenase (EC 1.5.1.9) catalyzes

N6-(L-1,3-Dicarboxypropyl)-L-lysine + NAD^+ + H_2O <=>

<=>L-Glutamate + L-2-Aminoadipate 6-semialdehyde + NADH + H^+

MAB21 EC 2.7.7.86 (Cyclic GMP-AMP synthase) catalyzes (http://enzyme.expasy.org/EC/2.7.7.86)

ATP + GTP <=> 2 Diphosphate + Cyclic GMP-AMP

Cyclic GMP-AMP synthase (shortly, GAS) plays as a trigger in type I interferons and other cytokines. Even through the evolution, it stays conserved from fish to human, and involved into synthesis of cyclic di-nucleotides. Investigation of this protein gives totally new outlook on mechanism of immune signaling. Even viruses, bacteria, parasites, etc. could trigger synthesis of cyclic GMP-AMP, and thus, provide strong immune response (Sun, Wu et al. 2013). Due to common metabolites in immune signaling pathway and purine nucleotide cycle, there is a hypothesis of MAB21 participation in amino acid catabolism and whole protein turnover.

1.7 Transcriptome assay

Amino acid catabolism is extremely difficult to study on, due to lack of the information about pathways and due to limit of tools. Previously, studies on this field were mostly based on indirect estimations: measuring level of catabolic products, blood and urine amino acid estimations, and quantitative methods with labeling of amino acids by tracers.

Upcoming techniques and technologies give a possibility to study the molecules, which constitute the organism. New wave brings 'omics' science in laboratory. 'Omics' technologies include genomics (study of genes), transcriptomics (study of RNA), proteomics (study of proteins), and metabolomics (study of metabolites). 'Omics' have a wide range of application: it helps in understanding of all physiological processes, as well as, in disease processes occurring in organism, marker investigation, drug discovery (Horgan and Kenny 2011).

Transcriptomics is a study of complete set of RNA transcripts, which are received from genome transcription in particular conditions of from a particular cell or tissue. Study of transcriptome identify genes, which are differentially expressed in under different conditions, or in different cells.

Reverse transcription PCR approach goes ahead in studies of differential expression of genes. This type of PCR gives opportunity to detect and measure products, amplified during each cycle of reaction. Well-designed oligonucleotide probe is hybridized to the target sequence. While Taq polymerase cleaves the probe, probe produces fluorescence detected by machine. This fluorescence indicates amplification of target-specific product. Ct value, which is established through the analysis, is a number of PCR cycle, where reporter fluorescence overcomes threshold (Heid, Stevens et al. 1996). Lately, Ct value is used in comparative analysis of gene differential expression between test sample and control sample.

1.8 Objectives of the study

The objective of experiment was to see the metabolic responses in Nile tilapia on feeding intake through performing the linkages between hepatic enzymes' transcriptional level, plasma free amino acids, ammonia excretion and digested protein.

The hypothesis is hepatic enzymes control protein turnover in Nile tilapia.

2 MATERIALS AND METHODS[1]

2.1 Background information from Bajgai and Hoque (2014)

The experiment was performed at Fish Nutrition Laboratory of the Norwegian University of Life Sciences, Ås, Norway. Formulation and preparation of the diet, feeding, fish growth performance, ammonia and nitrite measurements, digestibility evaluation, and feed and protein utilization are described in detail by Bajgai and Hoque (2014).

Briefly, GIFT tilapia (Eknath et al., 1993) from the 12th generation of selection for rapid growth were the experimental animals. The fish were placed in 10 indoor tanks (70×50×50 cm) equipped with recirculated freshwater, and exposed to 24 hours photo light regime. Each tank contained 30 fish (mean weight ± S.E.M; 24.03 ± 0.1 g). Oxygen level within tanks was measured daily and was approximately 7.5 mg l^{-1} and the average water temperature was 27.5 °C. Water flow was kept at 180 l h^{-1}. The experiment lasted for 45 days.

One plant protein based diet was prepared. Table A1 (Appendix) represents contents of the formulated diet. Ground ingredients were mixed and extruded at 54 °C with a pasta machine (P55DV, Italgy, Carasco, Italy) to pellets of 2 mm length and 2 mm diameter. Dried pellets were stored at -20 °C. The fish were divided into three feeding groups with three tank replicates in each. The first group was fed twice daily (10:00h and 20:00h) for 70 min in access of appetite. The second group had same feeding regime as previous one but with 90% of average day eaten feed of the first group. The third group was had fed four times a day (8:00h, 12:00h, 16:00h and 20:00h) for 35 min of feeding with the same amount of feed as second group. Fish were fed by automatic belt feeder. Uneaten feed was gathered by a strainer from the water outlet and dried at 105 °C.

Ammonia concentration in water was measured at day 45 in a closed system, allowing ammonia to accumulate over time. Measurements were done at one hour before last meal, 2 hours after last meal, 4 hours , 6 hours, 8 hours and 10 hours after last feeding, from the inlet and outlet water of the tanks. Analyses were done spectrophotometrically by a Spectroquant®

[1] All fish were anesthetized with tricanine methanesulfonate (MS-222, 0.1 g l-1 water, buffered with NaHCO3, 0.1 g l-1 water, Western Chemical Inc., Washington, USA) before being euthanized. Experimental procedures were approved by The Norwegian National Committees for Research Ethics.

NOVA 60 (Merck Millipore, Darmstadt, Germany) kits. The amount of 50 ml of water was obtained for each analysis. Ammonium (NH_4-N) measurement was followed by the Spectroquant® Protocol #14752. Thereafter, it was recalculated to NH_4^+, and NH_3 based on the following formulas:

$NH_3 = NH_3\text{-}N * 1.22$ (1),

$NH_{4^+} = NH_4\text{-}N * 1.29$ (2).

Fifteen fish were taken from each tank at the day of 41 for digestibility assessment. They were dissected; feces were collected from intestine at around 10 cm from the rectum. Crude protein and yttrium oxide contents were both measured in feces and in feed. Apparent digestibility was calculated following the formula:

$$Apparent\ digestibility = 100 - 100 \times \frac{nutrient\ in\ feces\ \% \times yttrium\ in\ feed\ \%}{nutrient\ in\ feed\ \% \times yttrium\ in\ feces\ \%}$$

Blood plasma sampling was performed on 41st day. Three randomly chosen fish were analyzed from each tank. Blood of three fish from the same tank was collected from the caudal vein into 5 ml tubes and a heparinized blood sample was centrifuged 1500 g for 15 min at room temperature to obtain plasma. About 1 ml of plasma was obtained for each tank, for 5 time points (2, 4, 6, 8 and 10 hours after last feeding). Samples were stored at -80℃ until analysis.

Table 1. Performance of the fish (Bajgai and Hoque 2014)

	Minimum	**Maximum**
Start weight, g fish^{-1}	24.0	24.0
End weight, g fish^{-1}	66.9	70.7
Weight gain, g fish^{-1}	42.9	46.7
Feed conversion ratio, g DM intake/(g gain $^{-1}$)	0.97	1.06
Protein digestibility, %	97.6	98.5
Liver weight, g	3.6	4.7
Retention, kg^{-1}		
protein, g	405	450
energy, g	410	438

TAN excretion, mmol (gBW*h) $^{-1}$	71.31	2061.38

2.2 Relationships between hepatic gene expressions, plasma amino acid clearance and digested protein

All work described from here on were done specifically for this thesis.

2.3 Sampling for hepatic transcriptional analysis

The whole liver of each fish was placed in a 10 ml tube and mixed with dry ice. Liver samples were stored at -80 ^{0}C. For qRT-PCR liver samples of two time points were used: 2 and 10 hours after last feeding.

2.4 RNA isolation, cDNA synthesis

RNA isolation and further manipulations with it were performed in Qingdao, China, at Yellow Sea Fisheries Research Institute.

For RNA isolation 16 samples totally were used: 8 RNAs from liver samples, taken at 2 hours after last feeding (encoded from 11-19), and 8 RNAs from liver samples, taken at 10 hours after last feeding (encoded from 51-59). Samples from tank 8 were not analysed, due to feeding mistake right before experiment. The total RNA was extracted by trizol, according to Zymo Research Direct-zol RNA MiniPrep (R2050). After isolation RNA was evaluated on NanoDrop spectrophotometry (Nanodrop 1000 Spectro- photometer, Thermo Scientific, Loughborough, UK) for its quality and concentration, and gel electrophoresis was performed (Table A2).

cDNA synthesis was performed according Transcriptor First Strand cDNA Synthesis Kit manual (Roche Applied Science) in two replicates, with random primers and oligo$(dT)_{18}$ anchored primers. The amount of 8 µg of total RNA mixed with 1 µl of 2.5 µM anchored-oligo $(dT)_{18}$ Primer (50 pmol/µl) or 2 µl of 60 µM Random Hexamer Primer (600 pmol/µl) for total volume of 13 µl. After denaturation step at 65 °C for 10 min, the rest of the components were added for the final volume of 20 µl. They were 4 µl of 5X Transcriptor Reverse Transcriptase Reaction Buffer, 0.5 µl of 40U/ µl Protector RNase Inhibitor, 2 µl of 10 mM Deoxynucleotide Mix, and 0.5 µl of 20U/ µl Transcriptor Reverse Transcriptase. Reverse transcriptase reaction was incubated at 55 °C for 30 min for anchored-oligo $(dT)_{18}$ Primer; and 10 min at 25 °C,

followed by 30 min at 55 °C for Random Hexamer Primer. The Transcriptase was inactivated by heating reaction at 85 °C for 5 min. cDNA were stored at -20 °C for further qRT-PCR.

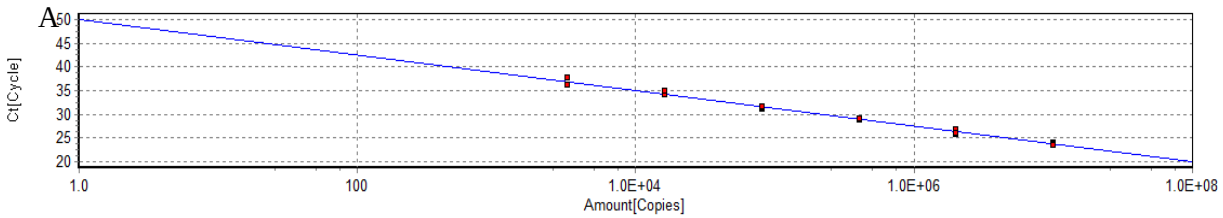
2.5 Identification of primers' annealing temperature

Primers had been checked for appropriate annealing temperature at 58, 59, 60, 61 and 62 ^{0}C, and 60 ^{0}C was chosen as annealing temperature in qRT-PCR reactions. Conclusion is based on PCR, running with different annealing temperatures and gel electrophoresis (Figure A4).

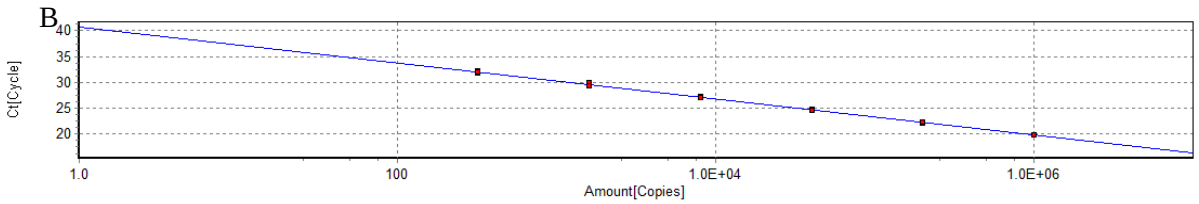
2.6 Standard curve

Due to low amount and low concentration of RNA in experimental samples, cDNA, obtained from farmed Nile tilapia (Qingdao, China) was used for obtaining standard curve.

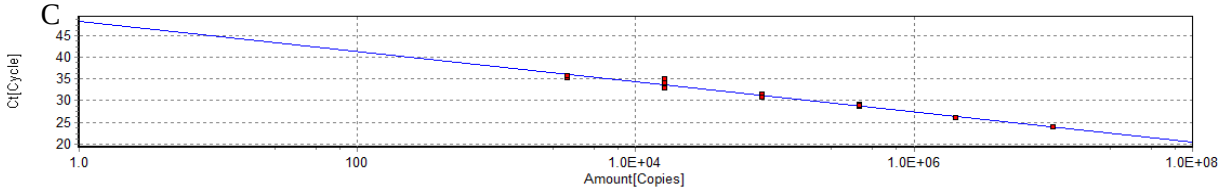
Standard curve was made for each primer pair in a line of several dilutions. Value of PCR efficiency was used in further calculations. Figures 2.1 (A-F) below represent standard curves for all six target genes. Amplification and melting curves are presented in Appendix.



Slope: -3.755
Y-Intercept: 50.01
Efficiency: 0.85
R^2: 0.991



Slope: -3.495
Y-Intercept: 40.7
Efficiency: 0.93
R^2: 0.999



Slope: -3.486
Y-Intercept: 48.3
Efficiency: 0.94
R^2: 0.986

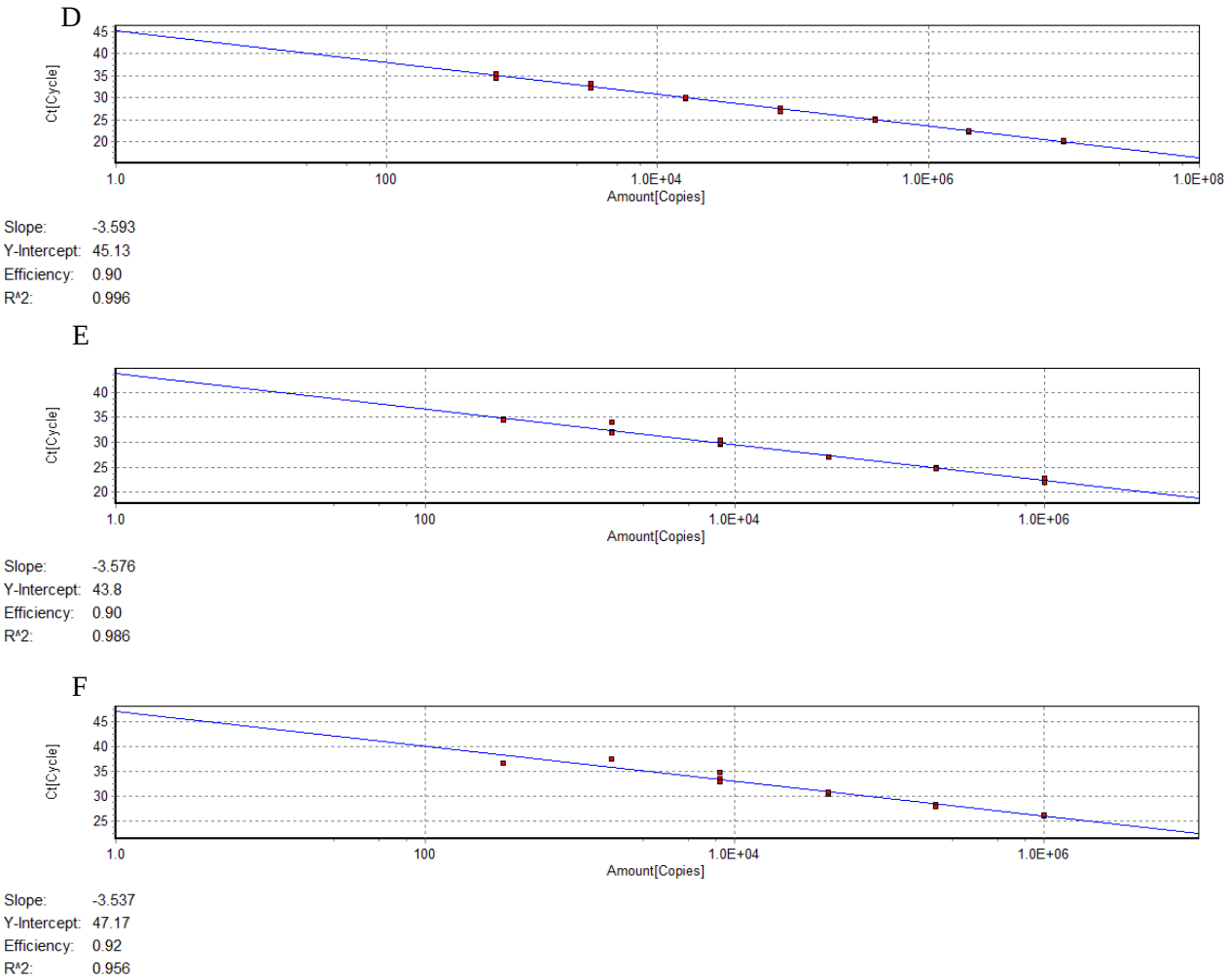


Figure 4. Standard curves: A – AASS, B – ALAT, C – AMPD2, D – ASAT, E – GDH, F – MAB target genes.

2.6.1 qRT-PCR

Quantitative real time PCR was performed on Eppendorf Mastercycler ep realplex real-time PCR system and using FastStart Essential DNA Green Master (Roche Applied Science). Six sequence tags were chosen for quantitative analysis. They are ALAT (alanine aminotransferase), ASAT (aspartate aminotransferase), GDH (glutamate dehydrogenase), AMPD2 (adenosine monophosphate deaminase 2), MAB (MAB-21 domain), AASS (aminoadipate-semialdehyde synthase). β-actin was chosen as housekeeping gene. The primers for target sequence tags are placed in supplement data (Table A3).

The PCR reactions were performed as follows: 10 minutes at 95 ^{0}C, and then 40 cycles of 10 sec at 95 ^{0}C, 15 sec of 60 ^{0}C, 20 sec at 72 ^{0}C, and melting step 15 sec at 95 ^{0}C, 15 sec at 60 ^{0}C, 15 sec at 95 ^{0}C.

Samples were tested in triplicate for each cDNA sample. As mentioned previously, two types of cDNA were synthesized. cDNA, synthesized by random primers, was used for qRT-PCR of AASS, ASAT, AMPD2, β-actin and by oligo(dT)$_{18}$ anchored primers for ALAT, MAB, GDH, β-actin.

Relative expression of target tags was calculated, according to Pfaffl's mathematical model (Pfaffl, 2001). Equation (7) shows calculation, based on the PCR efficiency (E) and the Ct of a sample versus the control, and expressed in comparison to the reference gene (β-actin).

$$\text{Ratio}=\frac{(E_{target})^{\Delta CP target(control-sample)}}{(E_{act})^{\Delta CP act(control-sample)}} \quad (7),$$

E is a real time PCR efficiency of target gene (E_{target}), and reference gene (E_{act}). CP is defined as a crossing point. Crossing point represents the number of the cycles, where fluorescence crosses fluorescence threshold. ΔCP represents the deviation of CP between control and sample for target gene ($\Delta CP_{target}(control - sample)$, and for reference gene ($\Delta CP_{act}(control - sample)$. Control was RNA of liver from the first tank.

2.6.2 Amino acid concentration

Plasma free amino acids were analyzed in China by reverse-phase high performance liquid chromatography (SGS-CSTC Standards Technical Services (Shanghai) Co., Ltd). HPLC setup and running conditions.

2.6.3 Digested protein

Digested protein intake per body weight was calculated by following formula. Feed intake data was estimated at day 41.

$$Digested\ protein = \frac{Feed\ intake \times Diet\ protein \times Digestibility}{Body\ weight \times 100},$$

Where feed intake and body weight are in grams, diet protein and protein digestibility are in per cent.

2.6.4 Calculation and statistical analysis

Amino acid clearance was calculated as a difference in between concentration at two time points. Totally there were three clearance ranges for each of amino acid: 2-6 hours, 6-10 hours and 2-10 hours after last feeding.

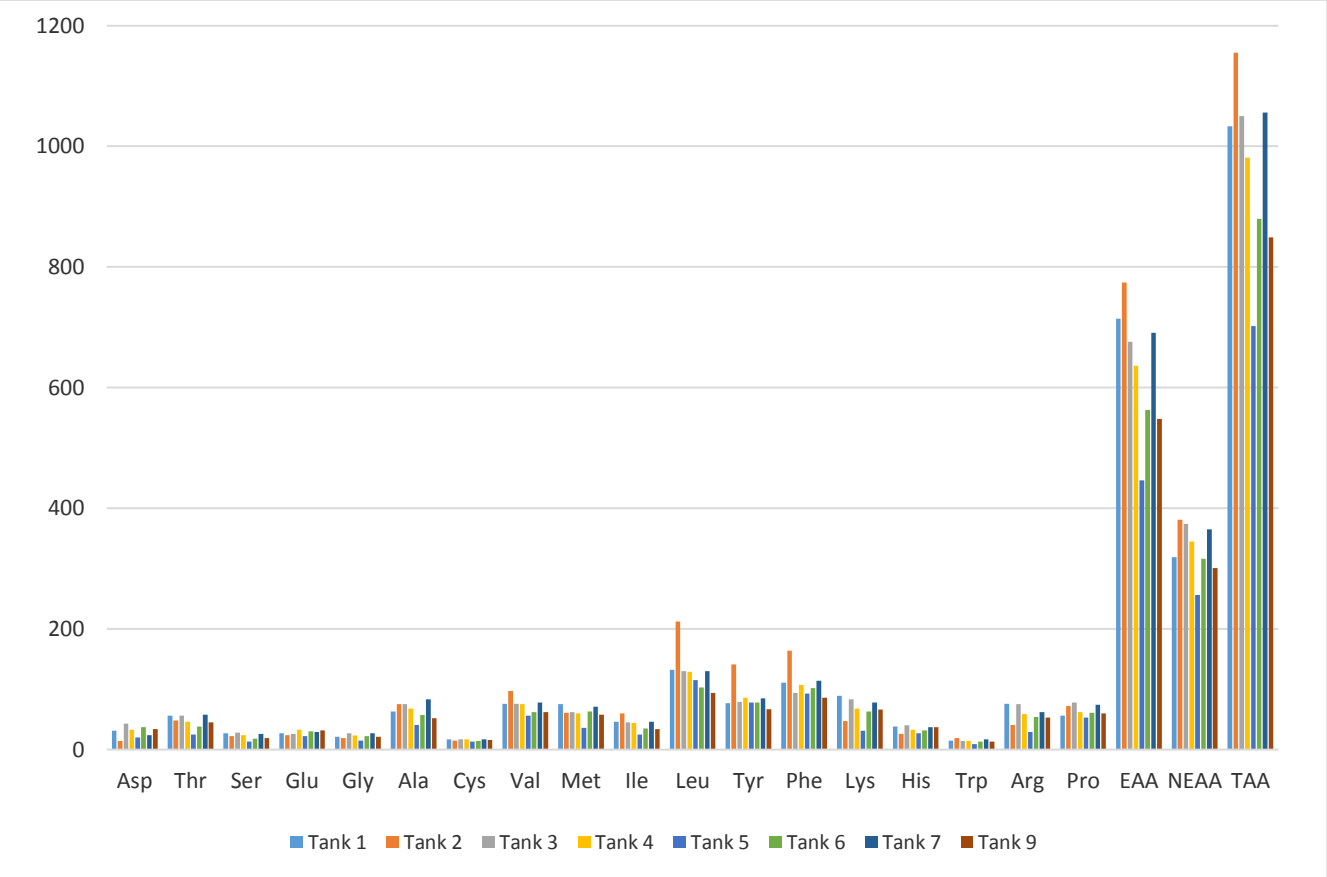
Statistical regression analysis was performed in Proc GLM by the SAS Statistical Software. Correlations between free amino acids in blood plasma and expression rate of transaminases had linear and polynomial character. Pearson correlation coefficients were estimated for free amino acids with different clearances. Statistically significant results were considered for $P<0.05$, trends for $0.05 \leq P<0.10$.

3 RESULTS

3.1 Amino acid concentration in blood plasma

Free amino acid concentration in blood plasma was measured at three time point, at 2, 6 and 10 hours after last feeding. Figures 5, 6 and 7 represents histograms with individual amino acid concentration, EAA (essential amino acids), NEAA (non-essential amino acids), TAA (total amino acids), all values presented as mg per kg body weight. Tables in Appendix show numerical data of the same estimation.

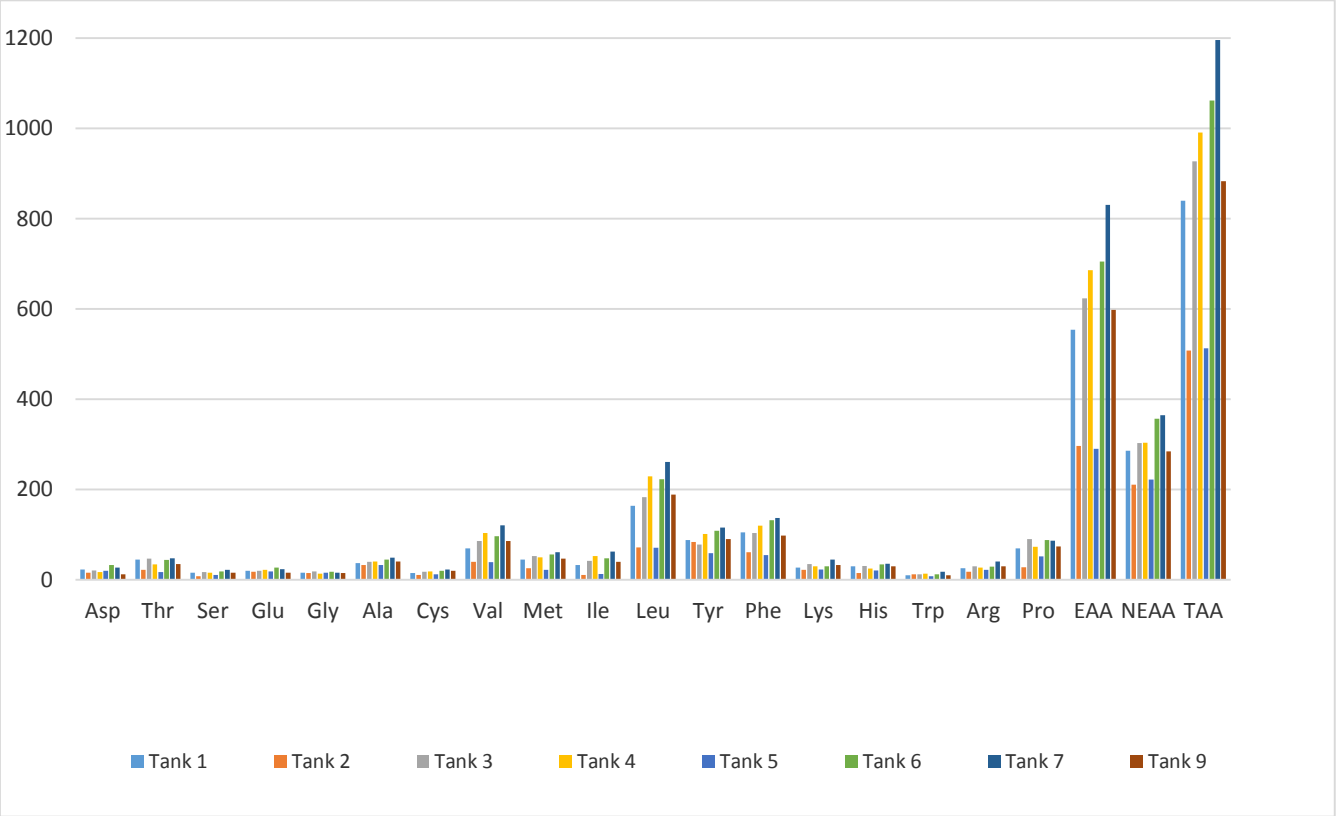
Figure 5. Free amino acid in blood plasma of Nile tilapia 2 hours after last feeding.



At 2 hours after last feeding, cysteine and tryptophan has lowest concentration in blood plasma, varying from 9 mg/kg for tryptophan in tank 5, to 19 mg/kg for tryptophan in tank 2. The highest concentration in blood plasma was for leucine, with the highest point of 212 mg/kg in tank 2. In average. The concentration of EAA was almost twice higher than the concentration of NEAA.

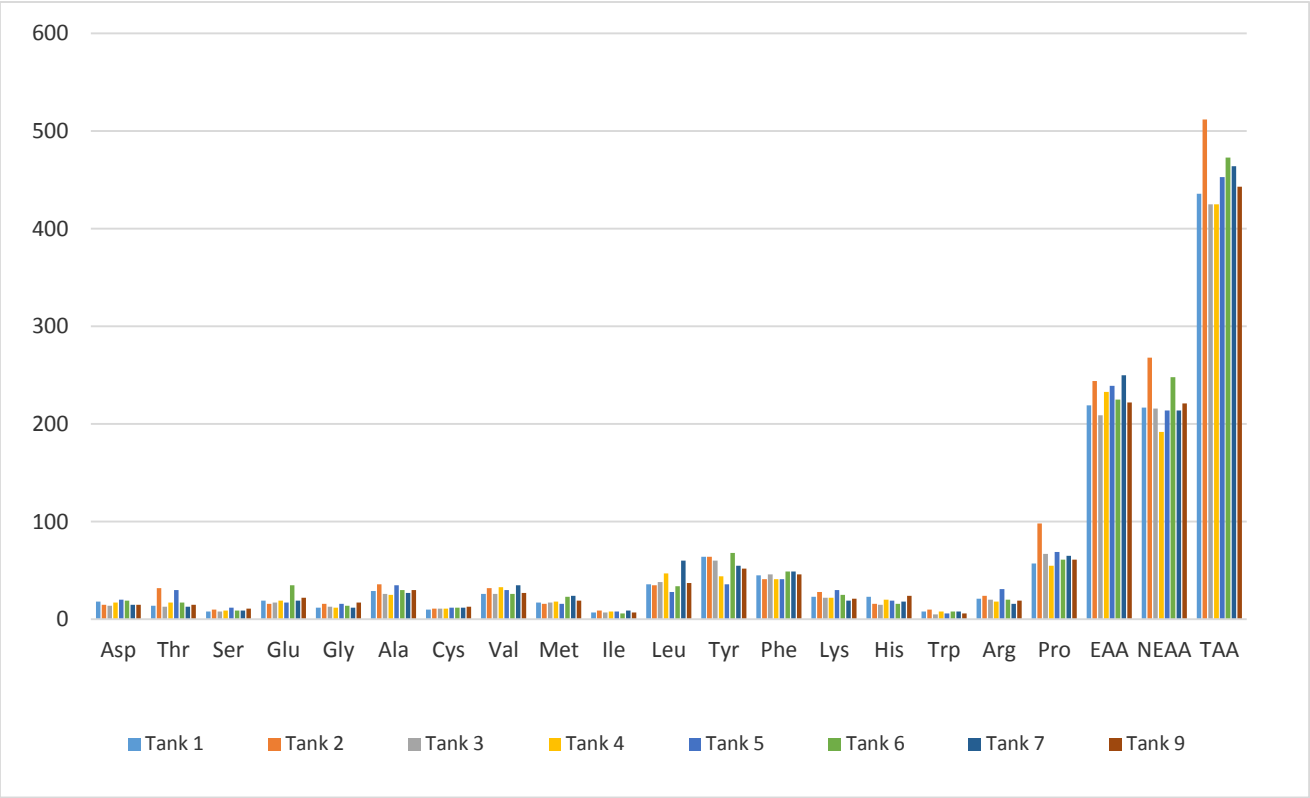
The AA concentration 6 hours after last feeding is presented in figure 6. Leucine still had the highest concentration level with a maximum of 261 mg/kg in tank 7. The majority of AA concentrations were reduced when compared to the values obtained at 2 h (Figure 5).

Figure 6. Free amino acid in blood plasma of Nile tilapia 6 hours after last feeding



At 10 hours, concentrations of all AA were decreased. Total amino acid concentration has fallen almost twice comparing to previous estimated levels (from 865 mg/kg to 464 mg/kg), and few amino acids reached 500 mg per kg body eight in the plasma.

Figure 7. Free amino acid in blood plasma of Nile tilapia 10 hours after last feeding



3.2 Pearson correlations coefficients for free amino acids in blood plasma

To measure the strength between two variables Pearson correlation coefficients were estimated. Table 2 represents the results for Pearson correlation coefficients for amino acids clearance from 2 to 6 hours after the last feeding. First line in a cell represents Pearson coefficient, second line is a P value, and the third line is a number of data pairs. Pearson coefficient varies from -1 to 1, which shows variables change correspondingly. Minus is for negative correlation, when one variable is increasing and the second is decreasing.

Table 2. Pearson correlation coefficients for amino acid clearance from blood plasma at 2-6 hours after last feeding in Nile tilapia.

Pearson Correlation Coefficients
Prob > |r| under H0: Rho=0
Number of Observations

	TAA	EAA	NEAA	ARG	HIS	Ile	Leu	Met	Phe	Thr	Val	Trp	Lys	Ala	Glu	Gly	Asp	Cys	Ser	Tyr	Pro
TAA	1.00000 8	0.99441 <.0001 8	0.93513 0.0006 8	0.03207 0.9399 8	0.75003 0.0321 8	0.97021 0.0003 7	0.95918 0.0002 8	0.86306 0.0058 8	0.95717 0.0002 8	0.80316 0.0164 8	0.94283 0.0015 7	0.85768 0.0065 8	-0.13141 0.7564 8	0.49877 0.2083 8	-0.09866 0.8162 8	-0.40091 0.3250 8	-0.25188 0.5473 8	0.90363 0.0021 8	0.74523 0.0338 8	0.95848 0.0002 8	0.91856 0.0013 8
EAA	0.99441 <.0001 8	1.00000 8	0.89250 0.0029 8	0.01317 0.9753 8	0.71155 0.0478 8	0.99316 <.0001 7	0.97164 <.0001 8	0.88350 0.0036 8	0.96000 0.0002 8	0.75615 0.0299 8	0.97955 0.0001 7	0.87415 0.0045 8	-0.14114 0.7389 8	0.43281 0.2841 8	-0.14224 0.7369 8	-0.47542 0.2338 8	-0.29430 0.4792 8	0.92587 0.0010 8	0.70047 0.0530 8	0.95026 0.0003 8	0.89645 0.0026 8
NEAA	0.93513 0.0006 8	0.89250 0.0029 8	1.00000 8	0.09284 0.8269 8	0.81648 0.0134 8	0.61076 0.1452 7	0.83715 0.0095 8	0.72228 0.0430 8	0.86762 0.0052 8	0.89376 0.0028 8	0.54131 0.2095 7	0.73069 0.0395 8	-0.08778 0.8363 8	0.67845 0.0644 8	0.05584 0.8955 8	-0.11728 0.7821 8	-0.08844 0.8350 8	0.75343 0.0309 8	0.83315 0.0102 8	0.90590 0.0019 8	0.91595 0.0014 8
ARG	0.03207 0.9399 8	0.01317 0.9753 8	0.09284 0.8269 8	1.00000 8	0.32406 0.4336 8	0.24565 0.5954 7	-0.19206 0.6486 8	0.20234 0.6308 8	-0.24564 0.5576 8	0.03708 0.9305 8	0.07467 0.8736 7	0.22781 0.5874 8	0.96409 0.0001 8	0.42602 0.2926 8	0.19748 0.6392 8	0.36205 0.3781 8	0.48607 0.2220 8	0.12262 0.7724 8	0.60199 0.1143 8	-0.16968 0.6879 8	-0.24988 0.5506 8
HIS	0.75003 0.0321 8	0.71155 0.0478 8	0.81648 0.0134 8	0.32406 0.4336 8	1.00000 8	0.64718 0.1161 7	0.61765 0.1027 8	0.59055 0.1232 8	0.59759 0.1177 8	0.78257 0.0217 8	0.56161 0.1895 7	0.64340 0.0852 8	0.16905 0.6890 8	0.42410 0.2950 8	0.43501 0.2814 8	-0.10237 0.8094 8	0.35941 0.3819 8	0.72874 0.0403 8	0.82782 0.0112 8	0.65844 0.0758 8	0.59554 0.1193 8
Ile	0.97021 0.0003 7	0.99316 <.0001 7	0.61076 0.1452 7	0.24565 0.5954 7	0.64718 0.1161 7	1.00000 7	0.91612 0.0037 7	0.71184 0.0728 7	0.84886 0.0157 7	0.28397 0.5371 7	0.98366 <.0001 7	0.72795 0.0636 7	0.14034 0.7641 7	-0.12799 0.7845 7	-0.11673 0.8032 7	-0.66428 0.1036 7	0.09812 0.8342 7	0.95604 0.0008 7	0.49990 0.2533 7	0.83011 0.0208 7	0.56813 0.1833 7
Leu	0.95918 0.0002 8	0.97164 <.0001 8	0.83715 0.0095 8	-0.19206 0.6486 8	0.61765 0.1027 8	0.91612 0.0037 7	1.00000 8	0.78291 0.0216 8	0.97863 <.0001 8	0.67271 0.0675 8	0.97053 0.0003 7	0.77901 0.0227 8	-0.35345 0.3904 8	0.30176 0.4676 8	-0.24632 0.5565 8	-0.60353 0.1131 8	-0.37326 0.3624 8	0.89769 0.0025 8	0.54728 0.1603 8	0.97697 <.0001 8	0.90150 0.0022 8
Met	0.86306 0.0058 8	0.88350 0.0036 8	0.72228 0.0430 8	0.20234 0.6308 8	0.59055 0.1232 8	0.71184 0.0728 7	0.78291 0.0216 8	1.00000 8	0.80560 0.0158 8	0.72964 0.0399 8	0.64589 0.1171 7	0.90759 0.0018 8	0.14674 0.7288 8	0.45683 0.2552 8	-0.04725 0.9115 8	-0.28536 0.4933 8	-0.37224 0.3638 8	0.83100 0.0106 8	0.68259 0.0621 8	0.70763 0.0496 8	0.73943 0.0360 8
Phe	0.95717 0.0002 8	0.96000 0.0002 8	0.86762 0.0052 8	-0.24564 0.5576 8	0.59759 0.1177 8	0.84886 0.0157 7	0.97863 <.0001 8	0.80560 0.0158 8	1.00000 8	0.76204 0.0280 8	0.91876 0.0035 7	0.77991 0.0224 8	-0.38574 0.3453 8	0.37473 0.3604 8	-0.17746 0.6742 8	-0.48237 0.2261 8	-0.43110 0.2863 8	0.83748 0.0095 8	0.54295 0.1643 8	0.96623 <.0001 8	0.96384 0.0001 8
Thr	0.80316 0.0164 8	0.75615 0.0299 8	0.89376 0.0028 8	0.03708 0.9305 8	0.78257 0.0217 8	0.28397 0.5371 7	0.67271 0.0675 8	0.72964 0.0399 8	0.76204 0.0280 8	1.00000 8	0.22059 0.6346 7	0.63541 0.0904 8	-0.04976 0.9069 8	0.67702 0.0651 8	0.28235 0.4981 8	0.12064 0.7760 8	-0.14055 0.7399 8	0.61490 0.1047 8	0.76093 0.0283 8	0.71780 0.0450 8	0.85221 0.0072 8
Val	0.94283 0.0015 7	0.97955 0.0001 7	0.54131 0.2095 7	0.07467 0.8736 7	0.56161 0.1895 7	0.98366 <.0001 7	0.97053 0.0003 7	0.64589 0.1171 7	0.91876 0.0035 7	0.22059 0.6346 7	1.00000 7	0.64202 0.1200 7	-0.03280 0.9443 7	-0.24060 0.6033 7	-0.19730 0.6715 7	-0.76048 0.0472 7	-0.00945 0.9840 7	0.94417 0.0014 7	0.36040 0.4271 7	0.87695 0.0095 7	0.63427 0.1260 7
Trp	0.85768 0.0065 8	0.87415 0.0045 8	0.73069 0.0395 8	0.22781 0.5874 8	0.64340 0.0852 8	0.72795 0.0636 7	0.77901 0.0227 8	0.90759 0.0018 8	0.77991 0.0224 8	0.63541 0.0904 8	0.64202 0.1200 7	1.00000 8	0.14178 0.7377 8	0.32061 0.4388 8	0.10657 0.8017 8	-0.37407 0.3613 8	-0.10224 0.8096 8	0.77786 0.0230 8	0.64189 0.0862 8	0.71767 0.0450 8	0.70270 0.0519 8
Lys	-0.13141 0.7564 8	-0.14114 0.7389 8	-0.08778 0.8363 8	0.96409 0.0001 8	0.16905 0.6890 8	0.14034 0.7641 7	-0.35345 0.3904 8	0.14674 0.7288 8	-0.38574 0.3453 8	-0.04976 0.9069 8	-0.03280 0.9443 7	0.14178 0.7377 8	1.00000 8	0.33271 0.4207 8	0.25650 0.5397 8	0.45488 0.2575 8	0.43811 0.2776 8	-0.03582 0.9329 8	0.44992 0.2633 8	-0.36129 0.3792 8	-0.38575 0.3453 8
Ala	0.49877 0.2083 8	0.43281 0.2841 8	0.67845 0.0644 8	0.42602 0.2926 8	0.42410 0.2950 8	-0.12799 0.7845 7	0.30176 0.4676 8	0.45683 0.2552 8	0.37473 0.3604 8	0.67702 0.0651 8	-0.24060 0.6033 7	0.32061 0.4388 8	0.33271 0.4207 8	1.00000 8	-0.08071 0.8493 8	0.50957 0.1971 8	-0.15128 0.7206 8	0.27333 0.5125 8	0.80251 0.0165 8	0.41575 0.3056 8	0.53151 0.1752 8
Glu	-0.09866 0.8162 8	-0.14224 0.7369 8	0.05584 0.8955 8	0.19748 0.6392 8	0.43501 0.2814 8	-0.11673 0.8032 7	-0.24632 0.5565 8	-0.04725 0.9115 8	-0.17746 0.6742 8	0.28235 0.4981 8	-0.19730 0.6715 7	0.10657 0.8017 8	0.25650 0.5397 8	-0.08071 0.8493 8	1.00000 8	0.39277 0.3358 8	0.69881 0.0538 8	-0.16344 0.6990 8	0.12412 0.7697 8	-0.22311 0.5953 8	-0.09680 0.8196 8
Gly	-0.40091 0.3250 8	-0.47542 0.2338 8	-0.11728 0.7821 8	0.36205 0.3781 8	-0.10237 0.8094 8	-0.66428 0.1036 7	-0.60353 0.1131 8	-0.28536 0.4933 8	-0.48237 0.2261 8	0.12064 0.7760 8	-0.76048 0.0472 7	-0.37407 0.3613 8	0.45488 0.2575 8	0.50957 0.1971 8	0.39277 0.3358 8	1.00000 8	0.24333 0.5614 8	-0.59154 0.1224 8	0.14136 0.7385 8	-0.49137 0.2162 8	-0.25511 0.5420 8
Asp	-0.25188 0.5473 8	-0.29430 0.4792 8	-0.08844 0.8350 8	0.48607 0.2220 8	0.35941 0.3819 8	0.09812 0.8342 7	-0.37326 0.3624 8	-0.37224 0.3638 8	-0.43110 0.2863 8	-0.14055 0.7399 8	-0.00945 0.9840 7	-0.10224 0.8096 8	0.43811 0.2776 8	-0.15128 0.7206 8	0.69881 0.0538 8	0.24333 0.5614 8	1.00000 8	-0.20679 0.6232 8	0.13991 0.7411 8	-0.30202 0.4672 8	-0.40769 0.3161 8
Cys	0.90363 0.0021 8	0.92587 0.0010 8	0.75343 0.0309 8	0.12262 0.7724 8	0.72874 0.0403 8	0.95604 0.0008 7	0.89769 0.0025 8	0.83100 0.0106 8	0.83748 0.0095 8	0.61490 0.1047 8	0.94417 0.0014 7	0.77786 0.0230 8	-0.03582 0.9329 8	0.27333 0.5125 8	-0.16344 0.6990 8	-0.59154 0.1224 8	-0.20679 0.6232 8	1.00000 8	0.67068 0.0687 8	0.84942 0.0076 8	0.71091 0.0481 8
Ser	0.74523 0.0338 8	0.70047 0.0530 8	0.83315 0.0102 8	0.60199 0.1143 8	0.82782 0.0112 8	0.49990 0.2533 7	0.54728 0.1603 8	0.68259 0.0621 8	0.54295 0.1643 8	0.76093 0.0283 8	0.36040 0.4271 7	0.64189 0.0862 8	0.44992 0.2633 8	0.80251 0.0165 8	0.12412 0.7697 8	0.14136 0.7385 8	0.13991 0.7411 8	0.67068 0.0687 8	1.00000 8	0.61122 0.1074 8	0.58352 0.1289 8
Tyr	0.95848 0.0002 8	0.95026 0.0003 8	0.90590 0.0019 8	-0.16968 0.6879 8	0.65844 0.0758 8	0.83011 0.0208 7	0.97697 <.0001 8	0.70763 0.0496 8	0.96623 <.0001 8	0.71780 0.0450 8	0.87695 0.0095 7	0.71767 0.0450 8	-0.36129 0.3792 8	0.41575 0.3056 8	-0.22311 0.5953 8	-0.49137 0.2162 8	-0.30202 0.4672 8	0.84942 0.0076 8	0.61122 0.1074 8	1.00000 8	0.93307 0.0007 8
Pro	0.91856 0.0013 8	0.89645 0.0026 8	0.91595 0.0014 8	-0.24988 0.5506 8	0.59554 0.1193 8	0.56813 0.1833 7	0.90150 0.0022 8	0.73943 0.0360 8	0.96384 0.0001 8	0.85221 0.0072 8	0.63427 0.1260 7	0.70270 0.0519 8	-0.38575 0.3453 8	0.53151 0.1752 8	-0.09680 0.8196 8	-0.25511 0.5420 8	-0.40769 0.3161 8	0.71091 0.0481 8	0.58352 0.1289 8	0.93307 0.0007 8	1.00000 8

Out of 210 coefficients being calculated (Table 2), 77 Pearson correlation coefficients are statistically significant (P<0.05) and represents strong correlation between amino acids, 15 coefficients has P value varying from 0.05 to 0.1.

Table 3. Pearson correlation coefficients for amino acid clearance from blood plasma at 2-10 hours after last feeding in Nile tilapia.

Pearson Correlation Coefficients, N = 8
Prob > |r| under H0: Rho=0

	TAA	EAA	NEAA	ARG	HIS	Ile	Leu	Met	Phe	Thr	Val	Trp	Lys	Ala	Glu	Gly	Asp	Cys	Ser	Tyr	Pro
TAA	1.00000	0.97517 <.0001	0.84112 0.0089	0.67003 0.0691	0.48604 0.2220	0.94524 0.0004	0.45750 0.2544	0.83779 0.0094	0.52941 0.1772	0.72496 0.0419	0.90041 0.0023	0.88634 0.0034	0.62024 0.1009	0.88078 0.0039	0.46472 0.2460	0.62670 0.0964	0.17062 0.6862	0.77099 0.0251	0.88410 0.0036	0.20145 0.6324	0.24168 0.5642
EAA	0.97517 <.0001	1.00000	0.70046 0.0530	0.60264 0.1138	0.37570 0.3590	0.97130 <.0001	0.56224 0.1469	0.85993 0.0062	0.61699 0.1032	0.65329 0.0790	0.95265 0.0003	0.85931 0.0062	0.55295 0.1552	0.77493 0.0239	0.35617 0.3865	0.48789 0.2200	0.08202 0.8469	0.71473 0.0463	0.80802 0.0152	0.22303 0.5955	0.08302 0.8451
NEAA	0.84112 0.0089	0.70046 0.0530	1.00000	0.68753 0.0595	0.64884 0.0817	0.67410 0.0668	0.10126 0.8114	0.59980 0.1160	0.19927 0.6361	0.74087 0.0355	0.57517 0.1358	0.75779 0.0294	0.64843 0.0820	0.94596 0.0004	0.62784 0.0956	0.82816 0.0111	0.34958 0.3960	0.73917 0.0361	0.87583 0.0044	0.10451 0.8055	0.57614 0.1350
ARG	0.67003 0.0691	0.60264 0.1138	0.68753 0.0595	1.00000	0.79037 0.0196	0.44073 0.2744	-0.29267 0.4818	0.84966 0.0076	-0.23267 0.5792	0.95961 0.0002	0.35421 0.3893	0.62608 0.0968	0.98849 <.0001	0.72855 0.0404	0.29273 0.4817	0.84543 0.0082	0.71527 0.0461	0.79914 0.0173	0.89164 0.0029	-0.57015 0.1400	0.79504 0.0184
HIS	0.48604 0.2220	0.37570 0.3590	0.64884 0.0817	0.79037 0.0196	1.00000	0.24656 0.5561	-0.33341 0.4196	0.48049 0.2281	-0.36859 0.3689	0.78149 0.0220	0.19243 0.6480	0.51581 0.1907	0.76235 0.0279	0.70243 0.0520	0.08593 0.8397	0.85182 0.0073	0.76470 0.0271	0.43477 0.2817	0.74382 0.0344	-0.52931 0.1773	0.80006 0.0171
Ile	0.94524 0.0004	0.97130 <.0001	0.67410 0.0668	0.44073 0.2744	0.24656 0.5561	1.00000	0.67665 0.0653	0.74551 0.0337	0.75023 0.0320	0.51080 0.1958	0.97802 <.0001	0.82290 0.0121	0.38162 0.3509	0.74058 0.0356	0.33914 0.4112	0.38270 0.3494	-0.04358 0.9184	0.58841 0.1249	0.68807 0.0592	0.41111 0.3116	-0.06064 0.8866
Leu	0.45750 0.2544	0.56224 0.1469	0.10126 0.8114	-0.29267 0.4818	-0.33341 0.4196	0.67665 0.0653	1.00000	0.13113 0.7569	0.92502 0.0010	-0.24559 0.5577	0.77581 0.0236	0.30739 0.4589	-0.36371 0.3758	0.11071 0.7941	0.09444 0.8240	-0.32799 0.4277	-0.53539 0.1715	0.07489 0.8601	0.05889 0.8898	0.81095 0.0146	-0.72184 0.0432
Met	0.83779 0.0094	0.85993 0.0062	0.59980 0.1160	0.84966 0.0076	0.48049 0.2281	0.74551 0.0337	0.13113 0.7569	1.00000	0.24074 0.5657	0.85700 0.0065	0.68355 0.0616	0.76202 0.0280	0.83862 0.0093	0.70924 0.0488	0.27114 0.5160	0.62567 0.0971	0.32561 0.4313	0.80276 0.0165	0.84676 0.0080	-0.24395 0.5604	0.38916 0.3407
Phe	0.52941 0.1772	0.61699 0.1032	0.19927 0.6361	-0.23267 0.5792	-0.36859 0.3689	0.75023 0.0320	0.92502 0.0010	0.24074 0.5657	1.00000	-0.14303 0.7355	0.77901 0.0227	0.38258 0.3496	-0.27996 0.5019	0.25775 0.5377	0.12795 0.7627	-0.18533 0.6604	-0.63956 0.0877	0.11011 0.7952	0.11101 0.7936	0.83501 0.0099	-0.63971 0.0876
Thr	0.72496 0.0419	0.65329 0.0790	0.74087 0.0355	0.95961 0.0002	0.78149 0.0220	0.51080 0.1958	-0.24559 0.5577	0.85700 0.0065	-0.14303 0.7355	1.00000	0.41288 0.3093	0.77837 0.0229	0.97297 <.0001	0.80983 0.0148	0.39096 0.3382	0.85967 0.0062	0.58810 0.1252	0.75606 0.0300	0.89762 0.0025	-0.46768 0.2426	0.75173 0.0315
Val	0.90041 0.0023	0.95265 0.0003	0.57517 0.1358	0.35421 0.3893	0.19243 0.6480	0.97802 <.0001	0.77581 0.0236	0.68355 0.0616	0.77901 0.0227	0.41288 0.3093	1.00000	0.77777 0.0231	0.28766 0.4897	0.63056 0.0937	0.30327 0.4653	0.25008 0.5503	-0.08406 0.8431	0.54356 0.1638	0.62438 0.0980	0.45192 0.2609	-0.18645 0.6584
Trp	0.88634 0.0034	0.85931 0.0062	0.75779 0.0294	0.62608 0.0968	0.51581 0.1907	0.82290 0.0121	0.30739 0.4589	0.76202 0.0280	0.38258 0.3496	0.77837 0.0229	0.77777 0.0231	1.00000	0.62821 0.0953	0.82519 0.0117	0.54200 0.1652	0.57235 0.1382	0.16386 0.6982	0.61598 0.1039	0.78251 0.0217	0.10862 0.7979	0.25946 0.5349
Lys	0.62024 0.1009	0.55295 0.1552	0.64843 0.0820	0.98849 <.0001	0.76235 0.0279	0.38162 0.3509	-0.36371 0.3758	0.83862 0.0093	-0.27996 0.5019	0.97297 <.0001	0.28766 0.4897	0.62821 0.0953	1.00000	0.70035 0.0531	0.32172 0.4371	0.83630 0.0097	0.67097 0.0685	0.77804 0.0230	0.86136 0.0060	-0.60896 0.1091	0.81006 0.0148
Ala	0.88078 0.0039	0.77493 0.0239	0.94596 0.0004	0.72855 0.0404	0.70243 0.0520	0.74058 0.0356	0.11071 0.7941	0.70924 0.0488	0.25775 0.5377	0.80983 0.0148	0.63056 0.0937	0.82519 0.0117	0.70035 0.0531	1.00000	0.42857 0.2894	0.88023 0.0039	0.30215 0.4670	0.66102 0.0743	0.89392 0.0028	0.03342 0.9374	0.55378 0.1544
Glu	0.46472 0.2460	0.35617 0.3865	0.62784 0.0956	0.29273 0.4817	0.08593 0.8397	0.33914 0.4112	0.09444 0.8240	0.27114 0.5160	0.12795 0.7627	0.39096 0.3382	0.30327 0.4653	0.54200 0.1652	0.32172 0.4371	0.42857 0.2894	1.00000	0.27461 0.5104	0.00433 0.9919	0.64566 0.0838	0.46877 0.2413	0.26919 0.5191	0.24097 0.5654
Gly	0.62670 0.0964	0.48789 0.2200	0.82816 0.0111	0.84543 0.0082	0.85182 0.0073	0.38270 0.3494	-0.32799 0.4277	0.62567 0.0971	-0.18533 0.6604	0.85967 0.0062	0.25008 0.5503	0.57235 0.1382	0.83630 0.0097	0.88023 0.0039	0.27461 0.5104	1.00000	0.55264 0.1554	0.62585 0.0969	0.85387 0.0070	-0.37643 0.3580	0.85889 0.0063
Asp	0.17062 0.6862	0.08202 0.8469	0.34958 0.3960	0.71527 0.0461	0.76470 0.0271	-0.04358 0.9184	-0.53539 0.1715	0.32561 0.4313	-0.63956 0.0877	0.58810 0.1252	-0.08406 0.8431	0.16386 0.6982	0.67097 0.0685	0.30215 0.4670	0.00433 0.9919	0.55264 0.1554	1.00000	0.35209 0.3924	0.45588 0.2563	-0.73074 0.0395	0.76870 0.0258
Cys	0.77099 0.0251	0.71473 0.0463	0.73917 0.0361	0.79914 0.0173	0.43477 0.2817	0.58841 0.1249	0.07489 0.8601	0.80276 0.0165	0.11011 0.7952	0.75606 0.0300	0.54356 0.1638	0.61598 0.1039	0.77804 0.0230	0.66102 0.0743	0.64566 0.0838	0.62585 0.0969	0.35209 0.3924	1.00000	0.88282 0.0037	-0.13356 0.7525	0.49826 0.2089
Ser	0.88410 0.0036	0.80802 0.0152	0.87583 0.0044	0.89164 0.0029	0.74382 0.0344	0.68807 0.0592	0.05889 0.8898	0.84676 0.0080	0.11101 0.7936	0.89762 0.0025	0.62438 0.0980	0.78251 0.0217	0.86136 0.0060	0.89392 0.0028	0.46877 0.2413	0.85387 0.0070	0.45588 0.2563	0.88282 0.0037	1.00000	-0.18704 0.6574	0.61927 0.1016
Tyr	0.20145 0.6324	0.22303 0.5955	0.10451 0.8055	-0.57015 0.1400	-0.52931 0.1773	0.41111 0.3116	0.81095 0.0146	-0.24395 0.5604	0.83501 0.0099	-0.46768 0.2426	0.45192 0.2609	0.10862 0.7979	-0.60896 0.1091	0.03342 0.9374	0.26919 0.5191	-0.37643 0.3580	-0.73074 0.0395	-0.13356 0.7525	-0.18704 0.6574	1.00000	-0.69153 0.0574
Pro	0.24168 0.5642	0.08302 0.8451	0.57614 0.1350	0.79504 0.0184	0.80006 0.0171	-0.06064 0.8866	-0.72184 0.0432	0.38916 0.3407	-0.63971 0.0876	0.75173 0.0315	-0.18645 0.6584	0.25946 0.5349	0.81006 0.0148	0.55378 0.1544	0.24097 0.5654	0.85889 0.0063	0.76870 0.0258	0.49826 0.2089	0.61927 0.1016	-0.69153 0.0574	1.00000

In table 3 for the long term clearance (2-10 hours), there are 78 Person correlation coefficients being calculated with high statistical significance ($P<0.05$), and there are 26, which are trends ($0.05 \leq P<0.10$).

Last table with Pearson correlation coefficients (Table 4) has been estimated for amino acid clearance from blood plasma at 6-10 hours after last feeding. Comparing to previous two calculations, there are 144 correlation coefficients being measured with $P<0.05$, and 12 coefficients – with P value varying from 0.05 to 0.10.

Table 4. Pearson correlation coefficients for amino acid clearance from blood plasma at 6-10 hours after last feeding in Nile tilapia.

Pearson Correlation Coefficients, N = 8
Prob > |r| under H0: Rho=0

	TAA	EAA	NEAA	ARG	HIS	Ile	Leu	Met	Phe	Thr	Val	Trp	Lys	Ala	Glu	Gly	Asp	Cys	Ser	Tyr	Pro
TAA	1.00000	0.99732 <.0001	0.97639 <.0001	0.92963 0.0008	0.82951 0.0109	0.99412 <.0001	0.98847 <.0001	0.96047 0.0001	0.96955 <.0001	0.89253 0.0029	0.99297 <.0001	0.83934 0.0092	0.87941 0.0040	0.99385 <.0001	-0.10257 0.8090	0.57305 0.1376	0.56789 0.1420	0.98137 <.0001	0.96790 <.0001	0.71684 0.0454	0.88010 0.0039
EAA	0.99732 <.0001	1.00000	0.95795 0.0002	0.93780 0.0006	0.82431 0.0118	0.99176 <.0001	0.98976 <.0001	0.97095 <.0001	0.97194 <.0001	0.90083 0.0023	0.99490 <.0001	0.83165 0.0105	0.88417 0.0036	0.99308 <.0001	-0.12497 0.7681	0.54832 0.1594	0.56206 0.1471	0.98572 <.0001	0.96194 0.0001	0.70579 0.0504	0.85608 0.0067
NEAA	0.97639 <.0001	0.95795 0.0002	1.00000	0.87623 0.0043	0.81871 0.0129	0.96974 <.0001	0.95349 0.0002	0.89926 0.0024	0.93192 0.0007	0.83990 0.0091	0.95597 0.0002	0.83556 0.0098	0.83762 0.0094	0.96479 0.0001	-0.03326 0.9377	0.62794 0.0955	0.56717 0.1426	0.93761 0.0006	0.95499 0.0002	0.72686 0.0411	0.92323 0.0011
ARG	0.92963 0.0008	0.93780 0.0006	0.87623 0.0043	1.00000	0.77382 0.0242	0.92831 0.0009	0.89598 0.0026	0.89676 0.0025	0.86503 0.0055	0.85975 0.0062	0.93098 0.0008	0.90066 0.0023	0.98401 <.0001	0.94984 0.0003	0.01409 0.9736	0.42339 0.2959	0.48799 0.2199	0.95979 0.0002	0.92328 0.0011	0.68851 0.0590	0.72922 0.0401
HIS	0.82951 0.0109	0.82431 0.0118	0.81871 0.0129	0.77382 0.0242	1.00000	0.79621 0.0181	0.77843 0.0229	0.81205 0.0143	0.77446 0.0240	0.81046 0.0147	0.79974 0.0172	0.74571 0.0337	0.73825 0.0365	0.81944 0.0128	-0.10634 0.8021	0.75167 0.0315	0.86311 0.0058	0.77124 0.0250	0.87405 0.0045	0.39086 0.3384	0.75242 0.0312
Ile	0.99412 <.0001	0.99176 <.0001	0.96974 <.0001	0.92831 0.0009	0.79621 0.0181	1.00000	0.98961 <.0001	0.94106 0.0005	0.96756 <.0001	0.84467 0.0083	0.99725 <.0001	0.85819 0.0064	0.88148 0.0038	0.99547 <.0001	-0.09019 0.8318	0.51764 0.1889	0.51981 0.1867	0.98606 <.0001	0.94586 0.0004	0.77706 0.0233	0.85048 0.0074
Leu	0.98847 <.0001	0.98976 <.0001	0.95349 0.0002	0.89598 0.0026	0.77843 0.0229	0.98961 <.0001	1.00000	0.94894 0.0003	0.97711 <.0001	0.85421 0.0069	0.99298 <.0001	0.77928 0.0226	0.82937 0.0109	0.97868 <.0001	-0.21121 0.6156	0.48766 0.2203	0.51092 0.1957	0.97641 <.0001	0.92536 0.0010	0.74754 0.0330	0.86380 0.0057
Met	0.96047 0.0001	0.97095 <.0001	0.89926 0.0024	0.89676 0.0025	0.81205 0.0143	0.94106 0.0005	0.94894 0.0003	1.00000	0.92763 0.0009	0.95280 0.0003	0.95526 0.0002	0.77960 0.0225	0.84833 0.0078	0.95506 0.0002	-0.12410 0.7697	0.59982 0.1160	0.54253 0.1647	0.94949 0.0003	0.93186 0.0008	0.53838 0.1686	0.84420 0.0084
Phe	0.96955 <.0001	0.97194 <.0001	0.93192 0.0007	0.86503 0.0055	0.77446 0.0240	0.96756 <.0001	0.97711 <.0001	0.92763 0.0009	1.00000	0.84493 0.0083	0.96475 0.0001	0.74326 0.0346	0.78225 0.0218	0.95221 0.0003	-0.13650 0.7472	0.55541 0.1530	0.60155 0.1147	0.93205 0.0007	0.93887 0.0005	0.74680 0.0333	0.79812 0.0176
Thr	0.89253 0.0029	0.90083 0.0023	0.83990 0.0091	0.85975 0.0062	0.81046 0.0147	0.84467 0.0083	0.85421 0.0069	0.95280 0.0003	0.84493 0.0083	1.00000	0.85822 0.0064	0.69379 0.0563	0.81513 0.0137	0.87241 0.0047	-0.08213 0.8467	0.66636 0.0712	0.60245 0.1140	0.86319 0.0058	0.92262 0.0011	0.36119 0.3794	0.82953 0.0109
Val	0.99297 <.0001	0.99490 <.0001	0.95597 0.0002	0.93098 0.0008	0.79974 0.0172	0.99725 <.0001	0.99298 <.0001	0.95526 0.0002	0.96475 0.0001	0.85822 0.0064	1.00000	0.84482 0.0083	0.88152 0.0038	0.99470 <.0001	-0.13687 0.7466	0.49806 0.2091	0.51182 0.1948	0.99138 <.0001	0.93742 0.0006	0.75043 0.0319	0.84747 0.0079
Trp	0.83934 0.0092	0.83165 0.0105	0.83556 0.0098	0.90066 0.0023	0.74571 0.0337	0.85819 0.0064	0.77928 0.0226	0.77960 0.0225	0.74326 0.0346	0.69379 0.0563	0.84482 0.0083	1.00000	0.93995 0.0005	0.88697 0.0033	0.30198 0.4673	0.51089 0.1957	0.44310 0.2715	0.86477 0.0056	0.84125 0.0088	0.68100 0.0630	0.65447 0.0783
Lys	0.87941 0.0040	0.88417 0.0036	0.83762 0.0094	0.98401 <.0001	0.73825 0.0365	0.88148 0.0038	0.82937 0.0109	0.84833 0.0078	0.78225 0.0218	0.81513 0.0137	0.88152 0.0038	0.93995 0.0005	1.00000	0.91393 0.0015	0.12976 0.7594	0.41099 0.3118	0.41889 0.3016	0.92388 0.0010	0.88022 0.0039	0.64616 0.0834	0.69335 0.0565
Ala	0.99385 <.0001	0.99308 <.0001	0.96479 0.0001	0.94984 0.0003	0.81944 0.0128	0.99547 <.0001	0.97868 <.0001	0.95506 0.0002	0.95221 0.0003	0.87241 0.0047	0.99470 <.0001	0.88697 0.0033	0.91393 0.0015	1.00000	-0.05127 0.9040	0.54306 0.1642	0.53177 0.1750	0.99066 <.0001	0.95711 0.0002	0.73514 0.0377	0.84863 0.0077
Glu	-0.10257 0.8090	-0.12497 0.7681	-0.03326 0.9377	0.01409 0.9736	-0.10634 0.8021	-0.09019 0.8318	-0.21121 0.6156	-0.12410 0.7697	-0.13650 0.7472	-0.08213 0.8467	-0.13687 0.7466	0.30198 0.4673	0.12976 0.7594	-0.05127 0.9040	1.00000	0.30620 0.4608	-0.02900 0.9457	-0.12009 0.7770	0.04211 0.9211	-0.00446 0.9916	-0.20408 0.6278
Gly	0.57305 0.1376	0.54832 0.1594	0.62794 0.0955	0.42339 0.2959	0.75167 0.0315	0.51764 0.1889	0.48766 0.2203	0.59982 0.1160	0.55541 0.1530	0.66636 0.0712	0.49806 0.2091	0.51089 0.1957	0.41099 0.3118	0.54306 0.1642	0.30620 0.4608	1.00000	0.78605 0.0207	0.43837 0.2773	0.69945 0.0535	0.07908 0.8523	0.61330 0.1059
Asp	0.56789 0.1420	0.56206 0.1471	0.56717 0.1426	0.48799 0.2199	0.86311 0.0058	0.51981 0.1867	0.51092 0.1957	0.54253 0.1647	0.60155 0.1147	0.60245 0.1140	0.51182 0.1948	0.44310 0.2715	0.41889 0.3016	0.53177 0.1750	-0.02900 0.9457	0.78605 0.0207	1.00000	0.44756 0.2662	0.69326 0.0566	0.19654 0.6409	0.46033 0.2511
Cys	0.98137 <.0001	0.98572 <.0001	0.93761 0.0006	0.95979 0.0002	0.77124 0.0250	0.98606 <.0001	0.97641 <.0001	0.94949 0.0003	0.93205 0.0007	0.86319 0.0058	0.99138 <.0001	0.86477 0.0056	0.92388 0.0010	0.99066 <.0001	-0.12009 0.7770	0.43837 0.2773	0.44756 0.2662	1.00000	0.92432 0.0010	0.73858 0.0364	0.83430 0.0100
Ser	0.96790 <.0001	0.96194 0.0001	0.95499 0.0002	0.92328 0.0011	0.87405 0.0045	0.94586 0.0004	0.92536 0.0010	0.93186 0.0008	0.93887 0.0005	0.92262 0.0011	0.93742 0.0006	0.84125 0.0088	0.88022 0.0039	0.95711 0.0002	0.04211 0.9211	0.69945 0.0535	0.69326 0.0566	0.92432 0.0010	1.00000	0.62864 0.0950	0.83990 0.0091
Tyr	0.71684 0.0454	0.70579 0.0504	0.72686 0.0411	0.68851 0.0590	0.39086 0.3384	0.77706 0.0233	0.74754 0.0330	0.53838 0.1686	0.74680 0.0333	0.36119 0.3794	0.75043 0.0319	0.68100 0.0630	0.64616 0.0834	0.73514 0.0377	-0.00446 0.9916	0.07908 0.8523	0.19654 0.6409	0.73858 0.0364	0.62864 0.0950	1.00000	0.49308 0.2144
Pro	0.88010 0.0039	0.85608 0.0067	0.92323 0.0011	0.72922 0.0401	0.75242 0.0312	0.85048 0.0074	0.86380 0.0057	0.84420 0.0084	0.79812 0.0176	0.82953 0.0109	0.84747 0.0079	0.65447 0.0783	0.69335 0.0565	0.84863 0.0077	-0.20408 0.6278	0.61330 0.1059	0.46033 0.2511	0.83430 0.0100	0.83990 0.0091	0.49308 0.2144	1.00000

Figure A8 in Appendix represents the results with patterns of clearance distribution for free amino acids in blood plasma at three time intervals: 2-6, 2-10 and 6-10 hours after last feeding.

3.3 Regression analysis of plasma clearance rate of individual amino acids on gene expression levels

Results presented in Table n shows the correlations between amino acid clearance and gene expression levels of hepatic transaminases and deaminases in Nile tilapia.

Table 5 represents results for non-essential amino acids and gene expression, measured at 2 hours after last feeding. The majority of regression patterns are linear. Minimum clearance range in plasma is 0,071 g/kg for proline in 6-10 time interval. Maximum clearance range is 0,076 g/kg for tyrosine in 2-10 time interval. Totally there are 22 regression patterns, while nine of them refers to ALAT, three – to AASS, six – to AMPD2, three – to MAB, just one to ASAT and

none – to GDH. Mostly gene expression correlates to amino acid clearance in 2-10 time interval (12 regression patterns), then in 2-6 time interval (7 regression patterns) and in 6-10 time interval (3 regression patterns).

In the table 6, there are also results for non-essential amino acids, but for gene expression, measured at 10 hours after last feeding. Totally there are 10 regression patterns, six of them refers to MAB, three – to AMPD2, and one – to AASS. Minimum clearance range in plasma is -0,026 g/kg for proline in 2-10 time interval, while maximum is 0,042 g/kg for alanine in 2-6 time interval. Majority of correlations are for amino acid clearance in 2-10 time interval (7 patterns), then in 2-6 time interval (3 patterns).

Next two tables represent data for essential amino acids. Table 7 shows the results for gene expression, measured at 2 hours after last feeding. Minimum clearance range in plasma is -0,043 g/kg, for valine in 2-6 time interval. The maximum clearance range in plasma is 0,066 g/kg for lysine in 2-10 time interval. Half of all regression patterns are for amino acid clearance in 2-10 time interval (12 patterns), then in 2-6 (11 patterns), and just one pattern in 6-10 time interval. Totally, there are 24 patterns. Distribution of regression patterns between genes is varied: seven patterns – to MAB, five – to ALAT, four – to AMPD2 and to GDH, two - to AASS and ASAT.

Table 8 gives the results from gene expression, measured at 10 hours after last feeding. Minimum clearance range is -0,043 g/kg for valine in 2-6 time interval, while maximum is 0,201 for leucine in 6-10 time interval. Half of all patterns refers to 6-10 time interval (6), three patterns are for 2-6 and the other three – for 2-10 time intervals; out of them four patterns – with AMPD2, three patterns – with MAB, two – with ALAT, and one – with AASS, ASAT and GDH.

Table 5. Statistically significant results (P<0.05) or trends (0.05≤P<0.10) in regression analysis of blood clearance rate of individual non-essential amino acids on expression of hepatic transaminases and deaminases in Nile tilapia. Gene expression was measured in liver dissected from the fish 2 hours after last feeding.

Amino acid	Clearance period, h	Clearance range in plasma, g/kg	Hepatic enzyme expressed[2]	Regression	R^2	P(linear)≠0	P(2^{nd} degree)≠0
ALANINE	2-6	0.009…0.42	ALAT	$0.796+14.1*ALA-247.4*ALA^2$	0.93	0.003	0.005
			AASS	$0.930+2.07*ALA$	0.46	0.096	
	2-10	0.006…0.056	ALAT	$0.893+2.0*ALA$	0.69	0.021	
			AASS	$0.914+1.92*ALA$	0.71	0.018	
GLUTAMINE	2-6	0.003…0.016	ALAT	$0.834+38.2*GLU-2210*GLU^2$	0.69	0.092	0.164
			ALAT	$0.91+9.59*GLU$	0.47	0.089	
	2-10	-0.005…0.013	AMPD2	$0.965+25.8*GLU+1576*GLU^2$	0.58	0.093	0.289
GLYCINE	2-6	-0.002…0.012	ALAT	$0.922+7.67*GLY$	0.70	0.020	
			MAB	$1.02+20.6*GLY$	0.63	0.033	
	2-10	0.001…0.015	ALAT	$0.921+5.30*GLY$	0.62	0.035	
ASPARGINE	6-10	-0.003…0.014	AMPD2	$1.14-21.6*ASP$	0.86	0.003	
CYSTEINE	2-10	0.001…0.007	ALAT	$0.845+45.8*CYS-3462*CYS^2$	0.86	0.087	0.225
			MAB	$0.748+221*CYS-25306*CYS^2$	0.53	0.101	0.111
			ALAT	$0.892+17.2*CYS$	0.79	0.0078	
SERINE	2-10	0.008…0.020	ASAT	$0.887+15.0*SER-479.9*SER^2$	0.75	0.074	0.152
			ALAT	$0.892+5.55*SER$	0.82	0.005	
			AASS	$0.927+4.31*SER$	0.55	0.055	
TYROSINE	6-10	0.017…0.061	MAB	$0.970+4.82*TYR$	0.63	0.033	
	2-10	0.010…0.076	AMPD2	$0.736+14.1*TYR-116.4*TYR^2$	0.74	0.074	0.158
			AMPD2	$0.881+4.24*TYR$	0.55	0.055	
PROLINE	2-6	-0.027…0.045	AMPD2	$1.16+8.55*PRO-205.6*PRO^2$	0.92	0.003	0.009
	6-10	-0.071…0.026	AMPD2	$1.13-6.11*PRO-87.26*PRO^2$	0.67	0.057	0.135

[2] MAB – Mab-21 domain containing 2 (mb21d2), GDH – glutamate dehydrogenase, ALAT – alanine aminotransferase, AASS - alpha-aminoadipic semialdehyde synthase, ASAT – aspartate aminotransferase, AMPD2 - adenosine monophosphate deaminase 2.

Table 6. Statistically significant results (P<0.05) or trends (0.05≤P<0.10) in regression analysis of blood clearance rate of individual non-essential amino acids on expression of hepatic transaminases and deaminases in *Nile tilapia*. Gene expression was measured in liver dissected from the fish 10 hours after last feeding.

Amino acid	Clearance period, h	Clearance range in plasma, g/kg	Hepatic enzyme expressed	Regression	R^2	P(linear)≠0	P(2^{nd} degree)≠0
ALANINE	2-6	0.009…0.042	MAB	$0.840+29.3*ALA-527.4*ALA^2$	0.65	0.067	0.089
ASPARGINE	2-6	-0.003…0.022	AMPD2	$1.12+8.88*ASP$	0.77	0.010	
	2-10	-0.001…0.030	AASS	$0.936+11.1*ASP-622.8*ASP^2$	0.85	0.090	0.023
			AMPD2	$1.10+6.85*ASP$	0.59	0.045	
			MAB	$1.10+6.31*ASP$	0.53	0.064	
CYSTEINE	2-6	-0.006…0.006	AMPD2	$1.24-23.5*CYS-6063*CYS^2$	0.62	0.081	0.080
	2-10	0.002…0.007	MAB	$1.02+37.4*CYS$	0.68	0.023	
SERINE	2-10	0.002…0.020	MAB	$1.02+11.7*SER$	0.66	0.025	
PROLINE	2-10	-0.026…0.011	MAB	$1.16+8.16*PRO+237.6*PRO^2$	0.63	0.085	0.313
			MAB	$1.19+4.77*PRO$	0.50	0.075	

Table 7. Statistically significant results (P<0.05) or trends (0.05≤P<0.10) in regression analysis of blood clearance rate of individual essential amino acids on expression of hepatic transaminases and deaminases in Nile tilapia. Gene expression was measured in liver dissected from the fish 2 hours after last feeding.

Amino acid	Clearance period, h	Clearance range in plasma, g/kg	Hepatic enzyme expressed	Regression	R^2	P(linear)≠0	P(2^{nd} degree)≠0
LYSINE	2-6	0.008…0.062	MAB	$0.863 + 19.7 * Lys – 280.8 * Lys^2$	0.71	0.042	0.036
			GDH	$0.983-7.44*LYS+121.0*LYS^2$	0.78	0.048	0.028
	2-10	0.001…0.066	GDH	$0.942-6.22*LYS+98.15*LYS^2$	0.74	0.046	0.033
			ALAT	0.909+1.38*LYS	0.70	0.019	
ARGININE	2-10	-0.001…0.055	MAB	$0.861+24.9*ARG-433.0*ARG^2$	0.64	0.066	0.057
HISTEDINE	2-6	-0.002…0.011	AMPD2	$0.908+50.6*HIS-3496*HIS^2$	0.68	0.089	0.230
ISOLEUCINE	2-6	-0.017…0.013	MAB	$1.16-8.36*ILE-280.4*ILE^2$	0.70	0.037	0.449
			GDH	$0.835+4.74*ILE+495.5*ILE^2$	0.81	0.022	0.037
			MAB	1.12-7.55*ILE	0.65	0.028	
	2-10	0.017…0.050	AASS	$0.762+11.2*ILE-130.3*ILE^2$	0.77	0.074	0.137
			ALAT	$0.720+13.6*ILE-174.8* ILE^2$	0.64	0.093	0.132
METHIONINE	2-10	0.020…0.058	MAB	$0.389+41.6*MET-530.5*MET^2$	0.58	0.078	0.082
			GDH	$1.22-20.8*MET+295.9*MET^2$	0.95	0.002	0.002
PHENYLALANINE	2-6	-0.030…0.038	AMPD2	$1.05+4.65*PHE-37.65*PHE^2$	0.67	0.064	0.163
THREONINE	2-6	-0.005…0.026	AMPD2	$0.902+18.3*THR-378.5*THR^2$	0.65	0.083	0.306
			AMPD2	0.916+10.4*THR	0.53	0.062	
	2-10	-0.006…0.045	ALAT	0.914+0.90*THR	0.78	0.009	
	6-10	-0.013…0.035	ASAT	$0.901-5.44*THR+270.1*THR^2$	0.95	0.002	0.001
VALINE	2-10	0.026…0.064	ALAT	$0.593+16.1*VAL-162.4*VAL^2$	0.72	0.041	0.052
			AASS	$0.678+12.4*VAL-115.5*VAL^2$	0.71	0.078	0.114
			ALAT	0.914+1.90*VAL	0.78	0.009	
	2-6	-0.043…0.017	MAB	1.07-4.16*VAL	0.67	0.024	
TRYPTOPHAN	2-10	0.003…0.009	ASAT	$1.41-165*TRP+13926*TRP^2$	0.61	0.088	0.077
	2-6	-0.001…0.007	MAB	$1.19-86.7*TRP+10660*TRP^2$	0.67	0.054	0.087

Table 8. Statistically significant results (P<0.05) or trends (0.05≤P<0.10) in regression analysis of blood clearance rate of individual essential amino acids on expression of hepatic transaminases and deaminases in Nile tilapia. Gene expression was measured in liver dissected from the fish 10 hours after last feeding.

Amino acid	Clearance period, h	Clearance range in plasma, g/kg	Hepatic enzyme expressed	Regression	R^2	P(linear)≠0	P(2^{nd} degree)≠0
LYSINE	2-6	0.008…0.62	MAB	1.19+4.77*LYS	0.50	0.075	
	6-10	-0.007…0.026	AMPD2	$1.17+12.3*LYS-464.4*LYS^2$	0.66	0.049	0.087
HISTEDINE	2-10	0.008…0.024	AASS	0.648+51.2*HIS-1975*HIS2	0.98	0.001	0.0003
ISOLEUCINE	2-6	-0.017…0.013	AMPD2	$1.32-5.01*ILE-971.7*ILE^2$	0.80	0.071	0.018
LEUCINE	6-10	0.037…0.201	ALAT	$0.784+1.83*LEU-7.826*LEU^2$	0.71	0.035	0.035
METHIONINE	6-10	0.006…0.037	GDH	$0.770+8.42*MET-197.3*MET^2$	0.83	0.011	0.011
PHENYLALANINE	6-10	0.014…0.088	ALAT	$0.794+3.75*PHE-37.20*PHE^2$	0.65	0.052	0.052
			MAB	$0.916+12.9*PHE-117.8*PHE^2$	0.63	0.067	0.082
	2-10	0.040…0.123	ASAT	$1.64-18.5*PHE+96.80*PHE^2$	0.79	0.033	0.042
VALINE	2-6	-0.043…0.017	AMPD2	$1.24-7.30*VAL-225.6*VAL^2$	0.77	0.051	0.061
	2-10	0.026…0.065	MAB	$0.397+34.6*VAL-357.6*VAL^2$	0.57	0.092	0.106
TRYPTOPHAN	6-10	0.001…0.010	AMPD2	$0.952+95.1*TRP-7152*TRP^2$	0.73	0.044	0.070

3.4 Regression analysis of nitrogen excretion on gene expression levels

Regression analysis has been performed with NH_4, measured at 0, 2, 4 and 6 hours after last feeding.

Table 9. Statistically significant results (P<0.05) or trends (0.10<P<0.05) in regression analysis of ammonia rate on expression of hepatic transaminases and deaminases in Nile tilapia. Gene expression was measured in liver dissected from the fish 2 hours after last feeding.

Hepatic enzyme expressed	**Ammonia measurement, h after last feeding**	**Regression**	**R^2**	**P(linear)≠0**
MAB	4	-662+1277*MAB	0.924	0.0001
	6	-2003+2838*MAB	0.854	0.0010
	8	-2952+3839*MAB	0.854	0.0010
	10	-2720+3643*MAB	0.840	0.0014
GDH	2	$6.16+6.0*10^{-5}*GDH-0.034*GDH^2$	0.670	0.038
	4	$2.54-0.004*GDH-2*10^{-6}*GDH^2$	0.661	0.058
ALAT	2	$4.21-0.021*ALAT+3*10^{-5}*ALAT^2$	0.565	0.059

Three out of six expressed sequence tags gave response to ammonia excretion. MAB gave linear correlation to ammonia excretion with high value of R^2. GDH and ALAT has less significant correlation, and more as a trend with 0.038<P<0.058.

3.5 Regression analysis of digested protein and gene expression levels

For the calculation the feed intake data from 41st day was used. Feed contains 29.5% of protein. Digestibility of protein was approximately 98% (Bajgai and Hoque, 2014). Digested protein per body weight was correlated to expression of hepatic transaminases and deaminases in Nile tilapia at 2 and 10 hours after last feeding. There were no correlations detected at 10 hours after last feeding. At 2 hours after last feeding, there was a statistically significant linear correlation of digested protein and MAB gene. Correlation is described with the equation MAB=0.456+0.837*DPI, where DPI is digested protein intake (R^2=0.792. P=0.003).

3.6 Regression analysis of digested protein and ammonia excretion

Regression analysis has been performed with digested protein, measured at 41st day and ammonia measurement at 45th day in a stagnant system. There were totally 5 ammonia

measurements, but just 4 of them (at 4, 6, 8 and 10 hours after last feeding) were correlated to digested protein intake. Table 3.7 represent detected significant correlations.

Table 10. Statistically significant results (P<0.05) or trends (0.05≤P<0.10) in regression analysis of ammonia rate on digested protein in Nile tilapia.

Ammonia measurement, h after last feeding	**Regression**	**R^2**	**P(linear)≠0**
4	-0.251+6.81*DPI	0.631	0.019
6	-4.509+15.65*DPI	0.635	0.018
8	-9.137+22.89*DPI	0.752	0.005
10	-6.906+20.24*DPI	0.644	0.017

4 DISCUSSIONS

4.1 Plasma free amino acids in Nile tilapia

The plasma concentration of essential amino acids (EAA) measured at 2 hours after last feeding were almost twice as high as the concentration of non-essential amino acids (NEAA). As it was mentioned in previous studies, EAA display the nutritional status of fish (Larsen, Dalsgaard et al. 2012). They are the first to increase in concentration after the feeding (Harding, Allen et al. 1977, Wilson, Harding et al. 1977). The rate of EAA clearance from the blood is high from 2 to 10 hours, while the clearance rate for NEAA is much lower. At 10 hours after the last feeding, the levels for EAA and NEAA were almost equal. Changes in NEAA levels in blood plasma are less affirmed through the time. NEAA participate in several different metabolic processes and may be transformed into other compounds. An example is that NEAA is a carrier of nitrogen within the organism (Yamada, Simpson et al. 1981, Blasco, Fernández et al. 1991).

The total picture of plasma amino acid concentration shows that over time the concentrations of leucine, tyrosine and phenylalanine increased. Aoki et al. reported increased levels of valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, and histidine in arterial and hepatic blood levels after two hours from last feeding for the humans, fed with high protein contents in the food (Aoki, Brennan et al. 1976). Increased level of those amino acids could be due to reaction of fish body on a proteinous feed. Alternatively, Peng Li mentioned in a review article, that many amino acids, participating in various metabolic pathways, are highly significant to growth, development, reproduction and immunity of animal. These amino acids are called functional (Li, Mai et al. 2009). Leucine, tyrosine and phenylalanine belong to these. Leucine is a branch chained amino acid, which contribute to immune system through glutamine synthesis in skeletal muscle (Newsholme and Calder 1997). Glutamine synthesis goes with the formation of leucine metabolite b-hydroxy-b-methyl-butyrate (HMB). Although, speaking about tilapia, role of HMB was not confirmed in its immunity improvement (Nissen and Abumrad 1997, Li and Gatlin 2007). Meijer and Dubbelhuis suggests that leucine plays role in activating the mTOR signaling pathway, which induces protein synthesis and drags proteolysis (Meijer and Dubbelhuis 2004). Thus, increased concentration of leucine blood plasma could be a marker for active protein synthesis.

Tyrosine can be obtained from phenylalanine through action of tetrahydrobiopterin dependent phenylalanine hydroxylase (Li, Mai et al. 2009). Thus, phenylalanine regulates

tetrahydrobiopterin, which is a cofactor for nitric oxide synthesis. Nitric oxide is main vasodilator, and contributes to vascular tone and hemodynamics (Ignarro, Cirino et al. 1999, Wu and Meininger 2000, Shi, Meininger et al. 2004). While tyrosine participates in immune response regulation and cell metabolism, is a precursor for several hormones synthesis (epinephrine and norepinephrine, triiodothyronine and thyroxine, dopamine and melanin) (Kim*, Mateo et al. 2007, Li, Mai et al. 2009).

For the tilapias fed 2 meals a day restricted (2M) the EAA leucine, phenylalanine, valine, methionine, isoleucine, threonine, histidine and the semi-essential AA tyrosine and proline had their low extremum at 6 hours, and their profile of amino acid concentration for three times points is curving inward. The fully fed control (2MF) and the tilapias fed 4 meals (4M) a day had amino acid profile curved outward. Both, 2MF and 2M groups were fed twice in a day, but 2M were fed 90% of feed intake from control group. Harding and Wilson mentioned that EAA are affected by the nutritional state and feeding rate of the fish (Harding, Allen et al. 1977, Wilson, Harding et al. 1977). Difference between the 2MF and 2M fishes, thus, could be due to the higher amounts of feed eaten by the 2MF tilapias. The 2MF tilapias were fed in excess and allowed to up regulate blood clearance and subsequent AA metabolism. This is illustrated by the lower plasma AA concentration in this fish compared to the 2M treatment, and may indicate that up-regulation of the AA transporters into the cells may have been sufficient to decrease amino acid concentration in blood plasma gradually. The 4M fishes had the same amino acid concentration profile as the 2MF group, although they were fed 4 times in a day and again with 90% of feed, eaten by control group. It seems that this group is supposed to behave as 2M group with rapid decrease of amino acid concentration in blood plasma. 4M fishes behaved as control group and decreased it gradually, so, I may suppose, that amino acid metabolism could be controlled also by small parts of feed with higher frequency.

4.2 Relationships between free amino acid clearance patterns

The correlation analysis for clearance rate for individual AA revealed several interesting relationships. Most striking were the high correlations between arginine and lysine and between isoleucine and valine for all measured time intervals.

Both arginine and lysine use the same intercellular transport. Intercellular transport of both of these amino acids is realized through cationic amino acid transporter (y^+), which also transports also histidine, and ornithine (Shima, Maeda et al. 2006, Luiking and Deutz 2007). The correlations in pairs histidine-arginine and histidine-lysine is not significant at 2-6 hours

analysis, while at 6-10 hours and 2-10 hours both pairs show statistically significant interactions. If we compare histidine-arginine and histidine-lysine to arginine-lysine, their Pearson coefficients are comparably low to ones from arginine-lysine pair. Several sources noticed arginine-lysine antagonism (Jones, Petersburg et al. 1967, Austic and Scott 1975), so that excess of one amino acid could intercept with and limit metabolism of the other (Austic and Scott 1975, Luiking and Deutz 2007).

Two branch-chained amino acids isoleucine-valine form a second pair with statistically significant Pearson correlation coefficients. At 2-6 and 6-10 hours interval Pearson coefficient reached 0.984 ($P<0.0001$), at 2-10 hours it became 0.978 ($P<0.0001$). Such strong correlation in clearance patterns between these two amino acids is due to their metabolic pathways, run by a common set of enzymes (UMBARGER and DAVIS. 1962). Moreover, there is a locus in genome, which is responsible for formation of these three common enzymes in biosynthesis of isoleucine and valine (Ramakrishnan and Adelberg 1965). The biosynthesis pathways are identical for all organisms that it has been studied in (de Robichon-Szulmajster and Magee 1968). Based on our results, we might suppose that these metabolic pathways share common enzymes in fish also.

Next three amino acids gives various combinations between each other and highly significant results in the correlation coefficients. They are leucine, tyrosine and phenylalanine.

Tyrosine and phenylalanine are aromatic amino acids. Difference in chemical formula is hydroxyl group which tyrosine has and phenylalanine is lacking. Strong correlation between these two amino acids could be due to common metabolic pathways. Phenylalanine could be a precursor for tyrosine synthesis in mammalian livers (Udenfriend and Cooper 1952, Bender 2012). Tyrosine is non-essential amino acid because it could be synthesized from phenylalanine. On the other hand, tyrosine could not transform back into phenylalanine. Phenylalanine hydroxylase (EC:1.14.16.1) is one from enzyme system, which is involved in non-reversible tyrosine synthesis and phenylalanine catabolism simultaneously. At 2 hours after last feeding the average phenylalanine level was 0.109 g/kg, while tyrosine was 0.086 g/kg. Four hours later the levels of these amino acids became almost equal; and at 10 hours after last feeding average tyrosine level was slightly higher (0.055g/kg) than phenylalanine (0.045g/kg). It correlates with the literature that tyrosine synthesis depends on phenylalanine availability. If intake is lacking phenylalanine, thus it will limit tyrosine production through phenylalanine conversation. Additional amount of phenylalanine supplemented the diet in our experiment. Due to FAO nutrient requirements for Nile tilapia, the phenylalanine level in a diet should not go less than

1.05% of a diet (www.fao.org). Phenylalanine level in our experimental diet was 5.9% of a diet (phenylalanine from soybean meal, corn gluten meal, potato starch and synthetic supplement); and it exceeded almost 6 times a required level. That is why phenylalanine and tyrosine levels were slightly increased throughout 10 hours after last feeding, comparing to other amino acids. Phenylalanine converted to tyrosine to get rid of excess and to compensate tyrosine needs.

Tight relationship between branch chained leucine and aromatic phenylalanine and tyrosine could be explained from their functional role in protein metabolism. These amino acids are those from a group, which inhibit hepatic proteolysis in liver (Mortimore and Poso 1987).

4.3 Correlations between plasma clearance rate of individual amino acids and gene expression levels

Sequence tags, used for gene expression measurements, were chosen due to their function in amino acid catabolic pathways. As it was mentioned in introduction, amino acid degradation starts with removal of amino group with further transformation of carbon derivative into important metabolic intermediates (Berg, Tymoczko et al. 2011).

For better processing of the data, all correlations were organized based on metabolic intermediates, which could participate in gluconeogenesis or citric acid cycle. All of 20 known amino acids finish up in 7 intermediates. They are pyruvate, acetyl CoA, acetoacetyl CoA, α-ketoglutarate, succinyl CoA, fumarate and oxaloacetate (Berg, Tymoczko et al. 2011).

Pyruvate is a metabolic intermediate for alanine, cysteine, glycine, serine, threonine and tryptophan. All of these amino acids, except tryptophan, correlates to ALAT (alanine aminotransferase). This aminotransferase participates in arginine biosynthesis; alanine, aspartate and glutamate metabolism; carbon fixation in photosynthetic organisms; carbon metabolism; 2-oxocarboxylic acid metabolism; biosynthesis of amino acids. ALAT serves as interlink in intermediate metabolism between glucose and amino acids (Yang, Blaileanu et al. 2002).

Fumarate is intermediate for asparagine, phenylalanine, and tyrosine. α-Ketoglutarate is intermediate of arginine, glutamine, histidine, proline. Oxaloacetate is intermediate for asparagine and aspartate. The common thing between these three intermediates is that their amino acids correlate to AMPD expression pattern. And AMPD is supposed to be one of the enzymes, catalyzing aspartate deamination in purine nucleotide cycle (Braunstein 1957, Lowenstein 1972). α-ketoglutarate participates in transformation of α-amino acid in α-keto acid and releasing glutamate. Glutamate together with oxaloacetate will produce aspartate, which is

entering purine nucleotide cycle (Campbell 1973). Additionally to AMPD, these amino acid clearances correlate to MAB (phenylalanine, tyrosine), and to ALAT (glutamine). We might suppose, that not all amino acids could be catabolized through purine nucleotide cycle; and those, which could be catabolized through purine nucleotide cycle, might be alternatively degraded through other catabolic pathways.

Rest of metabolic intermediate (acetyl-CoA, acetoacetyl-CoA and succinyl-CoA) gave comprehensive output, and correlate to unlike set of expressed tags.

If we look from the side of the expressed tags, the most frequent is MAB. MAB, measured at 2 hours after last feeding, gave the majority of correlations with amino acids with different metabolic intermediates. MAB correlates to clearance pattern of branch-chained amino acids (leucine, valine, isoleucine), some non-polar amino acids (methionine, leucine, valine) and aromatic amino acids (phenylalanine, tyrosine), those which have acetoacetyl-CoA intermediate (tryptophan, lysine) and pyruvate intermediate (cysteine and glycine). It is important to notice that leucine gave just correlation with MAB. In paragraph 4.1, we have already mentioned leucine as one of the functional amino acids, which affects immune system (Newsholme and Calder 1997), and is suggested to play role in activating mTOR signaling pathway, which induces protein synthesis and drag proteolysis (Meijer and Dubbelhuis 2004). MAB expression level, measured at 2 hours after last feeding, didn't correlate to three carbon amino acids (alanine, serine), amino acids with oxaloacetate intermediate (asparagine) and α-ketoglutarate (glutamine, proline, histidine). Lately, MAB, measured at 10 hours after last feeding, gave correlation to these amino acids. We believe that frequent correlations of MAB with the majority of amino acids and especially leucine, has a direct influence in proteolysis and amino acid catabolism. It could serve as common enzyme, participating in one of reactions in turnover, or as indicator of catabolic reaction.

4.4 MAB forms tight correlation between digested protein and nitrogen excretion

Digested protein estimations are based on data obtained from day 41. Regression analysis ran for digested protein and gene expression levels. Lately, the same type of analysis was complete for digested protein and nitrogen excretion. Data from day 45 measurement in a closed system performed nitrogen excretion levels.

There was a statistically significant correlation between MAB expression level after 2 hours and digested protein. That was the only one significant correlation, which we have found for digested protein. MAB sequence has been chosen for quantitative PCR analysis as cyclic

GMP-AMP synthase. In introduction there is brief description about the reaction it catalyzes and immunological function in activation of I type interferons pathway (Sun, Wu et al. 2013). Immunological function of MAB seems less attractive, but while comparing interferon activation pathway and purine nucleotide cycle, we found that they share common metabolites – guanosine triphosphate (GTP) and adenosine monophosphate (AMP). Purine nucleotide cycle is an alternative way to trans- or deamination in amino acid catabolism. It ends up with release of NH_3. I suppose that MAB regulates transformation of metabolites, which are common for interferon activation pathway and purine nucleotide cycle, and contribute to protein turnover.

From table 9, there are just three expressed sequence tags GDH, ALAT and MAB, which gave correlation to ammonia excretion. Ammonia is a final product of protein degradation in fishes, and excreted ammonia could serve as a marker for protein digestion (Buttle, Uglow et al. 1995, Wright 1995). GDH and ALAT gave rather complicated trend lines with comparably low P value, while MAB represented highly significant linear correlation ($P<0.05$). MAB is the only expressed tag to give correlation to digested protein, also with a high significance. Moreover, MAB is directly proportional to nitrogen excretion and to digested protein, as well as digested protein is directly proportional to nitrogen excretion. With the increase of dietary protein, nitrogen excretion will be also increased (Buttle, Uglow et al. 1995). As shown by our experiment results, MAB will be proportionally increasing.

While choosing target sequence for primer design, we took MB21 domain 2, which was very conservative for brackish water fishes (Figure A1). Lately, while analyzing the data, we realized that cyclic GMP-AMP synthase is encoded by MB21 domain 1. Consequently, we made a mistake by choosing the wrong sequence tag for further quantitative PCR analysis. The primers were designed for MB21 domain 2 (MAB). Although, both of domain sequences are member of the same gene family, it doesn't mean that they are identical in their function.

Based on our hypothesis about MAB function in purine nucleotide cycle, the other enzymes are supposed to work as well. Throughout the experiment, we measured AMPD (AMP deaminase) expression level, which is very common for purine nucleotide, catalyzing its final step. Van den Berghe and his team reported that purine nucleotide cycle has a minor contribution as NH_3 source in liver (Van den Berghe, Bontemps et al. 1992). Moreover, we didn't find any correlation between AMPD and nitrogen excretion or digested protein. If primer design was ideal for AMPD, and due to literature, AMP deaminase activity is somehow a marker for purine nucleotide cycle, and then we could suppose that MAB is not a part of purine cycle, but something else.

5 CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

The hypothesis of study was verified and confirmed with the results. Hepatic enzymes' transcriptions correlate with plasma free amino acid clearence, nitrogen excretion and digested protein.

During experiment correlations with a novel sequence tag (MAB) has been established. MAB has been sequenced and is found at NCBI gene data base as MB21 domain 2, but its function stays unclear. Thus, it would be interesting to analyze this sequence tag in a deeper way. As a preliminary step, I suggest to perform qRT-PCR analysis for several fish individuals, and check whether it is differentially expressed in fish treated under specific conditions. Next step could be the identification of gene function. It could be computational analysis of searching homologous genes, or experimental, performed with mutated gene and the altered phenotype of cloned organism. Further studies will include protein analysis, which is encoded by MAB.

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Appendix

Appendix A1. Formulation and chemical composition of the experimental diet[3]

Ingredients, g kg-1	
Soybean meal	350
Corn gluten meal	200
Potato starch	355
Threonine	0.6
Methionine	4.6
Phenylalanine	0.4
Taurine	1.35
Lysine	3.20
Mono calcium phosphate	10
Rapeseed oil	45
Premix	10
Y2O3	0.08
Vit-C 35%	0.10
Sodium alginate	20
Feed composition, kg-1	
Dry matter, g	949
Crude protein, g	292
Starch, g	396
Lipid, g	44
Ash, g	43
Energy, MJ	20

[3] For detailed information: Bajgai and Hoque master thesis, 2014

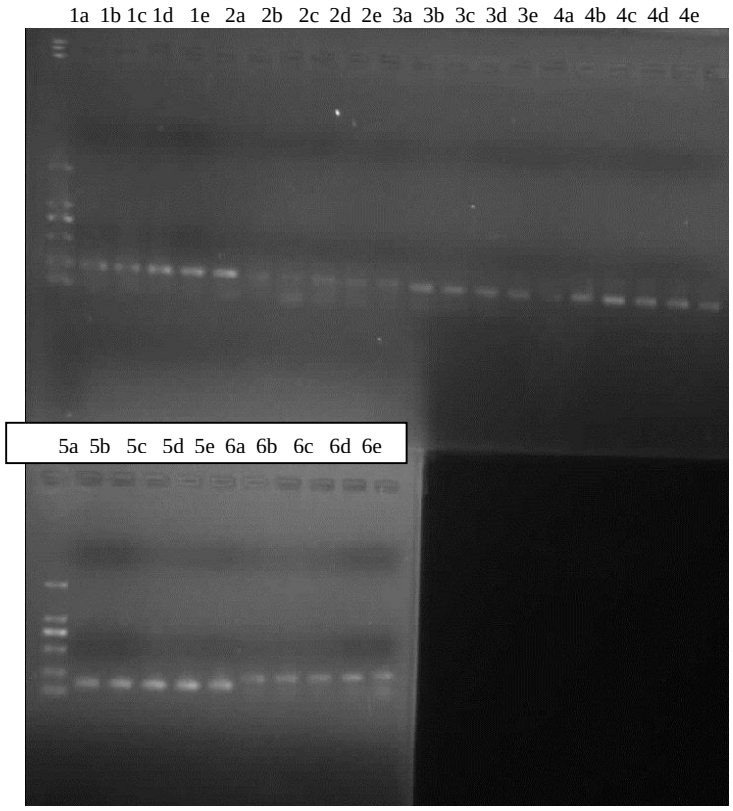
Appendix A2. RNA integrity by NanoDrop measurement.

Sample name	RNA concentration, ng/µl	A260/280	A260/230
11	99.9	1.81	0.46
12	287.5	1.60	0.73
13	316.4	1.62	0.72
14	96.4	1.66	0.51
15	146.2	1.82	0.77
16	224.0	1.68	0.73
17	124.8	1.73	0.58
19	212.2	1.77	0.76
51	77.3	1.71	0.54
52	102.6	1.70	0.75
53	322.0	1.70	0.69
54	91.2	1.70	0.63
55	176.9	1.76	0.69
56	235.4	1.76	0.67
57	98.0	1.74	0.55
59	168.4	1.75	0.71

Appendix A3. Primers for qRT-PCR.

Name	Forward	Tm	Reverse	Tm	Product size
ALAT	AGGTCCTGTTTGAGATGGGG	59	TAAGAAGGTTCCCCAGGCTG	59,01	244
ASAT	TCTCTGTCGGTCCTCCTGTA	59,01	ACCCCACACGACTTTACCAT	58,94	171
GDH	GCCAACAAGATCAAGGCCAA	59,03	GCAGGTGGTAGTTGGAGTCT	59,02	223
AMPD2	TGGACAAGGGAAGCCTAAGG	59,01	TCATGCTGCGTGTGAATAGC	58,99	224
MAB	CGGCACAGATTACGACATGG	59,07	TAGAACCAGTCAGCCACCAG	59,02	237
AASS	ACGATGTGGGGATTCGCTAT	58,91	TCCTTCCTCTTTCAGCCTGG	59,01	227
ACTIN	CCAGCCTTCCTTCCTTGGTA	59,01	TCAGGTGGGGCAATGATCTT	59	216

Appendix A4. Gel electrophoresis of PCR products. 1- PCR product from amplification with ALAT, 2- with MAB, 3 – GDH, 4 – AASS, 5- ALAT, 6-AMPD2; a, b, c, d and e refers to different annealing temperatures, 58 °C, 59°C, 60°C, 61°C and 62 °C, respectively.



Appendix A5. Free amino acid in blood plasma of Nile tilapia at 2 hours after last feeding (g/kg).

	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 9
Asp	31	14	43	33	20	37	24	34
Thr	56	48	56	46	25	38	58	45
Ser	27	22	28	24	13	18	26	19
Glu	27	24	26	33	22	30	29	32
Gly	21	19	27	23	15	22	27	21
Ala	63	75	75	68	41	57	83	52
Cys	17	15	17	17	13	14	17	16
Val	76	97	76	75	56	62	78	62
Met	75	61	62	60	36	63	71	58
Ile	46	60	45	44	25	35	46	34
Leu	132	212	130	129	115	103	130	94
Tyr	77	141	79	86	78	78	85	67
Phe	111	164	94	107	93	102	114	86
Lys	89	47	83	68	31	63	78	66
His	38	26	40	33	27	32	37	37
Trp	15	19	14	14	9	13	17	13
Arg	76	41	75	59	29	54	62	53
Pro	56	72	78	62	53	61	74	60
EAA	714	774	676	636	446	563	691	548
NEAA	319	381	374	345	256	316	365	301
TAA	1033	1155	1050	981	702	880	1056	849

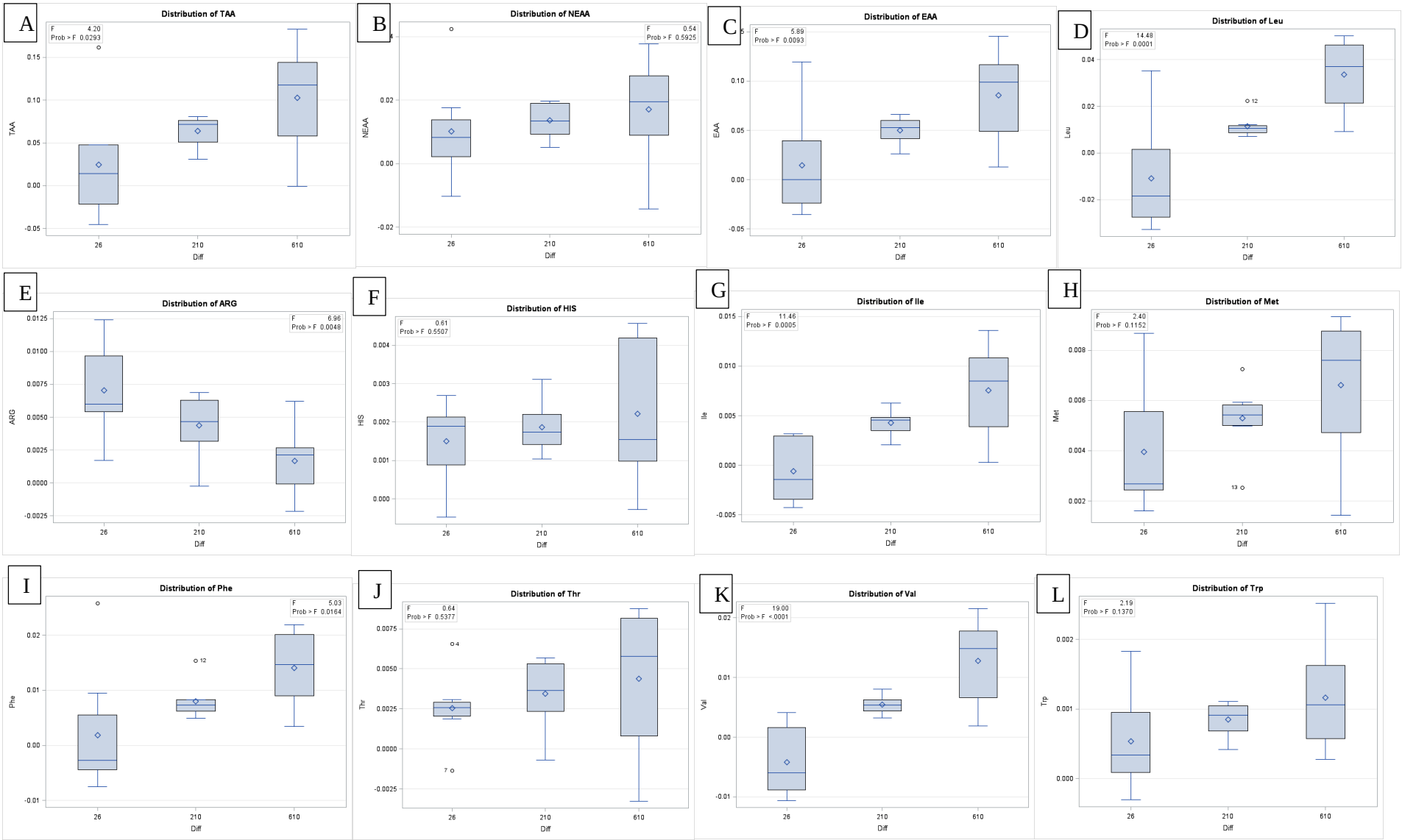
Appendix A6. Free amino acid in blood plasma of Nile tilapia at 6 hours after last feeding (g/kg).

	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 9
Asp	23	16	21	17	20	33	27	12
Thr	45	22	47	34	17	44	48	35
Ser	16	8	17	16	11	19	22	16
Glu	20	18	20	22	19	27	24	16
Gly	16	15	19	14	16	18	16	15
Ala	37	33	40	41	33	45	49	41
Cys	15	11	18	19	12	20	23	20
Val	70	40	86	104	39	97	121	86
Met	45	26	53	50	22	56	61	47
Ile	33	11	42	53	13	48	63	40
Leu	164	72	183	229	71	223	261	189
Tyr	88	84	78	102	59	109	116	90
Phe	105	61	104	120	55	132	137	98
Lys	27	22	35	30	23	30	45	33
His	30	15	31	25	21	34	36	30
Trp	10	12	12	14	8	12	18	10
Arg	26	18	30	27	22	29	41	30
Pro	70	28	90	73	52	88	87	74
EAA	554	297	624	686	290	705	831	598
NEAA	286	211	303	304	222	357	365	285
TAA	840	508	927	991	513	1062	1196	883

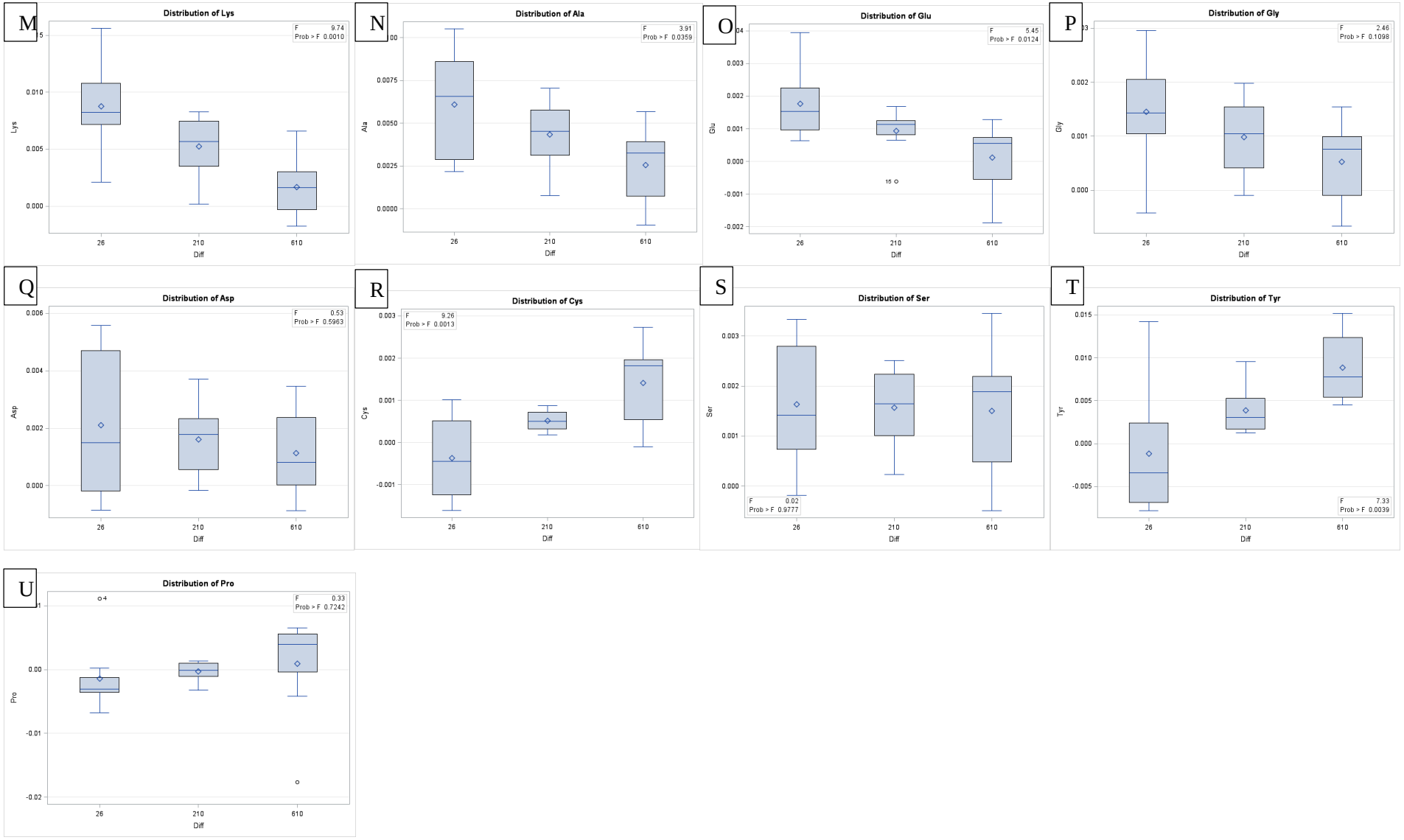
Appendix A7. Free amino acid in blood plasma of Nile tilapia at 10 hours after last feeding (g/kg).

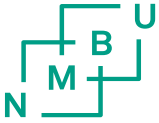
	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6	Tank 7	Tank 9
Asp	18	15	14	17	20	19	15	15
Thr	14	32	13	17	30	17	13	15
Ser	8	10	8	9	12	9	9	11
Glu	19	16	17	19	17	35	19	22
Gly	12	16	13	12	16	14	12	17
Ala	29	36	26	25	35	30	27	30
Cys	10	11	11	11	12	12	12	13
Val	26	32	26	33	30	26	35	27
Met	17	16	17	18	16	23	24	19
Ile	7	9	7	8	8	6	9	7
Leu	36	35	38	47	28	34	60	37
Tyr	64	64	60	44	36	68	55	52
Phe	45	41	46	41	41	49	49	46
Lys	23	28	22	22	30	25	19	21
His	23	16	15	20	19	16	18	24
Trp	8	10	5	8	6	8	8	6
Arg	21	24	20	18	31	20	16	19
Pro	57	98	67	55	69	61	65	61
EAA	219	244	209	233	239	225	250	222
NEAA	217	268	216	192	214	248	214	221
TAA	436	512	425	425	453	473	464	443

Appendix A8. Distribution of free amino acid clearances for three time intervals. 2-6. 2-10 and 6-10 hours after last feeding.



Appendix A8 (continuing). Distribution of free amino acid clearances for three time intervals. 2-6. 2-10 and 6-10 hours after last feeding.





Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no