Improved diet utilization of intensively cultured fish to address environmental sustainability - amino acid requirement in carp (*Cyprinus carpio*)

DISSERTATION

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By

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Abstract

The main goal of the present work was to improve diet utilization of intensively cultured fish to address environmental sustainability by addressing amino acid requirement in carp (*Cyprinus carpio*). This work includes five experiments designed to determine the requirement of the limiting amino acid, methionine (Met), using new semipurified diet formulations.

The first experiment described in **Chapter 2** tested how stomachless fish - koi carp respond to plant protein (pea)-based diet supplemented with one of the novel methionine derivatives (DD/LL/DL/LD-methionylmethionine and cyclic methionylmethionine).

This experiment showed that fish fed commercial (COM) and casein-gelatin based (CG) diets had the highest weight gains compared to other groups, and the Cyclic Met 0.6 group had significantly smaller weight gain compared to the Met- (negative control) and Met DL 0.6 group of fish.

The concentration of free Met in muscle tissues was the highest in fish fed CG diet compared to all other treatments except COM and FAA50 groups. The lowest level of free Met was observed in fish fed Met-, CycMet03 and CycMet06 diets compared to all other experimental groups with the exception of Met03. Free Met level in muscle of

CycMet03 and CycMet06 and negative control groups 3h after feeding was not different from Met level in the same group 48h after feeding (fasting group).

The second experiment described in **Chapter 3** was undertaken to gain better understanding and quantify amino acid (AA) loss during and post-ingestion of formulated diet containing significant proportion of free amino acids (FAA). In this experiment three diets were used: casein gelatin based diet (CG), casein gelatin based diets with 50% inclusion of FAA and commercial diet. This experiment showed that the level of Met released by fish fed FAA50 diet was the highest compared to COM and CG groups after 15 min, 2 and 4 hours. Similar trend was presented in other AAs such as: Leu, Ser, Pro, Tau, and Gln. In contrast, Tau and Gln were released in significantly higher amounts by fish fed COM diet (Otohime) compared to CG and FAA50 groups. The Lys level in the FAA50 group was comparable to other treatments 15 min after feeding, but excretion of this AA increased and its concentration in water was the highest 4 hours after a meal.

The third experiment described in **Chapter 4** tested how common carp will respond to casein-gelatin based diets with partial replacement of intact protein with FAA.

In this experiment reference diet based on casein and gelatin (CG) was used together with five diets with graded level of replacement 10% (FAA10), 20% (FAA20), 30% (FAA30), 40% (FAA40), and 50% (FAA50) of dietary protein with a mixture of FAA with identical AA profile. This experiment showed that the highest weight gains were obtained in fish fed the FAA10 diet compared to all other treatment groups. The lowest weight gain had fish that received the FAA40 diet but it was not different from

FAA30 and FAA50. The feed conversion ratio (FCR; feed/gain) was the lowest in the FAA10 group compared to all other treatments except CG and FAA20 groups.

The fourth experiment presented in **Chapter 5** evaluated common carp Met requirement at the near-maximal growth rate and analyzed postprandial free Met concentration in muscle tissues, indicative of dietary bioavailability.

In this study the reference diet based on casein and gelatin (CG) was used as well as five other diets in which 20% of dietary protein was replaced with a mixture of FAA with identical AA profile except Met. Met was added to the diets in form of DL-methionine at graded level: 0% (Met0), 0.2% (Met0.2), 0.4% (Met0.4), 0.8% (Met0.8), 1.6% (Met1.6), respectively, giving 0.9, 1.1, 1.3, 1.7, 2.5% DL-Met in the diets. The protein-bond Met level in CG diet was at the level of 1.14%.

This experiment showed the highest weight gains and the lowest FCR in fish fed the CG diet compared to all other treatment groups. The concentration of free Met in carp muscle tissues 6 hours after the last meal was the highest in Met 1.6 group compared to all other groups.

Finally, the fifth experiment described in **Chapter 6** determined how common carp respond to diets supplemented with piperine, curcumine and black pepper extract. Moreover, the influence of spices on fish appetite and FAA response in muscle was examined. In this experiment we used a reference diet based on casein and gelatin (CG), diet in which fraction of CG dietary protein was replaced with 20% of free amino acids (FAA) and supplemented with 0.4% DL-methionine (Met04), and three other diets identical to Met04 supplemented with 0.02% piperine (Pip), curcumine (Cur) and 0.2%

black pepper extract (BP), respectively. The addition of spices and crude extract of pepper did not show any influence of dietary Met on tissue Met level and food intake across the treatments. However, the Pip group of fish was characterized by the highest body fat level compared to other treatments. Phospholipids level was significantly lower in Pip and Curc compared to CG and Met 0.4. Neutral lipids presented opposite relationship.

This study addressed for the first time the possible regulatory mechanism and interaction between intake of methionine, transsulfuration via synthesis of taurine, and direct effects of these two spices on lipid deposition in fish.

Dedication

I dedicate this dissertation to my wife Dr. Karolina Kwasek.

She was my motivation and kept me moving forward when I wanted to give up.

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Vita

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Publications

Kwasek K., Dabrowski K., Nynca J., Wojno M., Wick M., 2014. The influence of dietary lysine on yellow perch maturation and the quality of sperm. North American Journal of Aquaculture 76, 119-126.

Kwasek K. Dabrowski K, Nynca J., Takata R., Wojno M., Wick M., 2014. The influence of dietary lysine on yellow perch (Perca flavescens) female reproductive performance and the quality of eggs. North American Journal of Aquaculture (in press).

Kwasek K., Wojno M., Terova G., Ostaszewska T., Wick M. and Dabrowski K., 2012. The effect of the dipeptide, Lys-Gly, supplement on growth, muscle proteins and PEPT1 gene expression in juvenile yellow perch. Reviews in Fish Biology and Fisheries 22: 797–812.

Yousef L.F., Wojno M., Dick W. A., Dick R. P., 2012. Lipid profiling of the soybean pathogen Phytophthora sojae using Fatty Acid Methyl Esters (FAMEs). Fungal Biology 116: 613-6119.

Dick R., Lorenz N., Wojno M. and Lane M., 2010. Microbial dynamics in soils under long-term glyphosate tolerant cropping systems. 19th World Congress of Soil Science, Soil Solutions for a Changing World 1 – 6 August 2010, Brisbane, Australia.

Fields of Study

Major Field: Environmental Science

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Chapter 1: GENERAL INTRODUCTION

Fish and fishery products represent valuable sources of protein and essential micronutrients such as indispensable amino acids (IDAA) required for balanced nutrition and health. The nutritional value of most fish proteins is similar to or better than that of terrestrial animals meat or milk protein (casein) (Friedman, 1996). Fish oils contain specific long chain n-3 polyunsaturated fatty acids (PUFA) in fish oils (i.e. decosahexaenoic acid, DHA 22:6n-3; eicosapentaenoic acid, EPA, 20:5n-3) that have equally high nutritional value for humans (Sargent, 1997). The beneficial effects of PUFA in cardiovascular disease have been widely reported in patients with not only preexisting syndromes but also in healthy individuals (Kris-Etherton *et al.*, 2003). Positive influence of PUFA have also been shown in mental development of children who received from mothers fish oil supplementation during pregnancy (Dunstan *et al.*, 2008).

Although the total consumption of fish in developing countries is much lower than in Western countries, the contribution of fish to animal protein intake is higher. In 2009 fish accounted for 6.5% of total protein consumed and 16.6% of the world population animal intake. Globally, fish provide protein to approximately 3 billion people which amounts to 20% of total animal protein intake (FAO, 2012).

Global capture fishery production leveled off since middle 1990s, reaching the oceans carrying capacity and higher fish production in the future would lead to overfishing. Aquaculture has become the driving engine of the rise in total fish production, filling the gap between available fishery products and its demand by fast growing human population. Overall world-wide production of aquaculture increased 12 fold since 1980 and evolved in terms of technological innovations and adaptation to changing environmental requirements. Recently, aquaculture fish production for human consumption reached nearly 50% of the total fish production, with an average 8.8% of annual growth - the highest trend compared to all other farmed animals (FAO, 2012).

Over 70% of capture marine fish production is used for human consumption; the remaining products are used for fish meal and fish oil production. Both products are highly valuable feed ingredients in farm animal nutrition. Most of the available fish meal and fish oil, however, is used in aquaculture sector, in formulated diets for farmed aquatic animals (Tacon & Metian, 2008).

High growth rates of produced fish in captivity require higher input of formulated diets, which in turn requires higher quantities of fish meal and fish oil. Therefore, fish meal and fish oil are limited resources and if their current use in aquaculture continues to increase in the future, it is apparent that the demand will exceed the annual global production. This means that the world aquaculture demand for wild fish resources might exceed the production capacity of the ocean (Hardy, 2010). Response to this major concern includes replacement of fish meal and fish oil with alternative ingredients

derived mainly from plants, such as grains, and/or byproducts recovered from livestock and poultry processing.

All processed-plant feedstuffs, however, have characteristics that place them at a disadvantage to fish meal in terms of their suitability for use in aquatic animal feeds (aquafeeds). The valuable alternative to fish meal must possess certain features such as: wide availability, competitive price, ease of handling and storage (Hardy, 2010). Furthermore, it must possess certain nutritional characteristics such as: low level of fiber, starch, low content of non-soluble carbohydrates and anti-nutritional factors. In addition, alternative feed ingredients must have a relatively high protein content, favorable indispensable amino acid profile, high nutrient digestibility and palatability characteristic meeting aquatic animal acceptance.

Production of freshwater fish has always been dominated by cyprinids with annual production around 24 million metric tons in 2010 that included common carp *Cyprinus carpio* production of 3.4 million tons compared to salmonid production of only 1.9 million metric tons (FAO, 2012). However, only 2-3% of cyprinids is cultivated intensively, the vast portion of the production still carried out in a traditional semi-intensive pond culture with use of low cost feeds (Tacon, 1993). Therefore, the intensification of carp culture requires optimization of nutritional requirements of that species to improve growth rate. An optimal balance of macro- and micronutrients in diet as well as their enhanced bioavailability will allow for better feed utilization ratio ultimately reducing the impact of carp farms on the environment. In addition, feeding

strategies should be modified to avoid waste (reduction of nitrogen and phosphorus discharge) and to maximize protein accretion.

The main goal of the project

The main goal of the present work was to improve diet utilization of intensively cultured fish to address environmental sustainability by addressing amino acid requirement in carp (*Cyprinus carpio*). This project includes five experiments designed to help to understand this phenomenon.

Chapter 2: DIETARY EFFICACY FOR GROWTH OF NOVEL METHIONINE DERIVATIVES COMPARED TO DL-MET IN KOI CARP (CYPRINUS CARPIO)

Summary

Many plant feedstuffs are used in aquaculture as a fish meal replacement. Most of them, however, are deficient in methionine, which must be supplemented to improve nutritional value of practical diets. In this experiment we tested how stomachless fish - koi carp respond to plant protein (pea)-based diet supplemented with one of the novel methionine derivatives (DD/LL/DL/LD-methionylmethionine and cyclic methionylmethionine).

This experiment showed that fish fed COM and CG diets had the highest weight gains compared to other groups, and the Cyclic Met 0.6 group had significantly smaller weight gain compared to the Met- (negative control) and Met DL 0.6 group of fish.

The concentration of free Met in muscle tissues was the highest in fish fed CG diet compared to all other treatments except commercial and FAA50 groups. The lowest level of free Met was observed in fish fed Met-, CycMet03 and CycMet06 diets compared to all other experimental groups with the exception for Met03. Met level in CycMet03 and CycMet06 and negative control 3h after feeding was not different from Met level in the same groups 48h after feeding (starved group).

Introduction

Many plant feedstuffs are used in aquaculture as a fish meal replacement, among them soybean is the most widely used (Gatlin et al., 2007). In Canada and Europe, however, soybean production is limited, mostly due to unfavorable climatic conditions. Therefore, a more sustainable plant protein derived from pea (*Pisum satium*) is broadly cultivated in these geographical regions. The attractiveness of pea protein increased recently mostly because of the genetic improvement which has led to higher protein yields, enhanced oil content, and better starch quality (Gouveia and Davies, 1998, Burel et al., 2000). The content of protein in pea seeds is not high, up to 25% of dry matter, but the level of complex carbohydrates and antinutritional factors is low compared to other legumes (Bond and Duc, 1993, Castell et al., 1996, Knudsen, 1997, Francis et al., 2001), which can be even further reduced with high temperature during extrusion process (Melcion and Van der Poel, 1993). However, pea protein concentrate is also characterized by low level of sulfur containing amino acids compared to other plant proteins such as those derived from soybean (Keith et al., 1977, Valencia et al., 2009). Since the optimal supply of indispensable amino acids (IDAA) in a diet is essential for maximizing growth and proper physiological conditions of fish, a supplementation with deficient IDAAs is often required to improve the nutritive value of plant protein meals (Murai et al., 1989, Davies et al., 1997, Regost et al., 1999, Cheng et al., 2003). Traditionally methionine deficiency has been mitigated by direct addition of chemically synthesized DL-methionine to a diet, which is a 1:1 mixture of D-, and L-methionine. Although, D- enantiomer is unnatural, it has been shown that it may be converted to

natural L- form by several fish species (Robinson et al., 1978; Kim et al., 1992; Schwarz et al., 1998; Sveier et al., 2001). While supplementation of aquafeeds with crystalline amino acids is common, the effectiveness of this process may be at risk due to their high solubility as they can be simply lost by leaching to water prior to ingestion (Yufera et al., 2002) or during feeding and mastication (Yamada and Yone, 1986). Moreover, supplemented free amino acids are absorbed faster in intestine than amino acids bound in dietary protein and consequently might result in high concentration of unbalanced amino acids in blood and sites of protein synthesis (Ogata, 1986). It has been shown that excretion trough the gills and kidneys occurs in fish (Murai et al., 1984b, Ng et al., 1996). Therefore, practical diets deficient in indispensable amino acids should be supplemented in chemically protected forms, which remain stable in the diet during feeding, is released slowly in the intestinal tract and are efficiently utilized by fish (Haeussner et al., 2009). Consequently the objectives of this study were: (i) to test how stomachless fish - koi carp will respond to plant protein (pea)-based diet supplementation with one of novel methionine derivatives (DD/LL/DL/LD-methionylmethionine and cyclic methionylmethionine), (ii) to evaluate postprandial free amino acids concentration in muscle tissues, mostly methionine as an indicator of its availability, and (iii) to examine if different dietary methionine sources may affect proximate carp body composition. These results may contribute to further development of plant based, sustainable diets for omnivorous fish in intensive aquaculture production.

Materials and methods

Diet formulations

Nine experimental diets were used in this feeding trial (Table 1). Two reference diets: casein-gelatin (CG) and casein-gelatin based diet with 50 % of protein in a form of free amino acids mixture (FAA50). Both diets were prepared according to the earlier description regarding rainbow trout alevins (*Oncorhynchus mykiss*) (Terjesen et al., 2006) and common carp larvae (*Cyprinus carpio*) (Kwasek et al., 2010). Casein and gelatin (5:1) were used as the main protein sources and dextrin was replaced with starch in comparison to the original formulations. Amino acids composition of both diets fulfilled amino acids requirement of common carp (NRC, 2011). In the present study, seven experimental diets were used to evaluate differences in supplemental derivatives of methionine (Met). Experimental diets were formulated based on Schwarz et al. (1998) studies with carp. Pea protein concentrate (PPC) was used as the major protein carrier (37%) combined with soybean protein concentrate (20%) and gelatin (10%). All ingredients used were characterized by deficiency in sulphur-containing amino acids (Valencia et al., 2009). Therefore, two levels of Met supplementation 0.3% and 0.6% were used for each derivatives, where higher supplementation level was close to Met requirement recommended by Murai et al. (1989) and Schwarz et al. (1998). We used three methionine derivatives: DL-methionine, DD/LL/DL/LD-Methionylmethionine and cyclic methionylmethionine (Met03, Met06, MetMet03, MetMet06, CycMet03 and CycMet06 treatments respectively). The seventh diet contained no Met supplementation and served as a negative control. In addition we used a commercial diet (Otohime B2,

Marubeni Nisshin Feed Co., Japan) containing 50% of crude protein, 10% crude fat, 3% crude fiber, 16% crude ash, 2.3% calcium, and 1.5% phosphorus as a practical control diet. The total (Table 2) and free (Table 3) amino acid composition of all diets are presented.

Fish maintenance and feeding experiment

Juvenile ornamental Japanese carp (*Cyprinus carpio*) were used in this experiment. Fish were bred from koi carp broodstock in the Aquaculture Laboratory at the Ohio State University, Columbus, OH, USA. The initial feeding included live brine shrimp naupli (Artemia sp.) and subsequent weaning to commercial starter diet (A, B1, B2, C1, Otohime, Japan). Full sibling fish at the size of 3.62 ± 0.04 g were randomly distributed into 30 L aquaria in semi-recirculating system, 30 fish per tank, in triplicates per dietary treatment. Each glass tank was considered as an independent experimental unit with constant water replacement at a rate of 0.35 L min^{-1} and temperature was maintained at 26.7±0.9 ºC. Supplemental aeration was provided to maintain dissolved oxygen level near saturation. Fish were fed restricted feeding rates in three meals per day at 9:00, 13:00, and 17:00, seven days per week for 35 days, all equal across treatments. The feeding rate gradually decreased from 8 to 4% of biomass and was estimated assuming daily biomass increase equal to food consumed. The feeding rate was adjusted to the actual fish biomass biweekly after fish weighing. All aquaria were siphoned daily and walls were scraped on regular basis in order to remove fecal material and prevent algae growth in the tanks. Feeding was stopped 24 h prior to each weighing.

The amount of food consumed by fish in one meal was evaluated in a satiation test for each diet, where fish were fed until clear food rejection. The amount of food consumed across all the experimental units was expressed as a percent of fish biomass for each treatment.

All procedures and handling of animals was conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University.

Statistical analysis

The experimental design was completely randomized for fish distribution and experimental diets assignment. The differences among dietary treatments were tested by one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at the value of $p < 0.05$.

Free amino acid analysis

Samples of fish muscles (~1% BM) were taken from each fish from dorsal part of the body between a dorsal fin and a head. Muscle tissue samples of three fish from each tank were combined and homogenized together with 0.1 mol/l HCl in 1:75 (w/v) containing 160 µmol/l norleucine internal standard according to Cohen et al. (1989). Samples were then spun at 12 000 g (4ºC, 15 min) and supernatants were filtered (Milipore, 10 kDa cutoff at 2000 g, 4ºC, 90 min). Blanks (0.1 M HCl + 160 µmol/l nLeu) (Terjesen et al., 2004) and external standards (Sigma acid/neutral and basic amino acids) were prepared along with the sample preparation. Samples, blanks and external standards were stored at -80^{o}C until the same concentration of glutamine in 0.1 M HCl as external

standard was prepared on the day of analysis and added to the basic amino acids standard. Amino acids were pre-column derivatised with phenylisothiocyanate (Cohen et al., 1989). Sample precipitates were removed by a 10-min centrifugation at 10 000 g (Terjesen et al., 2004). Free amino acids were quantified using a Waters Pico Tag RP-HPLC (Waters Corporation, Milford, MA, USA) equipped with an application-specific column (3.9 • 30 cm), a Waters 717 autosampler, 2 Waters 501 pumps, a Waters 441 absorbance detector at 254 nm and a column heater set at 46 C. Eluent 1 (composition of acetonitrile, triethylammonium-acetate, sodium acetate, and water) and eluent 2 (composition of acetonitrile, methanol, and water) purchased from Waters were used throughout the investigation. Each amino acid was identified by spiking with known amino acids and retention times of external standards. Free amino acid concentrations (expressed as mmol/kg wet body weight) were calculated using internal and external standards (Cohen et al., 1989).

Diets and whole body amino acid analysis

Total amino acid composition analysis was performed in Evonik (Evonik Industries, Essen, Germany) laboratory certified for accuracy and according to procedures corresponding to the official methods of EU Commission Directive 98/64/EC and the AOAC method 994.12. Briefly, samples were hydrolysed with 6 N HCl for 24 h at 110°C and neutralized with Na metabisulfite (Comision Directive, 1998) (Llames and Fontaine, 1994; Commission Directive, 1998). Amino acids profile in diets and fish whole body was analyzed by ion-exchange chromatography with postcolumn derivatization with ninhydrin. A presence of AA in the samples was detected by

measuring absorption of reaction products with ninhydrin at 570 nm and further quantified based on the internal standard. Tryptophan was measured after alkaline hydrolysis with barium hydroxide octahydrate for 20 h at 110°C (Directive, 1998) and then determined by HPLC with fluorescence detection (extinction 280 nm, emission 356 nm). Tyrosine was not determined. Dry matter content was measured by drying in an oven at 103^{o}C for 4 hours. Crude protein content was measured as N × 6.25 using a Leco FP-2000 analyzer (Leco Corp., St. Joseph, MI).

Proximate and mineral analyses of fish whole body.

Analyses of ash, moisture and total nitrogen were performed by standard procedures (AOAC, 1995). Crude protein was established by multiplying nitrogen content by 6.25 factor (N×6.25). Lipids were extracted with a chloroform and methanol mixture (v/v, 2/1) using the procedure described by Folch et al. (1957). Samples were prepared for analysis using the ashing procedure (AOAC, 1995). Mineral compositions of diets and whole-body were measured by the inductively coupled plasma (ICP) emission spectrophotometric method with the use of an ARI-3560 spectrometer (Applied Research Labs, Valencia, CA, USA), following Watson et al. (1990) methods. Minerals were then expressed per the whole body dry matter.

Results

Fish growth and food utilization

Final results of the experiment showed that the highest weight gains were obtained in fish fed commercial diet followed by CG diet compared to all other groups. On the contrary, fish that received the FAA50 diet had significantly lower weight

compared to commercial, CG, and Met0.6 diet fed fish but not different than MetMet06, CycMet03, CycMet06, and Met- diets (Figure 1). The satiation ration (amount of food consumed in one meal) was the lowest in the CG group compared to all other treatment groups. The FCR was the lowest in the control group followed by CG group compared to all other groups.

Muscle FAA

The concentration of free Met in koi carp muscle was the highest in fish fed CG diet compared to all other groups except commercial and FAA50 groups (Figure 2). The lowest level of free Met was observed in fish fed Met- (negative control), CycMet03 and CycMet06 diets compared to all other experimental groups with the exception for Met03. The difference between those supplemented with less than 0.6% Met and with less available Met derivative (CycMet) and control, amounted to 2-3 fold. Interestingly, Met level in these three groups 3h after feeding was not different from Met level in the same groups 48h after feeding (starved group). The concentration of free Tau was similarly the lowest in CycMet03, CycMet06 and Met- compared to all other treatment groups (12-16 fold difference). The level of free Tau 3h after a meal was even below the level of free Tau in starved fish. Free Tau concentration was the highest, however, in the control group compared to all other groups. No differences were found in the level of free Tau among CG, FAA50, MetMet03, and MetMet06 groups. The free Lys concentration was the highest in fish fed CG diet compared to all other groups except CycMet03. There were no differences in the level of free Lys in fish fed commercial, FAA50, Met03, Met06, MetMet03, and MetMet06 diets. The level of free Lys was higher in CycMet06

and Met- groups compared to all treatments except the CG group. The concentration of free Arg was the lowest in commercial, CG, and FAA50 groups compared to all other treatments. The concentration of Thr was the highest in CG group compared to all other groups but not different than CycMet03 group. The lowest level of IDAA was observed in fish that received commercial diet compared to all other groups with the exception of CG and FAA50 groups. The level of DAA in contrast to IDAA was the highest in FAA50 compared to all other treatment groups. The DAA concentration 3h after feeding was the lowest in the commercial diet fed group (but at the same level as in starved fish) compared to all other groups. All other free amino acid levels 3 h and 48 h after feeding are listed in Tables 4 and 5, respectively. There were no clean trends in differences in FAA muscle among treatment groups.

Whole body AA

There were no differences found in total Met concentration in koi carp whole body (Table 6). The level of Leu was higher in Met03 compared to CG, CycMet03, and Met-. The Lys level was higher in Met03 group compared to the CG group. The level of Phe showed the same trend. Similarly, Arg concentration was higher in Met03 group compared to CG and FAA50 groups. The concentration of Ser was highest in Met03 and Met06 compared to CG and Met- groups but not different than in other treatments. The level of Asp was the lowest in Met03 compared to CG, MetMet 06, and CycMet 03. The Glu concentration was higher in Met03 and MetMet06 compared to the CG diet. Pro was higher in FAA50 compared to all groups except CG group.

Discussion

In general, in the present experiment, weight gains of carp supplemented with 0.3 or 0.6% Met did not differ and there was no indication of increased attractiveness of food related to Met level (Figure 1). These results differ from weight gain obtained by Nwanna et al (2012) when carp was fed diets with either 0.45 or 0.86% Met supplementation. Although the effect of feeding strategy paralleled Met supplementation in Nwanna et al. (2012), the major difference between the latter and current experiments might have been high purity of pea protein concentrate (Nutri-Pea Ltd., Canada). Although Met was identified as one of the deterrent amino acid in carp taste assay (Kasumyam and Morsi 1996), we do not find evidence of reduced palatability for the feeds used in the present experiment (Figure 1)

During the last two decades, a significant amount of research has been conducted on fish meal replacement by plant protein sources in fish diets. Nevertheless, the outcome of using these alternative protein sources in respect to growth performance is highly variable among fish species and strongly dependent on the plant protein used. Pea protein concentrate is a potential alternative to other protein concentrates, however, similarly to soybean protein it requires supplementation with methionine to improve its nutritional value (Keith et al., 1977). Schulz et al. (2007) showed that 30% fish meal replacement with pea protein isolate without methionine supplementation in juvenile Nile tilapia (*Oreochromis niloticus*) did not suppress growth and FCR during 56 days of feeding trial. Further studies with use of pea protein in diets for Atlantic salmon (*Salmo salar*) (Øverland et al., 2009) and common carp (Davies and Gouveia, 2010) showed that Met

supplementation is unnecessary when fish meal remains at the level of 44-56% in diet formulations. In cases of diets based exclusively on plant proteins, such a soybean, a supplementation with methionine provided in coated form did not impact weight gains of carp (Murai et al. 1981) or channel catfish (*Ictalurus punctatus*) fingerlings after 6 weeks of feeding (Murai et al., 1982). The level of Met in the present study was estimated based on current recommendations for methionine requirements for juvenile common carp using different methods: (Dabrowski 1983), 2.1% (Nose 1978), 1.8% (Ogino 1980). Schwarz et al. (1998) determined Met requirement as 2.13% and 0.86% respectively in crude protein and diet dry matter in broodstock carp.

Nwanna et al. (2012) used very similar approach to our design in terms of the experimental diet composition. They used similar ratio of gelatin, pea and soybean protein concentrate (10, 37 and 22 % respectively) with two levels of DL-methionine, 0.45 and 0.86% based on dry matter, with 40% of crude protein content. Although much larger fish (239g) were used in their study, the impact of methionine supplementation on weight gain was significant after 82 days of feeding trial. In our study however, we did not observe any significant effect of methionine or methionine derivatives supplementation on weight gain of juvenile koi carp even though the weight gain was over 500% in the slowest growing treatment. It was probably caused by high crude protein content in our experimental diets (around 60%), resulting from our using pea protein concentrate of pea meal, as was used in earlier mentioned studies. The growth performance of fish fed with our reference diet (CG) was superior to that observed by Murai et al. (1984a) and Murai et al. (1983), 476 and 523%, respectively and FCR 0.86 in

common carp of similar size fed with 5% of body weight. Even though the protein concentration was lower (32%) the feeding trial was longer (42 days) and diets had higher proportion of casein compared to our experiment. Based on these references we can assume that the conditions provided for fish in our study were sufficient to support optimal growth of juvenile koi carp. The inferior performance of carp fed with FAA based diets compared to CG or other diets with protein bound amino acids was already reported in numerous studies (Aoe et al., 1970, Aoe et al., 1974, Nose et al., 1974). Data obtained in our experiment support this phenomenon. The satiation test (amount of food consumed) and FCR (except commercial diet) were the lowest in the CG group compared to all other treatment groups. However, based on the Nwanna et al. (2012) report, there is evidence that fish might consume significantly more diet supplemented with methionine than diet deficient in that essential amino acid. In rats fed a low soybean protein isolate diet, FCR was significantly improved by L-methionine supplementation (Hara et al., 1997). According to Baker (2006) excessive amount of dietary sulfur containing amino acids had a toxic effect on laboratory animals. Fau et al. (1987) showed that methionine affects daily food intake and weight gain in kittens. These animals fed high methionine diets decreased their food intake and lost weight in proportion to dietary methionine level. Friedman and Gumbmann (1988) also reported that excess of dietary methionine may have inhibitory effect on mice growth. In fish methionine deficiency, besides suppressing growth, may also have other pathological effects on fish. Keembiyehetty and Gatlin III (1993) have demonstrated that low dietary levels of methionine might increase susceptibility to fungal disease resulting in high mortality of juvenile hybrid striped bass

(Morone chrysops× Morone saxatilis). Moreover, dietary methionine deficiency can also lead to cataract formation in juvenile rainbow trout (Rumsey et al., 1983, Kim et al., 1992) and other salmonids (Poston et al. 1977).

Different methionine derivatives may be utilized with variable efficiency by different animals and fish species. Savolainen and Gatlin III (2010) have reported that soybean meal based diet supplemented with L-methionine and methionine hydroxyl analog (MHA) provided similar weight gain and FCR significantly greater compared to the unsupplemented diet fed fish (*Morone chrysops* × *M. saxatilis*) after 10 weeks of feeding. Keembiyehetty and Gatlin III (1997) have confirmed that sunshine bass (*Morone chrysops*× *Morone saxatilis*) utilize DL-methionine and acetyl-methionine as efficiently as L-methionine, while MHA calcium salt resulted in significantly reduced growth. In previous studies the same research team evaluated that MHA calcium salt was only 75% as effective as L-methionine (Keembiyehetty and Gatlin III, 1995). In contrast, juvenile red drum (*Sciaenops ocellatus*) used crystalline MHA as efficiently as L-methionine based on growth performance (Goff and Gatlin III, 2004). According to Robinson et al. (1978) weight gain and food efficiency data indicated that DL-methionine is utilized as efficiently as L-methionine by fingerlings channel catfish whereas MHA was inferior either to L- or DL-methionine. In growing pigs the bioavailability of DL-methionine-hydroxy analogue free acid was 75% as efficient as DL-methionine on equimolar basis (Kim et al., 2006, Shoveller et al., 2010). Friedman and Gumbmann (1988) have estimated the value of DD/LL/DL/LD-methionylmethionine as methionine derivatives based on mice growth performance. They reported that L-methionyl-L-methionine, L-

methionyl-D-methionine, D-methionyl-L-methionine, and D-methionyl-D-methionine are effective as 102.9%, 82.1%, 99.2%, 41.5% respectively, compared to L-methionine. We may therefore assume that DD/LL/DL/LD-methionylmethionine would give relative potency of 80% to replace L-methionine with mixture of all methionine enantiomers in equal proportions.

Our experiment showed that the concentration of free Met in muscle tissues was positively affected by DL-methionine and DD/LL/DL/LD-methionylmethionine at each level of supplementation. This observation may suggest that both of these forms are bioavailable for juvenile koi carp. The lowest level of free Met was observed in negative control and the treatments where cyclic methionylmethionine was used. For all these treatments the Met concentration was similar and almost identical for fed and starved fish. This fact may suggest that this chemical form of methionine is not effectively utilized by koi carp juveniles. Interestingly, lysine concentration that is considered as a second limiting AA, had inverse relationship with methionine in muscle tissues. The same association was observed by Schwarz et al. (1998) and Nwanna et al. (2012) in carp fed pea proteins based diets supplemented with DL-methionine, where methionine concentrations in carp blood plasma increased with supplementation with DL-methionine. Furthermore, in both reports lysine and arginine concentrations in blood plasma had inverse relationship with the level of methionine supplementation. In our study taurine concentration in muscle tissues showed the same trend across all the treatments as methionine reported also in blood plasma (Schwarz et al. 1998). The association between these AA may be explained by the fact that taurine is one of the

methionine metabolites (Ueki et al., 2012). Although Tau has been documented to be synthesized in carp in comparison to rainbow trout (Kim et al. 2008), the rate is clearly inadequate to maintain physiologically relevant concentration of this compound in muscle.

Yamada et al. (1982) found out that tilapia (*Oreochromis niloticus*) and carp were similar in respect to maximum postprandial level of plasma FAA at 4h following casein-gelatin based diets ingestion. In rainbow trout fed the same diet it occurred 24-36 h after a meal. The response of FAA profile in fish body to the diet may be presented in specific tissues in variable time and concentration. FAA pattern in the skeletal muscle, however, seems to be relatively stable compared to the blood plasma or hepatopancreas (Ogata, 1986, Murai and Ogata, 1990). Crystalline amino acids appear to be faster absorbed in carp from a diet and surge earlier in the blood stream and are consequently catabolized compared to AA derived from protein hydrolysis (Plakas et al., 1980). Although blood plasma AA concentration is commonly used for assessment of dietary protein biological value, there is strong evidence that postprandial FAA profile of muscle tissues is more robust indicator because it presents the actual FAA pool/profile available for protein synthesis.

There were no differences found in Met concentration in koi carp whole body across the treatments. These results go along with the commonly accepted opinion that regardless of a dietary treatment a whole body amino acid composition tends to maintain its profile. (Pérez-Jiménez et al., 2014, Peres and Oliva-Teles, 2005, Wilson and Cowey, 1985). Nevertheless, significantly higher levels of Lys, Arg, Leu, Phe, and Ser were

observed in fish fed diets supplemented with DL-methionine at the level of 0.3% compared to fish fed CG, Cyclic Met 0.3 and negative control.

In conclusion, our results suggest that koi carp is able to use DL-methionine as efficiently as DD/LL/DL/LD-methionylmethionine. Nevertheless, based on methionine concentration in muscle tissues we can conclude that the cyclic methionylmethionine has limited bioavailability in juvenile carp. In addition, we confirmed that pea protein concentrate is acceptable as alternative protein source in carp practical diets.

Table 1 Composition of experimental diets: casein-gelatin (CG), 50% free amino acid (FAA50), diets supplementation with three types of methionine derivatives (DL-Met, DD/LL/DL/LD-methionylmethionine; cyclic methionylmethionine) and negative control (-Met).

Ingredients (g/kg)	Diets								
	CG	FAA50	DL Met 0.3	DL Met 0.6	Met-Met 0.3	Met-Met 0.6	Cyclic Met 0.3	Cyclic Met 0.6	(-) Met
Casein[a]	400	200	-	-	-	-	-	-	-
Gelatin[b]	80	40	100	100	100	100	100	100	100
PPC (82%)[c]	-	-	370	370	370	370	370	370	370
SPC (45%)[d]	-	-	200	200	200	200	200	200	200
Free AA mixa[e]	-	240	-	-	-	-	-	-	-
CPSP 90[f]	50	50	50	50	50	50	50	50	50
Starch[g]	308.4	308.4	112.4	112.4	112.4	112.4	112.4	112.4	112.4
Fish oil[h]	30	30	30	30	30	30	30	30	30
Soy oil[i]	30	30	30	30	30	30	30	30	30
Vitamin mix[j]	20	20	20	20	20	20	20	20	20
Mineral mix[k]	40	40	40	40	40	40	40	40	40
NaH_2PO_4[l]	20	20	20	20	20	20	20	20	20
Vitamin C[m]	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
CMC[n]	20	20	20	20	20	20	20	20	20
Choline chloride[o]	1	1	1	1	1	1	1	1	1
DL Met[p]			3	6					
Met-Met[r]					3	6			
Cyclic Met[s]							3	6	
Glutamate[t]			3		3		3		6

[a] Casein vitamin free (MP Biomedicals, Solon, OH, USA).
[b]Gelatin type A (MP Biomedicals, Solon, OH, USA).
[c]Pea protein concentrate, 82% of protein (Nutri-Pea Limited, Manitoba, Canada)
[d]Soybean protein concentrate, 45% of protein.
[e]Compositions (g/240g; all L-form AA otherwise indicated); Arg free base, 9.1; His free base, 3.7; Ile, 4.8; Leu, 7.5; Lys monohydrochloride, 9.6; DL-Met, 5.4; Phe, 9.6; Thr ali o free, 4.3; Trp, 1.1; Val, 6.4; Pro, 59.5; Ser, 59.5; DL-Ala, 59.5.
[f]Soluble fish protein hydrolyzate (Sopropeche S.A., Boulogne Sur Mer, France).
[h]Cod liver oil (MP Biomedicals, Solon, OH, USA).
[i]Soybean oil (ICN Biomedicals, Aurora, OH, USA). Continued

Table 1 Continued

[j] Vitamin mixture (mg/kg diet) sources were Rovimix series: retinyl acetate, 2.00; cholecalciferol, 0.10; DL-α-tocopheryl acetate, 125.00; menadione niacinamide bisulfite, 5.00; nicotinic acid, 25.00; riboflavin, 20.00; pyridoxine hydrochloride, 15.00; D-calcium pantothenate, 50.00; biotin, 1.00; folic acid, 5.00; cyanocobalamin, 0.05; *myo*-inositol, 500.00; thiamine mononitrate, 10.00 (Aquaculture Research Group, DSM Nutritional Products France, Animal Nutrition & Health Research, Saint-Louis, France).
[k]Five milligram Se in the form of sodium selenite per kg Bernhart Tomarelli salt mixture (ICN Pharmaceuticals, Costa Mesa, CA, USA).
[l]Monosodium phosphate (Sigma-Aldrich , St. Louis, MO)
[m]Mg-L-ascorbyl-2-phosphate (ShowaDenko K. K., Tokyo, Japan).
[n]Carboxymethylcellulose (ICN Biomedicals, Solon, OH, USA).
[o]Choline chloride (MP Biomedicals, Solon, OH, USA)
[p]DL-Methionine (Evonik Industries, Essen, Germany)
[r]DD/LL/DL/LD-Methionylmethionine (Evonik Industries, Essen, Germany)
[s]cyclic Methionylmethionine (Evonik Industries, Essen, Germany)
[t]Glutamate (Sigma-Aldrich , St. Louis, MO).

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Table 2 The amino acid composition of control and experimental diets (expressed as % of crude protein)

in Dry Matter	DM	CP	MET	CYS	Met+Cys	LYS	THR	TRP	ARG	ILE	LEU	VAL	HIS	PHE	GLY	SER	PRO	ALA	ASP	GLU
Control	100	59.40	1.62	0.63	2.25	4.33	2.43	0.65	3.62	2.65	4.34	2.85	1.51	2.67	3.20	2.38	2.58	3.38	5.68	7.62
CG	100	51.05	1.32	0.20	1.54	3.72	1.95	0.52	2.40	2.28	4.21	2.94	1.30	2.35	3.21	2.68	5.79	2.26	3.70	9.95
FAA 50%	100	49.33	1.25	0.13	1.38	2.86	1.47	0.37	2.29	1.69	3.06	1.95	1.10	2.22	1.92	7.02	9.18	6.22	2.09	5.37
Met03	100	61.09	1.04	0.55	1.59	3.88	2.04	0.52	4.79	2.41	4.31	2.71	1.32	2.81	4.73	2.91	3.91	3.32	6.20	9.66
Met06	100	61.28	1.27	0.53	1.80	3.91	2.05	0.52	4.82	2.44	4.31	2.74	1.32	2.81	4.79	2.87	3.89	3.22	6.24	9.43
Met-Met03	100	61.58	1.06	0.56	1.62	3.91	2.06	0.50	4.81	2.43	4.34	2.74	1.34	2.82	4.77	2.89	3.81	3.20	6.27	9.73
Met-Met06	100	61.22	1.35	0.54	1.89	3.90	2.05	0.52	4.81	2.41	4.32	2.72	1.33	2.82	4.73	2.90	3.84	3.18	6.26	9.44
cyclic Met03	100	62.13	1.08	0.54	1.60	3.90	2.04	0.52	4.83	2.45	4.33	2.76	1.33	2.81	4.83	2.84	3.88	3.21	6.25	9.68
cyclic Met06	100	61.77	1.43	0.56	1.99	3.94	2.06	0.51	4.82	2.45	4.30	2.76	1.35	2.81	4.74	2.85	3.80	3.14	6.23	9.44
Met -	100	61.49	0.76	0.56	1.31	3.94	2.05	0.52	4.86	2.45	4.32	2.77	1.35	2.81	4.88	2.86	3.85	3.22	6.25	9.95

Table 3 The free amino acid composition of control and experimental diets.

in Dry Matter	MET	LYS	THR	ALA	ARG	GLU	GLY	HIS	ILE	LEU	PHE	PRO	VAL
Control		0.06	0.03	0.12	0.29		0.12	0.17					
FAA 50%	0.55	0.83	0.43	4.94	0.92			0.46	0.46	0.80	0.99	6.29	0.36
Met 0.3	0.29												
Met 0.6	0.55												
Met-Met 0.3	0.02					0.29							
Met-Met 0.6	0.01												
cyclic Met 0.3						0.29							
cyclic Met 0.6													
Met -						0.55							

Table 4 Free amino acid concentration in koi carp dorsal muscles three hours after feeding. Different letters indicate statistical difference within columns at $P<0.05$.

mM/kg	Control	CG	FAA 50	Met 03	Met 06	MetMet 03	MetMet 06	CycMet 03	CycMet 06	Met-
Ala	3.63±0.16^{be}	3.10±0.35^{cde}	6.02±0.26^{a}	2.53±0.12^{de}	2.73±0.3^{e}	2.93±0.56^{cde}	3.10±0.39^{cde}	3.82±0.23^{bc}	4.32±0.36b	3.84±0.20^{bc}
Asn	0.16±0.02abc	0.07±0.02bd	0.00±0.00d	0.09±0.05b	0.00±0.0	0.00±0.00	0.00±0.00	0.23±0.02	0.00±0.00	0.12±0.07
Asp	0.49±0.04^{a}	2.36±0.36^{c}	1.23±0.07^{b}	0.65±0.04^{a}	0.76±0.1^{ad}	0.79±0.12^{ae}	0.87±0.09^{ab}	1.04±0.04^{bde}	0.96±0.09^{bde}	1.16±0.10^{be}
Gln	2.60±0.20^{a}	2.66±0.19^{a}	3.27±0.23^{a}	4.24±0.39^{b}	4.07±0.2^{b}	5.69±0.37^{cd}	5.01±0.31d	6.42±0.30^{c}	6.21±0.21^{c}	5.70±0.14^{cd}
Glu	2.17±0.17^{ac}	1.90±0.10^{c}	1.77±0.06^{ac}	2.41±0.52ac	2.48±0.1^{ac}	3.16±0.53^{b}	2.65±0.22^{c}	2.76±0.05^{b}	2.92±0.27^{b}	2.72±0.16^{b}
Gly	3.15±0.22^{a}	3.92±0.14^{a}	7.08±0.69^{b}	11.76±0.96^{c}	8.69±0.8^{bd}	11.10±0.17^{cd}	11.93±2.37^{c}	13.45±0.97^{c}	13.39±1.47^{c}	13.03±0.42^{c}
His	15.18±0.20^{a}	13.91±1.09^{a}	15.62±1.60^{a}	22.34±1.85^{bc}	22.07±0.3^{c}	27.11±0.79^{b}	24.44±3.26^{bc}	23.51±1.71^{bc}	22.38±1.60^{bc}	23.30±2.14^{bc}
H-pro	0.65±0.06^{a}	2.36±0.35^{b}	2.00±0.28^{bc}	1.88±0.16^{bc}	1.70±0.1^{bc}	1.50±0.08^{c}	2.09±0.33^{bc}	1.49±0.24c	1.96±0.24^{bc}	2.04±0.43^{bc}
Ile	0.45±0.07^{abc}	0.36±0.04^{bc}	0.28±0.05^{c}	0.56±0.04^{ad}	0.57±0.0^{ad}	0.63±0.04^{d}	0.50±0.07^{abd}	0.67±0.05^{d}	0.61±0.08^{ad}	0.57±0.09^{ad}
Leu	0.75±0.05^{b}	0.73±0.09^{b}	0.43±0.08^{a}	0.82±0.04^{b}	0.75±0.1^{b}	0.94±0.08^{b}	0.79±0.07^{b}	0.91±0.14^{b}	0.90±0.05^{b}	0.78±0.06^{b}
Orn	0.44±0.03^{ac}	0.52±0.06^{ab}	0.30±0.02^{bcd}	0.37±0.09^{abcd}	0.53±0.0^{a}	0.34±0.13^{abcd}	0.44±0.05^{ad}	0.22±0.03^{bd}	0.18±0.00^{bd}	0.29±0.06^{bcd}
Phe/h-cys	0.15±0.02	0.22±0.03	0.15±0.02	0.24±0.05	0.26±0.0	0.26±0.05	0.22±0.05	0.23±0.04	0.24±0.05	0.24±0.04
Pro	1.58±0.15^{a}	21.53±0.59^{b}	25.34±1.09^{c}	4.44±0.25^{de}	3.44±0.2^{e}	3.54±0.54^{e}	4.85±0.83^{de}	4.44±0.57^{de}	6.06±0.91^{d}	4.67±0.40^{de}
Ser	1.37±0.09^{ab}	1.03±0.29^{b}	7.41±1.05^{c}	2.63±0.17^{ad}	2.92±0.6^{d}	3.46±0.42^{de}	4.71±0.43^{e}	3.62±0.22^{de}	4.20±0.43^{e}	3.57±0.30^{de}
Trp	0.08±0.02^{b}	0.07±0.01^{b}	0.02±0.00^{a}	0.08±0.02^{b}	0.06±0.0^{ab}	0.08±0.01^{b}	0.06±0.01^{ab}	0.07±0.01^{b}	0.07±0.01^{b}	0.09±0.01^{b}
Tyr	0.34±0.07	0.55±0.01	0.37±0.03	0.60±0.03	0.66±0.0	0.77±0.07	0.72±0.03	0.79±0.04	0.79±0.05	0.75±0.05
Val	0.69±0.07	0.66±0.05	0.51±0.16	0.80±0.05	0.75±0.0	0.91±0.07	0.83±0.05	1.03±0.09	0.92±0.07	0.82±0.06

Table 5 Free amino acid concentration of koi carp muscle 48 hours after the last feeding.

mM/kg	CG	Control	FAA50	Met03	Met-	Met06	MetMet03	MetMet06	CycMet03	CycMet06
Asp	0.59	0.50	0.56	0.32	0.50	0.47	0.63	0.33	0.54	0.61
Glu	1.66	1.32	1.35	1.15	1.51	1.02	1.10	1.00	1.35	1.53
H-pro	1.82	0.83	1.43	0.98	1.01	0.80	0.93	0.72	0.60	1.01
Ser	0.30	0.48	0.53	0.64	0.80	0.49	0.67	0.51	0.78	0.73
Asn	0.31	0.36	0.40	0.62	0.57	0.56	0.59	0.49	0.58	0.48
Gly	3.89	3.03	4.75	9.25	8.79	6.68	9.36	7.58	9.27	8.05
Gln	2.09	2.45	2.70	2.79	3.62	2.48	3.36	2.77	3.72	3.97
His	11.29	12.01	10.94	17.97	18.57	16.47	19.44	17.96	18.25	15.48
Ala	3.19	2.34	3.11	3.43	4.60	3.00	3.62	2.93	3.77	4.40
Pro	4.07	2.73	6.77	0.23	0.23	0.20	0.22	0.18	0.24	0.23
Tyr	0.11	0.17	0.04	0.08	0.26	0.26	0.33	0.32	0.34	0.22
Val	0.16	0.21	0.16	0.21	0.22	0.18	0.19	0.18	0.21	0.19
Ile	0.08	0.10	0.10	0.11	0.11	0.10	0.10	0.09	0.11	0.10
Leu	0.18	0.21	0.20	0.20	0.21	0.19	0.18	0.17	0.20	0.20
Phe/h-cys	0.02	0.02	0.03	0.03	0.03	0.04	0.01	0.03	0.04	0.04
Trp	0.05	0.04	0.04	0.04	0.04	0.04	0.05	0.04	0.03	0.06
Orn	0.08	0.04	0.04	0.03	0.04	0.04	0.04	0.03	0.03	0.03

Table 6 The whole body amino acid composition of experimental samples (n=3). Different letters indicate statistical difference at P<0.05.

Description	Initial sample	Commercial	CG	FAA 50	Met 03	Met 06	MetMet 03	MetMet 06	CycMet 03	CycMet 06	Met-
Crude Protein	67.50	58.12±2.22	56.15±1.85	56.84±0.32	59.92±1.64	58.0±0.33	57.80±1.25	58.82±0.58	55.95±1.29	57.25±2.42	56.55±0.91
Ala	3.91	3.38±0.15	3.26±0.16	3.25±0.05	3.39±0.06	3.28±0.04	3.28±0.10	3.34±0.03	3.22±0.02	3.22±0.14	3.24±0.04
Arg	4.03	3.45±0.16^{ab}	3.30±0.16^{a}	3.31±0.03^{a}	3.63±0.09^{b}	3.49±0.02^{ab}	3.51±0.07^{ab}	3.50±0.10^{ab}	3.42±0.03^{ab}	3.39±0.16^{ab}	3.41±0.05^{ab}
Asp	5.93	5.04±0.18^{abc}	4.79±0.12^{ac}	4.88±0.08^{abc}	5.15±0.09^{b}	5.02±0.03^{abc}	4.98±0.10^{abc}	5.07±0.06^{a}	4.83±0.08^{ac}	4.91±0.14^{abc}	4.79±0.08^{bc}
Cys	0.63	0.63±0.02	0.45±0.07	0.50±0.01	0.53±0.02	0.53±0.02	0.54±0.01	0.54±0.02	0.53±0.05	0.52±0.03	0.50±0.02
Glu	8.53	7.19±0.33^{ab}	6.83±0.11^{a}	7.00±0.10^{ab}	7.45±0.19b	7.27±0.05^{ab}	7.20±0.15^{ab}	7.32±0.06^{b}	7.01±0.09^{ab}	7.12±0.26^{ab}	7.03±0.08^{ab}
Gly	4.68	4.17±0.19	4.18±0.44	4.09±0.04	4.28±0.12	4.04±0.06	4.13±0.15	4.17±0.13	4.17±0.07	4.04±0.29	4.16±0.11
His	1.52	1.42±0.06	1.32±0.20	1.45±0.03	1.62±0.09	1.56±0.03	1.55±0.02	1.19±0.55	1.53±0.10	1.55±0.09	1.48±0.08
Ile	2.55	2.15±0.11	2.02±0.04	2.08±0.04	2.18±0.05	2.15±0.01	2.13±0.07	2.14±0.04	2.07±0.04	2.10±0.09	2.03±0.03
Leu	4.58	3.84±0.17^{ab}	3.63±0.04^{a}	3.74±0.05^{ab}	3.92±0.10^{b}	3.84±0.03^{ab}	3.78±0.09^{ab}	3.84±0.06^{ab}	3.70±0.09^{a}	3.74±0.13^{ab}	3.65±0.05^{a}
Lys	5.00	4.26±0.05^{ab}	3.97±0.24^{a}	4.16±0.04^{ab}	4.34±0.15^{b}	4.23±0.07^{ab}	4.24±0.13^{ab}	4.21±0.08^{ab}	4.09±0.10^{ab}	4.18±0.15^{ab}	4.14±0.10^{ab}
Met	1.74	1.43±0.09	1.32±0.06	1.39±0.03	1.42±0.10	1.42±0.03	1.41±0.04	1.40±0.01	1.37±0.04	1.43±0.06	1.36±0.04
Phe	2.55	2.15±0.08^{ab}	2.04±0.03^{a}	2.10±0.03^{ab}	2.21±0.05^{b}	2.16±0.02^{ab}	2.11±0.05^{ab}	2.14±0.03^{ab}	2.06±0.05^{a}	2.10±0.07^{ab}	2.05±0.04^{a}
Pro	3.12	2.99±0.19^{a}	3.06±0.38^{ab}	3.49±0.07^{b}	2.89±0.09^{a}	2.82±0.04^{a}	2.82±0.08^{a}	2.78±0.11a	2.82±0.05^{a}	2.79±0.16^{a}	2.84±0.08^{a}
Ser	2.64	2.19±0.06^{ab}	2.11±0.07^{a}	2.22±0.05^{ab}	2.31±0.07^{b}	2.23±0.03^{b}	2.19±0.06^{ab}	2.25±0.01^{ab}	2.17±0.05^{ab}	2.18±0.10^{ab}	2.14±0.02^{a}
Thr	2.57	2.19±0.05	2.10±0.05	2.13±0.04	2.23±0.08	2.19±0.03	2.17±0.04	2.20±0.03	2.13±0.06	2.13±0.08	2.10±0.03
Trp	0.64	0.52±0.03	0.48±0.01	0.51±0.01	0.52±0.01	0.51±0.02	0.51±0.02	0.52±0.02	0.49±0.02	0.49±0.02	0.49±0.01
Val	2.91	2.47±0.13	2.33±0.04	2.40±0.03	2.50±0.06	2.46±0.01	2.45±0.09	2.45±0.05	2.39±0.05	2.41±0.10	2.34±0.03

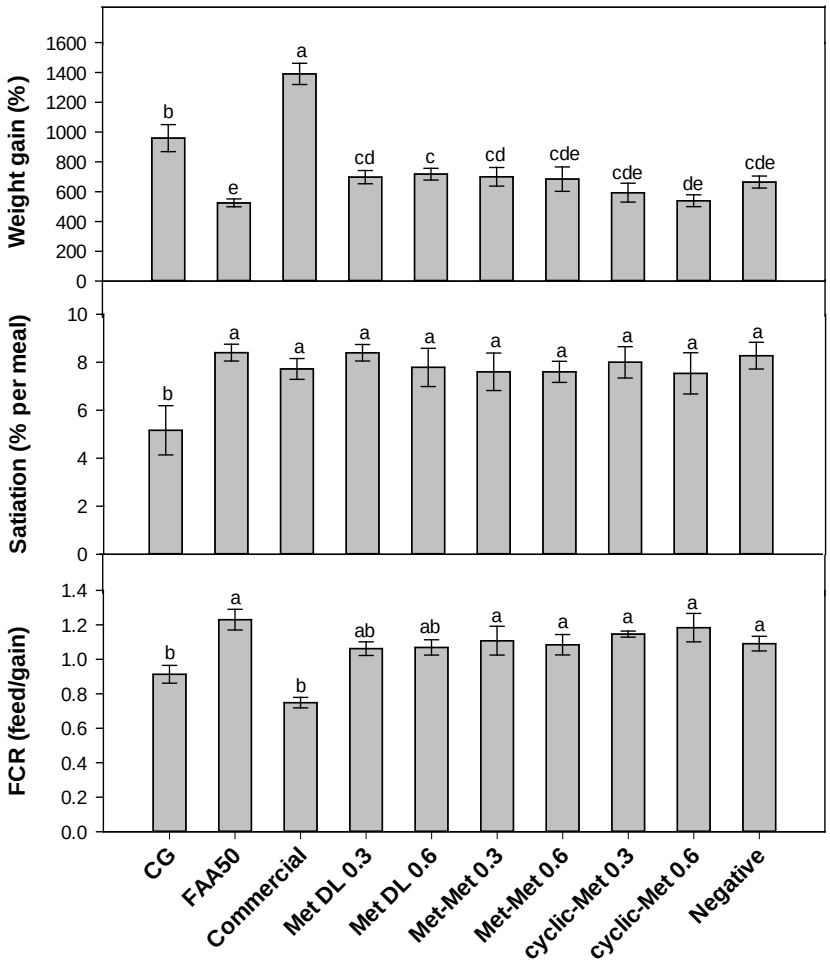

Figure 1 Weight gain, satiation, and FCR of koi carp. Different letters indicate statistical differences at $p<0.05$.

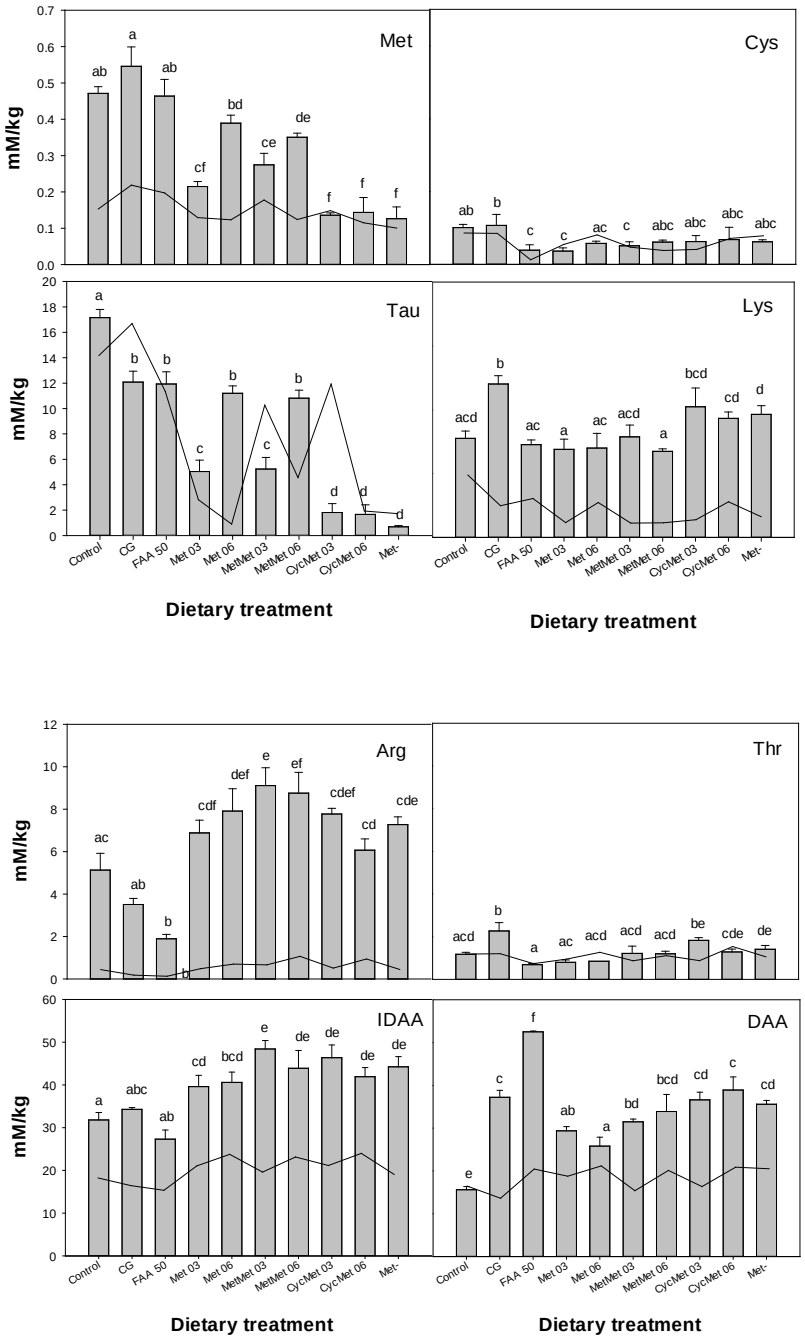

Figure 2 The concentration of free amino acids methionine, cysteine, arginine, threonine (a) and taurine, lysine, total indispensable amino acids and dispensable amino acids (b) of koi carp muscle three hours after feeding. The baseline indicates FAA levels in fish starved for 48 hours.

CHAPTER 3: NUTRITIOUS WASTE – HOW AND WHY ARE DIETARY FREE AMINO ACIDS EXCRETED IN FISH?

Summary

Synthetic free amino acids may be utilized by different fish species to various degree, and it is commonly accepted that diets supplemented with FAA are less suitable for fish growth, compared to these based on intact protein. The difference may be related to the use of FAA as energy source or excreted from fish body via gills or kidneys.
In this experiment we used three diets: casein gelatin based diet (CG), casein gelatin based diets with 50% inclusion of FAA and commercial diet. Before the FAA utilization test fish were fed with ⅓ of daily ration in one meal. Therefore, the present study was undertaken to gain better understanding and quantify AA loss during and post-ingestion of formulated diet containing significant proportion of FAA.

The level of Met released by fish fed FAA50 diet was the highest compared to commercial and CG groups after 15 min, 2 and 4 hours. Similar trend was presented in other AAs such as: Leu, Ser, Pro, Tau, and Gln. In contrast, Tau and Gln were released in significantly higher amounts by fish fed commercial diet (Otohime) compared to CG and FAA50 groups. The Lys level in the FAA50 group was comparable to other treatments

15 min after feeding, but excretion of this AA increased and its concentration in water was the highest after 2 and 4 hours.

Introduction

The major role of dietary protein is to supply amino acids for body protein accretion. This process may be optimal only when all indispensable amino acids are present at the protein synthesis sites above threshold concentrations (1-10 μM; Walton and Cowey 1982). Therefore, required profile of IDAA in dietary protein is critical to its effective utilization (Murai et al., 1984a). Amino acids in formulated diets can be provided in a few forms such as protein-bound, di- , tri-peptides and free amino acids (FAA) that differ in respect to absorption mechanisms (Dabrowski et al., 2010). Synthetic free amino acids may be utilized by different fish species to various degree (Yamada et al. 1982). However, many nutritional studies demonstrated that diets supplemented with FAA are less suitable for fish growth, compared to these based on intact protein (Aoe et al., 1970, Dupree and Halver, 1970, Nose et al., 1974, Mazid et al., 1978, Peres and Oliva-Teles, 2005, Zhang et al., 2006, Kwasek et al., 2010). The faster rate of intestinal absorption and different FAA profile in blood plasma observed in fish fed with diets supplemented with FAA compared to intact protein based diets was described in common carp (*Cyprinus carpio*) (Plakas et al., 1980), Nile tilapia (*Tilapia nilotica*) (Yamada et al., 1982), and white sturgeon (*Acipenser transmontanus*) (Ng et al., 1996). This fact is considered as a major reason for poor utilization of FAA by warm-water fish and leads to various speculations about the fate of excessive AA in fish blood plasma. According to Murai et al. (1984b) free amino acids not used for protein synthesis may be excreted from

fish body via gills or kidneys, or catabolized (Kaushik and Dabrowski, 1983). Yamada and Yone (1986) observed that some FAA as water soluble components, can leach out and be lost during mastication of ingested diet. Above mentioned reports, however, do not fully clarify the issue. Therefore, the present study was undertaken to gain better understanding and quantify AA loss during and post-ingestion of formulated diet containing significant proportion of FAA. These results may contribute to further development of experimental purified diets commonly used in dose-response fish nutritional requirements tests.

Materials and Methods

For details related to diet formulation, fish maintenance and feeding experiment performance the reader is referred to Chapter 2.

Prior to the experiment fish were maintained in an open system with a dechlorinated city water and fed with commercial diet (Finfish G, Zeigler Bros. Inc, Gardners, Pa). After moving fish to the experimental setup they were adapted to the new conditions for 5 days and fed with 5%/day body weight with experimental diets (commercial, CG, and FAA50). On the 6th day fish were starved for 24 hours. After this period all fish were moved to clean tanks, fed with 1.67% body weight (⅓ of daily ration in one meal). Each diet was tested in triplicate aquaria. Immediately after feeding, the incoming water was stopped. Water samples (120 ml) were taken from each 30 L tank at 15 min, 2 and 4 hours after the feeding for the evaluation of the released free amino acid concentrations. In addition, one more sample after 4 hours was taken from each tank to sealed container and kept for the next 4 hours in the same room temperature in order to

evaluate the rate of amino acid degradation in water. Water samples were placed in a biofreezer (-80°C) and eventually freeze-dried immediately after collecting. All procedures and handling of animals was conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University.

Statistical analysis

The experimental design was completely randomized for fish distribution and experimental diets assignment. The differences among dietary treatments were tested by one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$.

Free amino acid analysis

After sampling water samples were sealed in containers, and immediately placed on the shelf of a freezer in -80 ºC and left there until frozen. They were moved subsequently to the freeze-drier and dried. Freeze-dried water samples were dissolved in 0.1 mol/l HCl in 1:75 (w/v) containing 160 µmol/l norleucine internal standard according to Cohen et al. (1989). Samples were then spun at 12 000 g (4 ºC, 15 min) and supernatants were filtered (Milipore, 10 kDa cutoff at 2000 g, 4 ºC, 90 min). The residue from each container was then dissolved in 2 ml of 0.1 mol/l HCl in 1:75 (w/v) containing 160 µmol/l norleucine and then spun at 12 000 g (4 ºC, 15 min) and supernatants were filtered (Milipore, 10 kDa cutoff at 2000 g, 4 ºC, 90 min). Blanks (0.1 m HCl + 160 µmol/l nLeu) (Terjesen et al., 2004) and external standards (Sigma acid/neutral and basic amino acids) were prepared along with the sample preparation. Samples, blanks, and external standards were stored at -80^{o}C until the same concentration of glutamine in 0.1

M HCl as external standard was prepared on the day of analysis and added to the basic amino acids standard. Amino acids were pre-column derivatised with phenylisothiocyanate (Cohen et al., 1989). Sample precipitates were removed by a 10-min centrifugation at 10 000 g (Terjesen et al., 2004). Free amino acids were quantified using a Waters Pico Tag RP-HPLC (Waters Corporation, Milford, MA, USA) equipped with an application-specific column (3.9 • 30 cm), a Waters 717 autosampler, 2 Waters 501 pumps, a Waters 441 absorbance detector at 254 nm and a column heater set at 46^{o}C. Eluent 1 (composition of acetonitrile, triethylammonium-acetate, sodium acetate and water) and eluent 2 (composition of acetonitrile, methanol and water) purchased from Waters were used throughout the investigation. Each amino acid was identified by spiking with known amino acid and validated by retention times of external standards. Free amino acid concentrations (expressed as mg/g diet) were calculated using internal and external standards (norleucine) (Cohen et al., 1989).

Results

Figure 3 presents the data how the diets affected the growth of fish in each treatment group following 35 days of feeding (see Chapter 2 for details). These results show that fish weight gains significantly differed among commercial, casein-gelatin, and FAA50 diet fed groups.

The level of Met released by fish fed FAA50 diet was the highest compared to commercial and CG groups after 15 min, 2 and 4 hours. The amino acids such as: Leu, Ser, Pro, Tau, and Gln levels showed similar trends. However, Tau and Gln were released in significantly higher amounts by fish fed commercial diet (Otohime) compared to CG

and FAA50 groups. The level of released Arg was the lowest in the CG group compared to commercial and FAA50 groups. However, Arg released by FAA50 group was 5 fold higher than CG and commercial and it remained high throughout the 4 h period. The Lys level in the FAA50 group was not different after 15 min compared to COM and CG groups, but it increased after 2 hours and it was higher compared to the CG group and the highest after 4 hours compared to both CG and COM groups (Figure 4). The levels of all other free amino acids released into water after 15 min, 2 and 4 hours are listed in Table 7.

Figure 5 presents carp requirement for IDAA and the level of IDAA excretion into the fish surrounding water. No differences were found between Ile, Thr, and Trp levels in water among different treatment groups after 4 hours. The level of the released Leu was the highest in the FAA50 group compared to CG and commercial groups. Similarly, the concentrations of the released Lys, Met, Phe, Val, Arg, and His were the highest in the FAA50 group compared to CG and commercial groups. The Tyr level, however, was the highest in the CG group compared to both FAA50 and commercial diet fed fish.

Discussion

Nolles et al. (2009) stated that human or rats have a capacity to adapt their gastrointestinal tract to the presence of FAA in the diet. The adjustment is notable in a reduced oxidative loss after 3 weeks of adaptation period to high concentration of FAA in the consumed food. Based on our observation, however, we have not noticed any significant improvement of weight gain or feed efficiency ratio in fish fed with FAA50

during 35 days long feeding trial (Chapter 2; Figure 1). After 10 days of feeding, the FAA50 treatment resulted in significantly lower weight gain compared to CG and commercial diet, and this trend continued until the end of the experiment (Figure 1).

In terrestrial animal nutrition, it is generally assumed that all nutrients from a diet enter the digestive tract after food ingestion. In fish, however, it is possible that part of water soluble nutrients may be lost by diffusion into the aqueous medium. The nutrients wastage may be significant in fish such as cyprinids which possess pharyngeal teeth and masticate food. Yamada and Yone (1986) addressed this issue in their experiment with juvenile carp fed casein or FAA based diets with corresponding AA profile. Weight gain obtained for fish was 80 and 11% for casein and FAA based diets, respectively. According to these authors such disparity may be explained by water soluble components loss into the surrounding environment during mastication in pharyngeal apparatus. Yamada and Yone (1986) reported the decline in concentration of AA in consumed food (15 minutes after meal) reached 70% for most of the AA, with exception for cysteine and tyrosine which amounted to 30 %. The highest losses in our study were observed in respect to proline (80±8%), serine (54±9%), and methionine (53±6%). The lowest values were observed for phenylalanine (7±6%) and lysine (20±9%). The average loss of CAA to the water during first 15 minutes after food administration was 40%. The values, except for phenylalanine, were significantly higher for FAA50 treatment compared to the two others. The differences between our results and those of Yamada and Yone (1986) may be related to Murai et al. (1984b) findings that even small supplementation of casein (7.65%) with FAA reduces FAA loss up to four times. Moreover, Yamada and Yone

(1986) did not include information about food particles size. Therefore, it can be speculated that improperly large diet particles could cause more intense mastication and consequently greater losses of dietary FAA. In addition, the specific dietary binder used may have a significant effect on feed efficiency and growth performance of fish. According to Yamada and Yone (1986), agar was superior to carboxymethylcellulose (CMC) and caused over three times higher weight gain and improved feed efficiency. Furthermore, frequent feeding with FAA based diet may give significantly better weight gain compared to low frequency of feeding with the same quantity of a diet. According to Yamada et al. (1981) an increasing number of meals significantly improves growth performance and utilization of FAA based diets. These results may suggest that hampered growth of carp based on FAA diets is not exclusively caused by the nutrients loss during mastication and may be related to different absorption rate and pattern of FAA in blood plasma in fish fed FAA and intact protein based feeds. We may speculate that fish growth supported by frequent feeding with FAA based diets helps maintaining appropriate level of all IDAA in the protein synthesis sites. Murai et al. (1984b) observed that FAA may be excreted through gills and kidneys by juvenile carp. They noticed that the amount of the total nitrogenous substances excreted by fish during 24 hours post- feeding period was 36% and less than 1% for CAA and CG diets, respectively. They also concluded that poor utilization of FAA by carp resulted from excretion rather than catabolism of absorbed amino acids, what is consisted with our findings.

Different approach to explain weaker growth performance of white sturgeon (Acipenser transmontanus) on FAA based diet was proposed by Ng et al. (1996). They

measured the dynamic changes of FAA in blood plasma and urine after force feeding with FAA and intact protein based diets. Total IDAA in fish blood plasma reached maximum concentration 2 and 8 hours post- feeding, and then returned to initial values after 6 and 12 hours for FAA and casein-wheat gluten based diets, respectively. Moreover, DAA concentration in white sturgeon generated rapid increase 2 hours after feeding with FAA based diet and returned to initial level after 4 hours. Control treatment did not show significant changes in blood plasma FAA in post-feeding levels. Similarly, IDAA concentrations in urea 8 hours after feeding was five times higher than in control treatment in which FAA excretion was observed almost at the constant rate. Ammonia and urea excreted in urine were detected in higher concentration in control group. Nevertheless, the total loss of IDAA in urine was estimated to be less than 1%, making the result insignificant. Unfortunately the authors did not measure the loss of FAA through gills which together with changes in blood plasma and urine would provide us with more insight picture. Water solubility of FAA may have major influence on FAA losses from the diet as well as from fish body. In our experiment we observed the highest AA loss from FAA50 treatment for Met (229±36%), Leu (260±106%), Val (118±15%), and Trp (140±30%). Unfortunately, these values do not correspond with the hydrophobicity scale for amino acids residues (Eisenberg et al., 1982). All FAA lost in the highest quantities are reported as the least hydrophilic amino acid residues. Therefore, some other physiological mechanisms regulate excretion of excess of amino acids in the blood circulation of stomachless carp.

The high losses of IDAA observed in fish fed FAA50 diet were compared to requirement levels for these IDAA (Figure 5). This way of expressing the loss of IDAA may contribute to understanding of process governing utilization of FAA at major protein synthesis sites (liver; Cowey and Lugnet 1983). For instance, it may be explained by the fast absorption of FAA from intestine resulting in spike of FAA levels in the circulation and liver (hepatopancreas in the case of carp). Although, FAA appear quickly in high quantities in blood plasma and disappear equally fast, they compose different AA profile dependent of the diet (Plakas et al., 1980). The elevated losses of IDAA observed in our experiment could be related to high concentration of protein in CG and FAA50 diets (50% in dry matter) compared to those used by Nose et al. (1974) (37%). The different, unbalanced FAA composition may result in reduced protein synthesis (Todd et al., 1967) and even further destabilization causing even higher losses of supplemented, protein-bound and body AA.

Table 7 The concentration of free amino acids in water 15 min (15M), 2 hours (2H), and 4 hours (4H). The % of free amino acids in water expressed as proportion of free amino acid supplementation in the diet was calculated only for the FAA50 4H.

mg/g diet	Concentration of FAA released in water									FAA excreted (% added to diet)
	COM 15M	CG 15M	FAA50 15M	COM 2H	CG 2H	FAA50 2H	COM 4H	CG 4H	FAA50 4H	FAA50 4H
Ala	0.95±0.39	0.24±0.05	30.28±10.41	1.57±0.64	0.91±0.42	37.09±17.88	1.58±0.29	1.92±1.42	19.66±31.08	33.1±52.3
Asn	1.73±0.48[a]	0.31±0.14[b]	0.00	3.05±0.92[a]	0.71±0.13[b]	0.00±0.24[b]	3.12±0.04[a]	1.10±0.29[a]	0.95±0.87[b]	0.00
Asp	0.15±0.13	0.00	0.00	0.17±0.16	0.08±0.01	0.10±0.06	0.17±0.14	0.24±0.04	0.18±0.04	0.00
Gln	1.20±0.26	0.00	0.00	1.05±0.65a	0.00±0.13	0.10±0.25b	1.18±0.77	0.00	0.43±0.72	0.00
Glu	0.76±0.24[a]	0.23±0.01[b]	0.19±0.07[b]	0.96±0.29[a]	0.65±0.03[ab]	0.33±0.07[b]	0.81±0.05	0.87±0.15	0.50±0.15	0.00
His	0.90±0.63	0.00	1.19±0.46	1.34±0.25[a]	0.23±0.03[b]	3.08±0.49[c]	1.32±0.49[a]	0.68±0.09[a]	3.21±0.73[b]	85.6±19.5
Ile	0.17±0.01	0.04±0.04	1.66±0.42	0.90±0.68	0.82±0.53	5.01±2.67	0.91±0.77	1.41±0.32	2.65±1.64	54.9±34.0
Orn	0.28±0.29	0.03±0.05	0.22±0.38	0.40±0.23	0.06±0.06	0.43±0.24	0.46±0.31	0.06±0.01	0.52±0.37	0.00
Phe/h-cys	0.13±0.06	0.03±0.03	0.63±0.58	0.33±0.15	0.40±0.06	2.01±0.89	0.38±0.08	0.49±0.04	1.72±0.14	17.8±1.4
Thr	0.52±0.07[a]	0.37±0.23[a]	1.60±0.39[b]	1.08±0.65	1.51±1.27	1.80±0.38	0.92±1.03	4.59±4.42	1.65±0.81	38.5±19.0
Trp	0.00	0.00	0.00	0.02±0.04	1.09±1.66	1.29±0.50	0.07±0.11	3.28±4.97	1.50±0.32	140.4±30.1
Tyr	1.90±1.72	0.05±0.08	0.25±0.25	1.60±0.61	3.11±0.80	2.49±0.41	1.22±0.65[a]	5.64±1.51[b]	1.95±0.42[a]	0.00
Val	0.19±0.08[a]	0.03±0.06[a]	2.60±0.53[b]	0.56±0.29[a]	0.36±0.13[a]	8.39±2.02[b]	0.57±0.13[a]	0.84±0.17[a]	7.57±0.98[b]	117.8±15.3

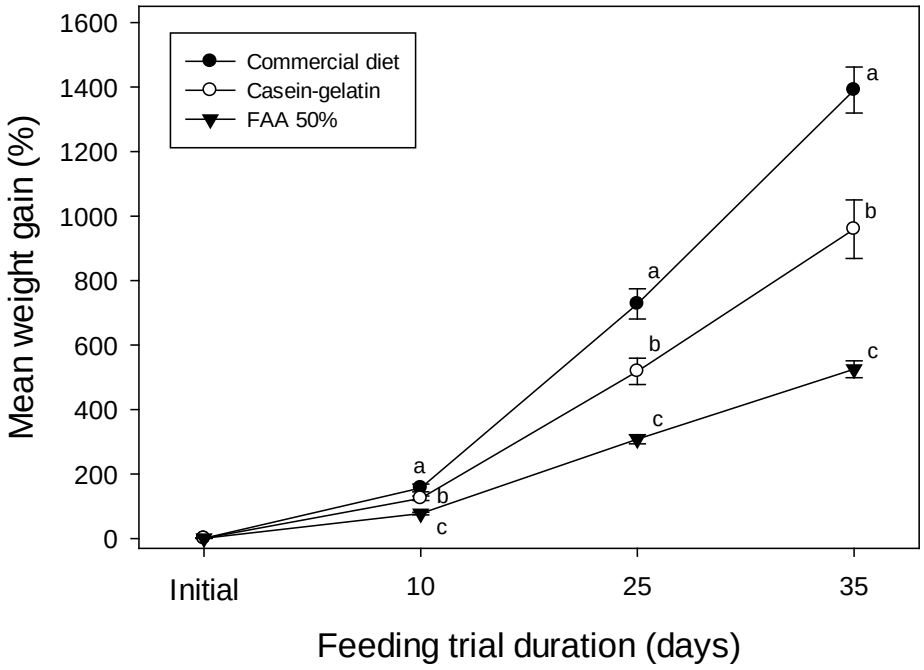

Figure 3 The growth curve expressed as mean weights of fish fed commercial, casein-gelatin, and FAA50 diets after 10, 25, and 35 days (see Chapter 1 for details).

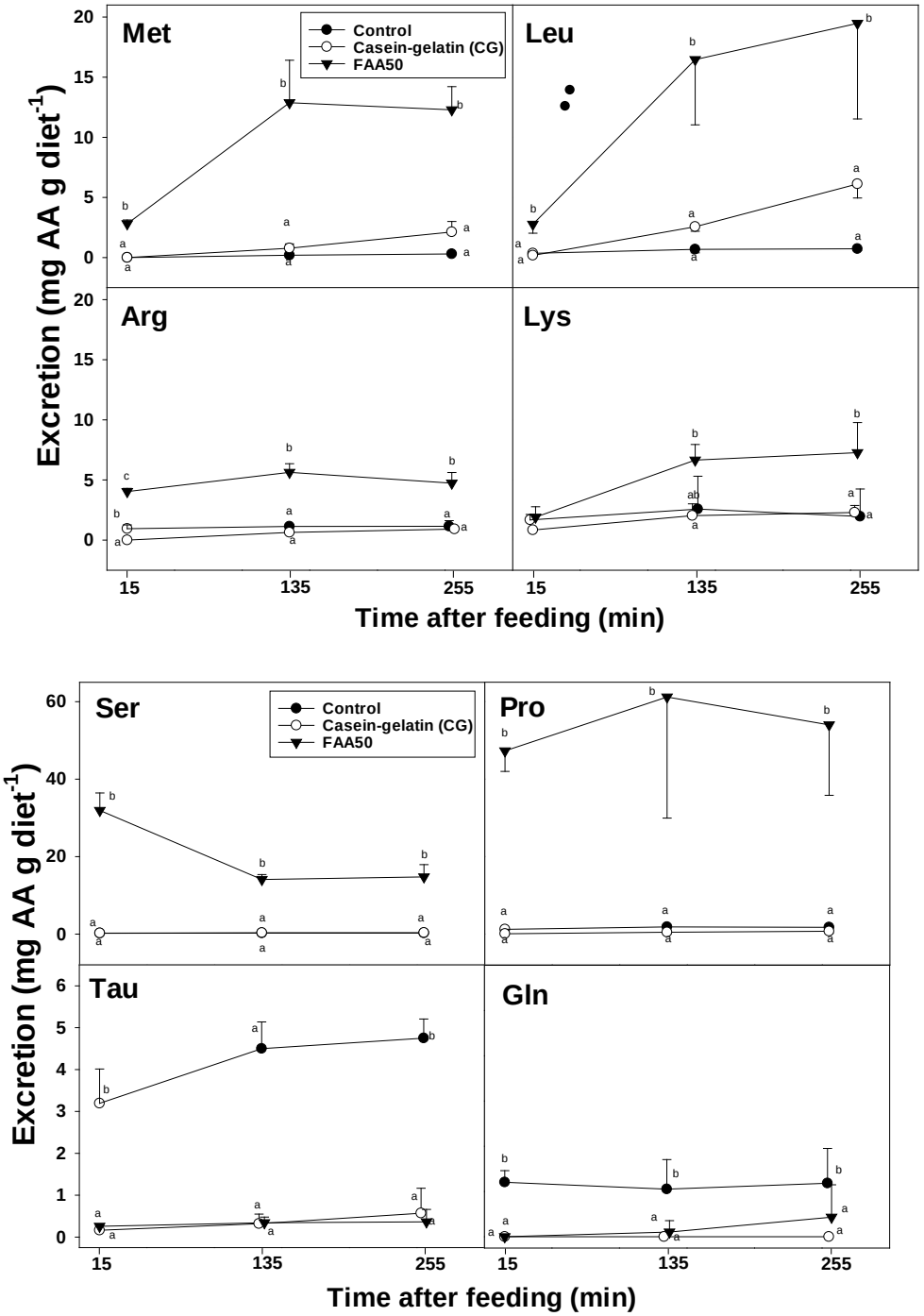

Figure 4 The concentration of free amino acids (expressed as excretion rate) in water after 15 min (15M), 2 hours (2H), and 4 hours (4H) in groups of carp receiving commercial (COM), casein-gelatin (CG) or 50% free amino acid-based (FAA50) diets.

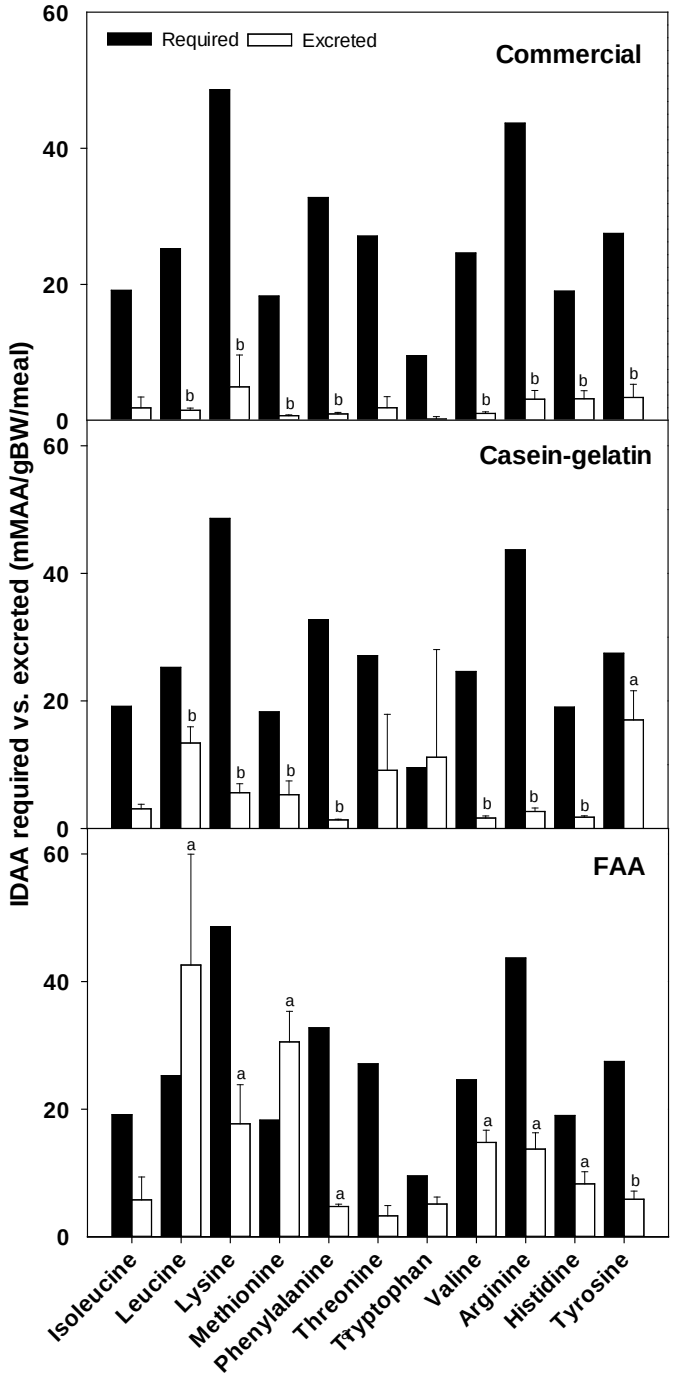

Figure 5 The indispensable amino acid ratio between required amino acid level and the level of excreted amino acids found in water. Different letters indicate statistical differences at $P<0.05$ between dietary treatments.

CHAPTER 4: EXPLORING THE MECHANISM OF PHYSIOLOGICAL CHANGES DUE TO REPLACEMENT OF PROTEIN WITH FREE AMINO ACIDS IN PURIFIED DIETS FOR COMMON CARP (CYPRINUS CARPIO)

Summary

Amino acids nutritional requirements are established in studies with semi-purified diets which consist of well-defined chemical composition. In such diets, proteins are replaced partly or totally with FAA mixture, giving maximal control over the nutrient under investigation. However, the ability to utilize dietary FAA for growth varies among the fish species. Therefore, one of the objectives of this study was to test how common carp will respond to casein-gelatin based diets with partial replacement of intact protein with FAA.

In this experiment we used a reference diet based on casein and gelatin (CG) and five diets with graded level of replacement 10% (FAA10), 20% (FAA20), 30% (FAA30), 40% (FAA40), and 50% (FAA50) of dietary protein with a mixture of FAA with identical AA profile.

Our experiment showed that the highest weight gains were obtained in fish fed the FAA10 diet compared to all other treatment groups. The lowest weight gain had fish that received the FAA40 diet but it was not different from FAA30, FAA50. The FCR was the

lowest in the FAA10 group compared to all other treatments except CG and FAA20 groups.

Introduction

Nutrient requirements are estimated in studies with semi-purified diets which consist of well defined chemical composition and provide maximal control over the nutrient under investigation (NRC, 2011). Halver et al. (1957) developed the first satisfactory free amino acid test diet for estimating essentiality of individual amino acids for chinook salmon (Oncorhynchus tshawytscha). His diet was designed based on amino acids (AA) profile of whole chicken egg protein and contained 70% of crystalline L-AA. This approach was subsequently summarized and improved by others (Mertz, 1972) and involved diets based on a mixture of casein, gelatin and additional free amino acids (FAA) containing graded levels of one amino acid at a time with identical concentration of other AA. A number of nutritional studies, however, demonstrated that diets supplemented with FAA are less suitable for fish growth, compared to the diet based on intact protein (Aoe et al., 1970, Dupree and Halver, 1970, Nose et al., 1974, Mazid et al., 1978, Cowey, 1995, Peres and Oliva-Teles, 2005, Zhang et al., 2006, Kwasek et al., 2010). The major reason for poor utilization of FAA by fish may be a higher rate of intestinal absorption compared to AA from intact protein, resulting in different FAA profile in blood plasma and leads to various speculations about the fate of excessive AA in fish blood plasma (Plakas et al., 1980, Yamada et al., 1982, Ng et al, 1996). Cowey and Walton (1988) showed that incorporation of amino acids labeled at carbon (14C) into tissue proteins of rainbow trout (*Oncorhynchus mykiss*) was greater when provided as

intact protein, whereas incorporation into lipids and glycogen was greater for FAA source. The efficiency of FAA utilization in common carp (*Cyprinus carpio*) may be improved by using a binder such as agar (Yamada and Yone, 1986), elevation of dietary pH to neutral level (Murai et al., 1983) or by increasing number of meals per day (Yamada et al., 1981a). Experimental diets which result in reduced growth performance of fish, may lead to overestimating indispensable amino acids (IDAA) requirement (Kim et al., 1992, Cowey, 1994). Therefore, before such studies a FAA inclusion should be compared to protein at the level which does not affect growth and feed utilization of fish species under investigation (Marcouli et al., 2004, Pérez-Jiménez et al., 2014). Moreover, a reference diet of known composition and nutritional value should be included in experimental design in order to verify the usefulness of FAA test diet (NRC, 2011). Consequently the objectives of this study were: (i) to test how stomachless fish - koi carp will respond to casein-gelatin based diets with partial replacement of intact protein with FAA, (ii) to estimate the highest level of FAA inclusion, which support growth rate comparable to reference diet, (iii) to evaluate postprandial free amino acids concentration in muscle tissues, as an indicator of their availability, and (iv) to examine if different level of dietary FAA inclusion may affect carp body composition. These results may contribute to further development of semipurified diet suitable for further re-evaluation IDAA requirement of common carp in intensive aquaculture production.

Materials and methods

Diet formulations and feeding strategy

Six isonitrogenous and isolipidic diets were used in this 60 day feeding trial (Table 8). A reference diet based on casein and gelatin (CG) and five diets with partial replacement of dietary protein with a mixture of FAA with identical AA profile. Casein and gelatin were replaced at the graded level of 10% (FAA10), 20% (FAA20), 30% (FAA30), 40% (FAA40), and 50% (FAA50) (Table 9). The diets were prepared according to the earlier experience with rainbow trout alevins (Terjesen et al., 2006) and common carp larvae (Kwasek et al., 2010) with minor modifications. Casein and gelatin (5:1) were used as main protein source and dextrin was replaced with starch in comparison to the original formulation. All the experimental diets were designed to meet the requirements for indispensable amino acids by common carp (NRC, 1993). In addition two "negative" control diets, Control30 and Control50, were included which had the FAA mixture replaced with starch. The pH of all of the diets was adjusted to 7 (± 0.1 unit) with 6 N NaOH during the mixing of dietary ingredients with FAA mixtures (Wilson et al., 1977, Murai et al., 1983). In order to reduce FAA leaching and increase absorption rate from intestine, 4% of inorganic commercial binder (Sipernat® 50S, Evonik, Germany) was added. In addition, all diets were supplemented with 5% of soluble fish protein concentrate (CPSP 90, Sopropeche S.A., Boulogne Sur Mer, France) to improve palatability. Dry ingredients were added together and mixed for extended time prior to addition of oil and water and mixed again. The complete mixture was processed through 2 mm die using the Hobart feed mixer (Troy, Ohio) with meat grinder attachment and

pellets subsequently freeze dried. Dry strands were then ground into appropriate particle size (2.5-3.5 mm). In order to examine carp's ability to adapt to a gradual increase of dietary CAA level and changes in FAA utilization efficiency (Nolles et al., 2009), additional "switch" treatment was included in the experimental design with triplicate groups of fish fed with CG diet for the first 10 days and then switched every 10 days to a diet with higher FAA concentration (10 to 50%).

Fish maintenance and feeding experiment

Common carp broodstock of European descent originated from Auburn University Experimental Station (Auburn, AL; courtesy of Dr. R.A. Dunham), were housed for two generations in the Aquaculture Laboratory at the Ohio State University, Columbus, Ohio, USA. Fish were bred out of the season following standard carp pituitary extract injection and spawned on March 5, 2012. The initial feeding included live brine shrimp naupli (Artemia sp.) and subsequent weaning to the commercial starter diet (A, B1, B2, C1, Otohime, Japan).

The experiment was conducted in a 4,000L semi-recirculation system that consisted of 28 fiberglass conical tanks (50 L), overhead tank with temperature controlling device (26 ± 0.2 ºC), and a biofilter. The freshwater replacement rate was up to 10 L/min for entire system.

Full sibling fish at size 2.2±0.04 g were randomly distributed into the tanks, 40 fish per tank in triplicate for each treatment. Each fiberglass tank was considered as an independent experimental unit with constant water replacement at a rate of minimum 1.5 L min^{-1}. Supplemental aeration was provided to maintain dissolved oxygen level near

saturation. Fish were fed by hand in one hour intervals, 10 times per day (Yamada et al., 1981b) at restricted rates of 5% of the biomass. The assumption was made that daily weight increase equals food consumed. The feeding trial was conducted during 60 days time period. Every 10 days total fish biomass was measured in each tank and feeding rate was re-adjusted. Food particles size was gradually increased during the feeding trial from 0.8-1.0, to 1.0-1.4, to 1.4-2.0 mm. Feeding was stopped 24 h prior to each weighing, tanks were brushed and siphoned on regular basis in order to remove fecal material and algae from the tanks. All procedures and handling of animals were conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University.

Sample collection and analysis

` Following the completion of feeding trial, juvenile common carp were anesthetized and killed with a blow to a head. Fish were sampled 6 and 48 h after feeding in order to obtain high postprandial and basal FAA concentrations, respectively, in dorsal muscles. For details related to method employed to determine muscle free amino acid, diet and whole body proximate and mineral analyses the reader is referred to experiment 1 (Chapter 2).

Statistical analysis

The experimental design was completely randomized for fish distribution and experimental diets assignment. The differences among dietary treatments were tested by one-way ANOVA followed by Tukey's multiple comparison test. Paired t-test with Shapiro-Wilk normality test was used to test differences between "Switch" group and

other treatments growth performance. Differences were considered significant at the value of $p < 0.05$.

Results

Our study showed that the highest weight gains were obtained in fish fed the FAA10 diet compared to all other treatment groups. Fish that received the FAA40 diet had weight gains not different from FAA30, FAA50, and Control 50 (CON2) groups but different (lower) compared to the other treatment groups (Figure 6). The FCR was the lowest in the FAA10 group compared to all other treatments except CG and FAA20 groups (Figure 6). No differences were found when the growth of fish from the SwTrt group was compared with all other treatments (Figure 7). The mortality of the fish was the highest in the CON1, CON2, SwTrt, and FAA10 compared to all other groups (Figure 8). Our study showed that the level of Tau in carp muscle 6 hours after a meal was significantly higher in the CG group compared to FAA20, FAA50, CON2, and SwTrt groups but not different than FAA10, FAA30, and FAA40 groups (Figure 9). The level of Arg was the highest in SwTrt, CON1, FAA40, FAA30, FAA20, and CG groups. The level of Thr was the highest in the CG group compared to all other treatments. Similarly, the level of Lys was the highest in the CG group but not different than FAA20, FAA30, and CON1 groups. The Lys level was the lowest in CON2 compared to all other groups. The Lys level in muscle of fish fed FAA10-FAA50 diets was lower at 24h than 6h after a meal. This was opposite to the response in postprandial level of Met (Figure 9). The level of Met was the highest in the FAA40 group although not different than in FAA20 and CON2 groups. In addition, the concentration of Leu was the lowest in FAA10 and SwTrt

groups compared to all other groups except CG, FAA30, and FAA50. The total level of IDAA was higher in the CG group compared to FAA10 and CON2 but not different than other treatment groups. The total level of DAA was higher in the CG group compared to all other groups except FAA50. Again, the level of DAA 48 h after feeding tended to be higher than after 6 h opposite to IDAA levels. All other free amino acid levels are listed in Tables 10 (6 hours after a meal) and 11 (48 hours after a meal).

We did not find any differences in proximate analysis of carp whole body except lipids which were lower in FAA50 and SwTrt compared to FAA10, FAA30, FAA40, CON1, and CON2 groups (Table 12). The lipid level was the highest in the FAA30 group compared to initial samples, CG, FAA50, and SwTrt groups (Figure 10).

Discussion

Nutritional studies designed to evaluate IDAA requirements in fish employ semipurified diets with graded levels of specific IDAA under investigation. Therefore intact protein must be partly or totally replaced with FAA mixture and AA requirement is established based on commonly accepted dose-response experimental design (NRC, 2011). In fish, however, it has been demonstrated that diets solely or partly based on FAA as nitrogen source do not sustain full potential of growth (Dupree and Halver, 1970, Mazid et al., 1978, Peres and Oliva-Teles, 2005). This inability to utilize FAA mixture diets has been shown particularly significant in agastric fish such as carp, where FAA based diets lead to extremely low growth rates (Aoe et al., 1970; Nose et al., 1974; Zhang et al., 2006; Kwasek et al., 2010). A number of possible explanations for this phenomenon have been suggested. According to Yamada and Yone (1986) FAA from

diet may leach to water before ingestion or during mastication of diet particles in pharyngeal teeth. Moreover, readily soluble FAA in the neutral pH of the intestine may build up high concentrations of FAA in blood plasma with different profiles compared to response when intact protein based diets are fed (Plakas et al., 1980; Yamada et al., 1982; Ng et al., 1996). This may subsequently result in reduced protein synthesis process (Todd et al., 1967). Free amino acids not used in protein synthesis may be catabolized (Kaushik and Dabrowski, 1983, Cowey and Walton, 1988) or excreted from fish body via gills and kidneys (Murai et al., 1984).

Yamada et al. (1981b) observed that carp growth performance could be improved by increasing frequency of feeding with diets exclusively based on FAA with maintaining the same daily feeding rate. These authors reported that 18 meals daily (every 30 minutes) led to 72% of growth rate obtained by fish fed with casein-gelatin-based diet, while 3 meals daily (4 hours intervals) resulted in approximately 20%. Moreover, utilization of FAA based diets by carp may be strongly improved by coating free amino acids with casein (Murai et al., 1980, Murai et al., 1982), using binders such as agar or carboxymethylcellulose (Yamada and Yone 1986), adjusting pH of diet to neutral (Murai et al., 1983), or by diet supplementation with casein below 10% (Murai et al., 1984). Therefore, based on all of these observations, in the design of our experiment we employed frequent feeding protocol - 10 times daily, every one hour. The major intact protein source in this case was casein-gelatin mixture. Furthermore, we neutralized pH of the diets and incorporated commercial binder (Sipernat® 50S, Evonik, Germany). Fish growth performance under experimental conditions fed with 40% FAA inclusion was

significantly inferior to carp given whole-protein based diets and diets with FAA inclusion at the level of 10-20%. In addition, fish from FAA40 treatment expressed growth performance significantly lower than fish from FAA50, which is difficult to explain. The highest growth rate and lowest FCR was observed in fish fed with 10% FAA inclusion. Appropriate supplementation level (cysteine; 10 mM) with FAA may elevate gustatory attractiveness and intake of CG based diet (Marui et al., 1983, Kasumyan and Morsi, 1996). Consequently, caused faster and complete ingestion and more efficient diet utilization. Therefore, there may be evidence that our approach in diet design mentioned earlier could have positive effect on diet utilization by carp with the exception of FAA10. Furthermore, surprising results were obtained after comparing the FAA30 and FAA50 with negative controls (CON1 and CON2, respectively). These results suggest the FAA mixture was not utilized efficiently, one of the explanations being that protein level in our diets was possibly higher than required for carp at this life stage in these experimental conditions.

The lowest FCR (the best utilization) among the treatments was found in fish fed the CG diet and diets supplemented with 10-20% FAA mixture. This might suggest that the supplementation of FAA at 20% protein replacement does support efficient body protein synthesis in carp, equivalent to protein-based diet fed fish with balanced indispensable amino acid composition. In other words, this diet formulation can be used in further experimentation addressing the need of specific indispensable amino acid; for instance methionine, the most commonly limited amino acid (Yurkowski and Tabachek 1979).

The available literature data on semi-purified diets use for evaluating IDAA requirement of juvenile carnivorous fish is not consistent. Peres and Oliva-Teles (2005) observed that 19% replacement of fishmeal with mixture of FAA sustained the same growth performance as fishmeal based diet in juvenile turbot (*Scophthalmus maximus*). However, increasing FAA inclusion over this value leads to poorer growth compared to the control group. Higher level of FAA inclusion in the diet was proven to be acceptable by gilthead seabream (*Spartus aurata*). Juvenile individuals of this species were able to utilize diet efficiently where fishmeal was replaced with FAA at the level of 55% (Marcouli et al., 2004). Rodehutscord et al. (1995) observed that rainbow trout is able to sustain acceptable growth rate when fed with diets based on wheat gluten protein concentrate supplemented with almost 50% of FAA, however, in this case control diet with plant protein did not provide maximum growth rate. In addition, Pérez-Jiménez et al. (2014) did not observe any differences in growth performance and feed efficiency in juvenile Senegalese sole (*Solea senegalensis*) after 55 days of feeding with diet where 50% of intact protein sources (steam dried fishmeal) was replaced with FAA. Moreover, increasing level of FAA inclusion (50-80%) did not have any effect on survival across treatments. At the end of our study overall fish mortality was low but there were significant differences among the treatments. In addition, there seemed to be a trend of the increased mortality in treatments that presented higher growth rates. This may suggest that “high performance” fish appeared to be more sensitive to rearing conditions including perhaps the nutritional imbalances of the present study. Current study suggests major differences in postprandial handling of FAA in muscle depending on their

essentiality, IDAA or DAA (Figure 9). Incorporation of IDAA was 10-13 fold higher than DAA in turbot (carnivorous, coldwater fish). However, there was no noticeable effect of protein level in the diet (6 or 50%). Therefore, Cowey and Sargent (1979) concluded that incorporation of IDAA is greater and most likely less IDAA were oxidized as energy source in comparison to DAA. However, the final judgment can only be made when the pool size of individual amino acids is examined and tissue compartmentation included in the analysis. In any case, fish amino acid metabolism contrasts with that in mammals that reduces IDAA utilization as energy source when the dietary protein level is diminished.

According to Ogata (1986) and Murai and Ogata (1990) postprandial FAA pattern in the skeletal muscle appear to be relatively stable compared to the blood plasma or hepatopancreas. Therefore, it is commonly used as the dietary protein biological value indicator presenting the actual FAA pool available for protein synthesis. Our study showed that six hours after feeding concentration of total IDAA in fish muscle tissues from FAA10 treatment, the fastest growing group of fish, was one of the lowest among the treatments. The low concentration of FAA in muscle tissues may be explained by increased protein synthesis and resulting fast growth rate of fish fed with 10% CAA inclusion. However, the fact that the concentration of total IDAA was similar to that in slow growing groups such as FAA40, made it unclear and may suggest that FAA from this latter group was lost during mastication, excreted from fish body and/or catabolized equally fast. Total DAA was significantly higher in FAA40 group compared to fish from FAA10 treatment (Figure 9). Interestingly, the level of IDAA and DAA in fish muscles

tissues was one of the highest in CG. The reason for this result may be explained based on differences in evacuation rate of FAA and CG-based diets in stomachless carp (Tanaka et al. 1977). The latter authors found that evacuation rates are 105 and 295 min, respectively. Earlier studies with juvenile turbot (Peres and Oliva-Teles, 2005) and gilthead seabream (Marcouli et al., 2004) did not show any differences in the whole body composition among fish fed with different FAA inclusion levels which is consistent with our findings. The only one exception was lipid concentration in carp whole body (Figure 10). Interestingly, there was no significant difference in body fat concentration between the fastest (FAA10) and slowest (FAA40) growing treatments. This may suggest that FAA were not utilized neither in protein synthesis nor as energy source. Dietary FAA were probably lost in one of the earlier mentioned ways, leaching or excretion. The negative control groups showed that there was no difference in fat concentration between FAA30 and CON1, but fish from CON2 treatment accumulated more fat in the body compared to FAA50. This suggests indeed that utilization of starch as an energy source. Nolles et al. (2009) stated that human or rats have capacity to adopt their gastrointestinal tract to the presence of high concentration of FAA in the diet and reduce its oxidative losses just after 3 weeks of adaptation. Based on our observation, however, we did not notice any significant improvement of weight gain or feed efficiency ratio when the growth of fish from the SwTrt group was compared with parallel treatments. It was also observed that gradual increase of FAA in the diet did not show consistent adaptation to its high level by carp although when simple t-test was used for groups FAA20-FAA40,

there were significant differences suggesting adaptation to increasing amounts of FAA in the diet in SwTrt group in the course of the experiment.

Table 8 A composition of experimental diets for common carp.

	Diets					
Ingredients (g/kg)	CG	FAA10	FAA20	FAA30	FAA40	FAA50
Casein	400	360	320	280	240	200
Gelatin	80	72	64	56	48	40
Free AA mix[a]	-	48	96	144	192	240
CPSP 90[b]	50	50	50	50	50	50
Starch	273.4	273.4	273.4	273.4	273.4	273.4
Fish oil[c]	30	30	30	30	30	30
Soy oil[d]	30	30	30	30	30	30
Vitamin mix[e]	20	20	20	20	20	20
Mineral mix[f]	40	40	40	40	40	40
NaH_2PO_4	20	20	20	20	20	20
Vitamin C[g]	0.6	0.6	0.6	0.6	0.6	0.6
Sipernat® 50[h]	40	40	40	40	40	40
Taurine	15	15	15	15	15	15
Choline chloride	1	1	1	1	1	1

[a]Compositions (g/240g; all L-form AA otherwise indicated); Arg free base, 9.1; His free base, 3.7; Ile, 4.8; Leu, 7.5; Lys monohydrochloride, 9.6; DL-Met, 5.4; Phe, 9.6; Thr ali o free, 4.3; Trp, 1.1; Val, 6.4; Pro, 59.5; Ser, 59.5; DL-Ala, 59.5.
[b]Soluble fish protein hydrolyzate (Sopropeche S.A., Boulogne Sur Mer, France).
[c]Cod liver oil (MP Biomedicals, Solon, OH, USA).
[d]Soybean oil (ICN Biomedicals, Aurora, OH, USA).
[e]Vitamin mixture (mg/kg diet) sources were Rovimix series: retinyl acetate, 2.00; cholecalciferol, 0.10; DL-α-tocopheryl acetate, 125.00; menadione niacinamide bisulfite, 5.00; nicotinic acid, 25.00; riboflavin, 20.00; pyridoxine hydrochloride, 15.00; D-calcium pantothenate, 50.00; biotin, 1.00; folic acid, 5.00; cyanocobalamin, 0.05; *myo*-inositol, 500.00; thiamine mononitrate, 10.00 (Aquaculture Research Group, DSM Nutritional Products France, Animal Nutrition & Health Research, Saint-Louis, France).
[f]Five milligram Se in the form of sodium selenite per kg Bernhart Tomarelli salt mixture (ICN Pharmaceuticals, Costa Mesa, CA, USA).
[g]Mg-L-ascorbyl-2-phosphate (ShowaDenko K. K., Tokyo, Japan).
[h]Evonik, Germany

Table 9 Amino acid profiles of CG and FAA50 experimental diets (examples). Amino acids were provided as protein (casein, gelatin) or in the free form (FAA).

AA (g/kg)	CG			FAA50			
	Casein*	Gelatin*	Sum	Casein	Gelatin	FAA	Sum
Arg	16.30	5.78	22.09	8.15	2.89	11.04	22.09
Val	23.85	1.76	25.61	11.92	0.88	12.80	25.61
His	11.45	0.61	12.06	5.72	0.31	6.03	12.06
Leu	40.50	2.14	42.64	20.25	1.07	21.32	42.64
Gly	7.07	18.91	25.98	3.53	9.45	12.99	25.98
Ile	20.68	0.90	21.59	10.34	0.45	10.79	21.59
Met	10.84	0.57	11.41	5.42	0.29	5.71	11.41
Phe	20.08	1.55	21.62	10.04	0.77	10.81	21.62
Ala	16.43	6.41	22.85	8.22	3.21	11.42	22.85
Tyr	21.68	0.45	22.13	10.84	0.22	11.06	22.13
Lys	33.17	3.09	36.26	16.59	1.55	18.13	36.26
H-lys	0.00	0.69	0.69	0.00	0.35	0.35	0.69
Trp	5.03	1.00	6.03	2.51	0.50	3.01	6.03
Thr	13.92	1.46	15.38	6.96	0.73	7.69	15.38
Cys	0.00	0.07	0.07	0.00	0.03	0.03	0.07
Glu	70.24	7.64	77.89	35.12	3.82	38.94	77.89
H-pro	0.00	8.97	8.97	0.00	4.49	4.49	8.97
Pro	46.83	11.36	58.19	23.41	5.68	29.10	58.19
Ser	13.70	2.34	16.04	6.85	1.17	8.02	16.04
Asp	28.23	4.29	32.51	14.11	2.14	16.26	32.51
SUM	400.00	80.00	480.00	200.00	40.00	240.00	480.00

* Source : MP Biomedicals, Solon, OH, USA

Table 10 The free amino acid concentration of common carp muscle 6 hours after the last meal. Different letters indicate statistical difference at $P<0.05$.

FAA	CG	FAA10	FAA20	FAA30	FAA40	FAA50	CON1	CON2	SwTr
Asp	2.15±0.33^{c}	1.13±0.01^{a}	1.46±0.28^{ab}	1.20±0.19^{ab}	1.22±0.06^{ab}	1.16±0.04^{ab}	1.48±0.14^{a}	0.91±0.14^{b}	1.23±0.14^{a}
Glu	2.34±0.34^{ab}	2.79±0.16^{b}	2.41±0.35^{ab}	1.94±0.17^{a}	2.73±0.21^{b}	2.57±0.35^{ab}	2.19±0.25^{ab}	2.82±0.26^{b}	2.34±0.14^{ab}
H-pro	1.35±0.08bc	2.08±0.23^{de}	1.89±0.11^{e}	1.94±0.21^{e}	2.58±0.10^{a}	2.48±0.04^{ad}	1.75±0.14^{be}	0.95±0.10^{cf}	2.09±0.28^{def}
Ser	1.11±0.20	1.27±0.06	1.91±0.29	1.22±0.15	1.90±0.25	1.87±0.58	1.62±0.09	1.34±0.39	1.46±0.39
Ala	2.35±0.28^{a}	1.76±0.05^{bc}	2.54±0.09^{a}	1.67±0.10^{bc}	2.08±0.19^{abc}	1.74±0.10^{b}	2.32±0.13^{ac}	1.77±0.25^{b}	1.32±0.06^{c}
Asn	2.25±0.14^{ab}	1.66±0.04^{c}	2.05±0.05^{abc}	1.82±0.01^{ac}	2.08±0.07^{abc}	1.88±0.22^{abc}	2.27±0.08^{ab}	2.38±0.44^{b}	1.69±0.07^{c}
Cys	0.07±0.01^{bc}	0.04±0.01^{ad}	0.05±0.01^{abd}	0.06±0.01^{abc}	0.05±0.00^{abd}	0.03±0.02^{d}	0.09±0.02^{c}	0.08±0.01^{bc}	0.02±0.01^{d}
Gln	2.15±0.21^{abc}	1.81±0.09^{abc}	2.25±0.30^{b}	1.74±0.09^{ac}	2.26±0.10^{b}	1.95±0.22^{abc}	2.19±0.03^{abc}	2.20±0.24^{ab}	1.69±0.08^{c}
Gly	2.87±0.26^{e}	3.11±0.16^{de}	3.37±0.21^{de}	4.37±0.29^{ab}	4.69±0.04^{a}	4.10±0.10^{bc}	3.58±0.25^{cd}	3.18±0.06^{de}	3.00±0.22^{e}
His	15.04±3.26^{ab}	12.75±0.79^{ab}	13.87±1.21^{ab}	13.50±4.29^{ab}	12.54±0.17^{ab}	13.13±0.98^{ab}	17.58±0.81^{a}	10.85±1.01^{b}	15.38±0.57^{ab}
Ile	0.24±0.05^{b}	0.08±0.01^{c}	0.17±0.04^{ab}	0.13±0.01^{ac}	0.16±0.00^{a}	0.10±0.01^{ac}	0.17±0.02^{ab}	0.24±0.04^{b}	0.08±0.02c
Orn	0.12±0.02^{ab}	0.08±0.01^{cd}	0.11±0.01^{abc}	0.14±0.03^{a}	0.09±0.00^{bcd}	0.06±0.02^{cd}	0.09±0.01^{bcd}	0.05±0.01^{d}	0.07±0.02^{cd}
Phe/h-cys	0.07±0.02^{b}	0.08±0.00^{ab}	0.07±0.01^{ab}	0.08±0.01^{ab}	0.09±0.01^{ab}	0.07±0.02^{ab}	0.08±0.01^{ab}	0.11±0.02^{a}	0.06±0.01^{b}
Pro	12.09±1.09^{a}	7.92±1.03^{c}	5.06±0.08^{b}	4.49±0.54^{b}	7.46±0.63^{c}	11.31±0.43^{a}	8.22±0.25^{c}	4.11±0.45^{b}	7.82±0.36^{c}
Trp	0.06±0.01^{a}	0.06±0.00^{a}	0.06±0.01^{ab}	0.06±0.00^{ab}	0.05±0.00^{ab}	0.05±0.01^{ab}	0.06±0.00^{ab}	0.06±0.01^{ab}	0.04±0.00^{b}
Tyr	0.29±0.06^{a}	0.29±0.01^{a}	0.34±0.02^{ab}	0.35±0.02^{ab}	0.43±0.05^{b}	0.44±0.03^{b}	0.60±0.06^{c}	0.37±0.06^{ab}	0.38±0.04^{ab}
Val	0.47±0.07^{c}	0.22±0.01^{e}	0.32±0.05^{abd}	0.26±0.01^{abe}	0.33±0.02^{abd}	0.23±0.02^{ae}	0.35±0.05^{bd}	0.37±0.03^{cd}	0.20±0.01^{e}

Table 11 The free amino acid concentration of common carp muscle 24 hours after the last meal.

FAA	CG	FAA10	FAA20	FAA30	FAA40	FAA50	CON1	CON2	SwTrt
Ala	2.47	2.30	2.46	2.58	2.67	2.20	2.57	2.41	2.50
Asn	2.09	1.92	1.87	1.88	1.70	1.55	1.89	1.93	1.70
Asp	2.24	1.36	1.21	0.69	0.94	0.99	1.61	1.48	1.40
Cys	0.01	0.04	0.03	0.05	0.05	0.03	0.04	0.01	0.05
Gln	1.96	2.24	2.19	2.05	1.78	1.88	1.65	1.96	1.89
Glu	1.90	2.39	2.65	1.95	1.14	2.07	1.34	0.89	1.84
Gly	3.51	3.90	4.45	4.43	5.05	4.14	3.70	3.69	4.12
His	13.30	13.10	14.76	17.10	15.14	11.33	19.60	12.36	15.27
H-pro	1.10	1.67	1.27	1.67	1.60	1.62	1.25	0.87	1.69
Ile	0.17	0.19	0.22	0.20	0.23	0.19	0.20	0.24	0.21
Orn	0.08	0.05	0.05	0.07	0.06	0.07	0.07	0.08	0.09
Phe/h-cys	0.09	0.10	0.10	0.11	0.09	0.11	0.08	0.10	0.10
Pro	5.03	5.20	1.95	3.50	2.54	3.02	6.34	2.91	3.26
Ser	0.66	0.65	0.97	1.06	0.77	0.77	0.73	1.01	0.90
Trp	0.05	0.07	0.06	0.07	0.05	0.07	0.06	0.05	0.08
Tyr	0.23	0.28	0.29	0.32	0.32	0.30	0.22	0.34	0.24
Val	0.28	0.31	0.35	0.31	0.33	0.29	0.30	0.39	0.33

Table 12 Proximate analysis of whole body common carp (n=3).

	Units	Initial	CG	FAA10	FAA20	FAA30	FAA40	FAA50	Control 1	Control 2	SwTr
Dry Matter		20.5	20.5±1.4	20.3±1.9	19.6±0.3	19.4±0.7	19.3±1	19.7±0.9	20.8±0.3	20.2±0.77	19.3±0.84
Crude Protein	%	66.2	68.5±2.1	66.9±1.2	70.6±0.6	69±1.7	70.7±0.9	71.4±0.6	68.1±0.7	65.8±1.13	68.5±2.17
Ash		19.3	19.3±1.1	14.6±0.7	16.0±0.1	15.9±1.0	15.2±0.4	17.5±0.4	14.8±2.1	14.6±1.19	19.6±6.97
P		29844	20398±1725	19741±3004	19133±1268	20828±1832	21261±1560	20803±3053	19370±1885	2005±2010	20844±2025
K		12469	11158±363	11550±179	11684±226	11191±215	11829±136	11566±259	11343±226	1085±288	11466±125
Ca		43506	29910±1640	28099±3644	26517±2770	30314±1683	29791±200	30180±5496	27236±3936	3014±2464	30300±2407
Mg		1764	897±46	852±50	881±60	811±14	898±59	906±54	905±50	825.±40	840±45.5
S		9035	8703±462	8855±840	8875±300	8976±529	9225±633	8994±667	8876±223	8381±55	90004±504
Cu	ug/g	6.1	17.5±22.6	43±72	2.5±1.2	23.4±19.0	452±662	11.4±17.1	2.8±1.4	13.5±19.7	33.1±53.4
Fe		138	82.2±57.3	64±6	71±27	65.7±3.3	91±38	86±10	59.6±3.6	76.4±25.7	75.5±22.6
Mn		4.7	10.1±1.1	9±0.4	8.6±1.4	9.1±0.8	10.4±2.5	11.5±1	9.1±2.0	8.8±0.78	8.94±0.55
Mo		0.7	0.5±0.1	0.5±0.0	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.54±0.06	0.47±0.12
Na		5544	6310±831	6418±1613	6469±208	7038±442	6411±251	6521±697	6009±132	6753±555	69773±1067
Zn		596	367±22	307±39	316±23	304±10	557±419	300±44	334±267	315±36.54	324±47

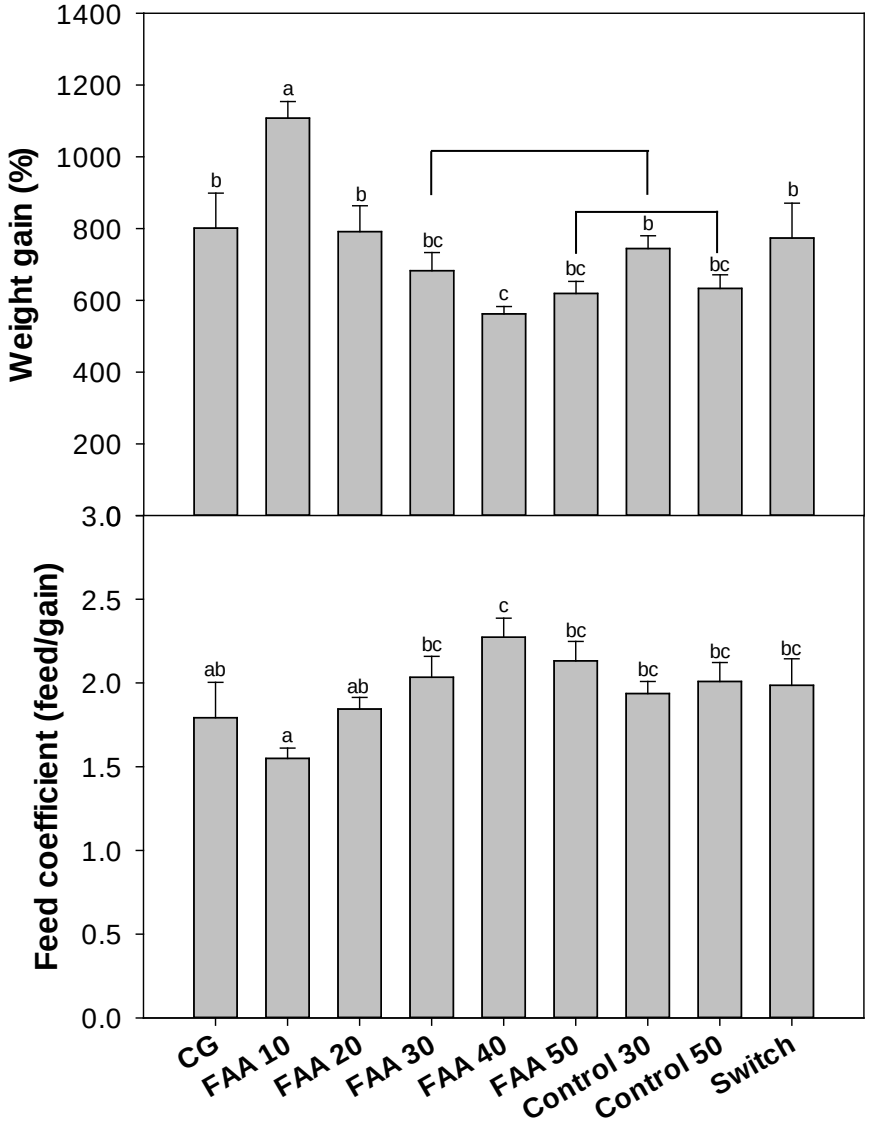

Figure 6 .The weight gains and feed conversion ratios of common carp after the feeding trial.

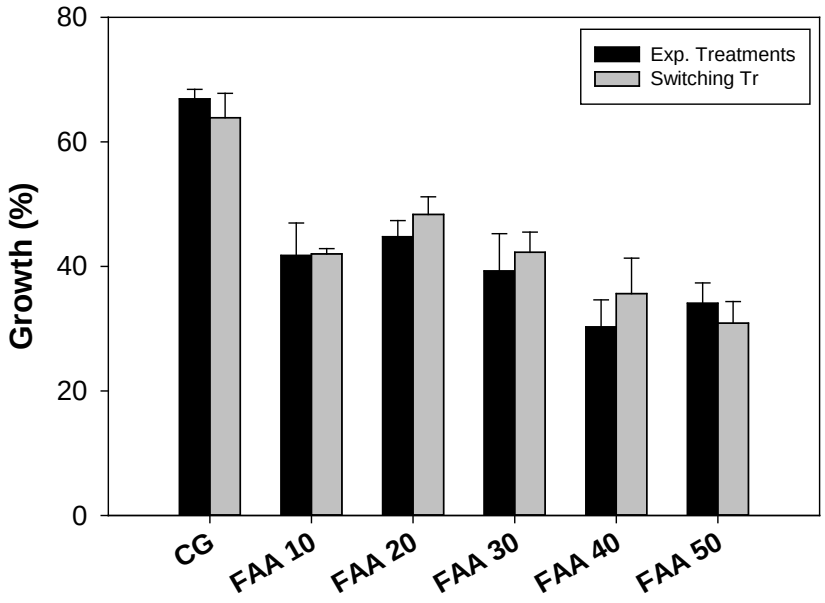

Figure 7 Growth performance of fish fed diets with increasing FAA levels (Switch treatment).

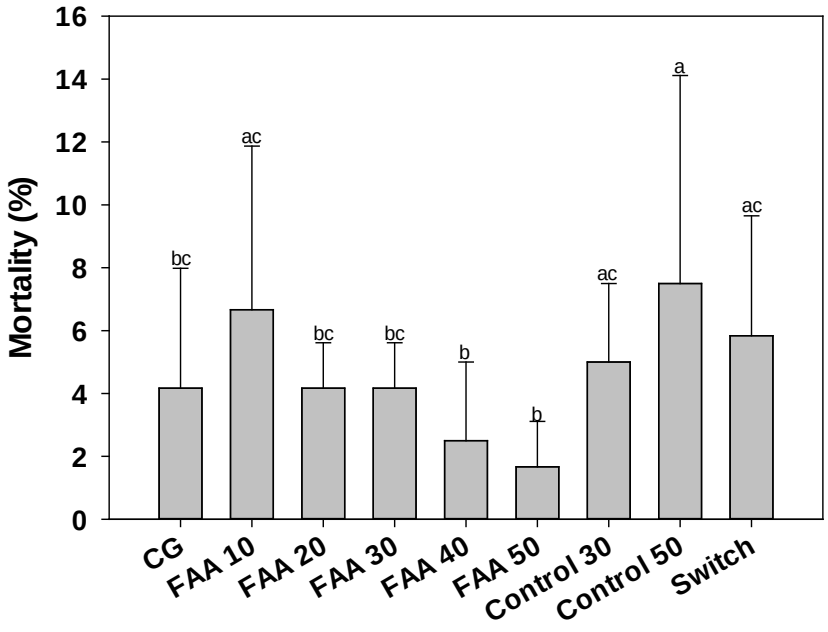

Figure 8 Carp juveniles performance on semi-purified diets with free amino acids mixture inclusion. Different letters indicate statistical difference at $P<0.05$.

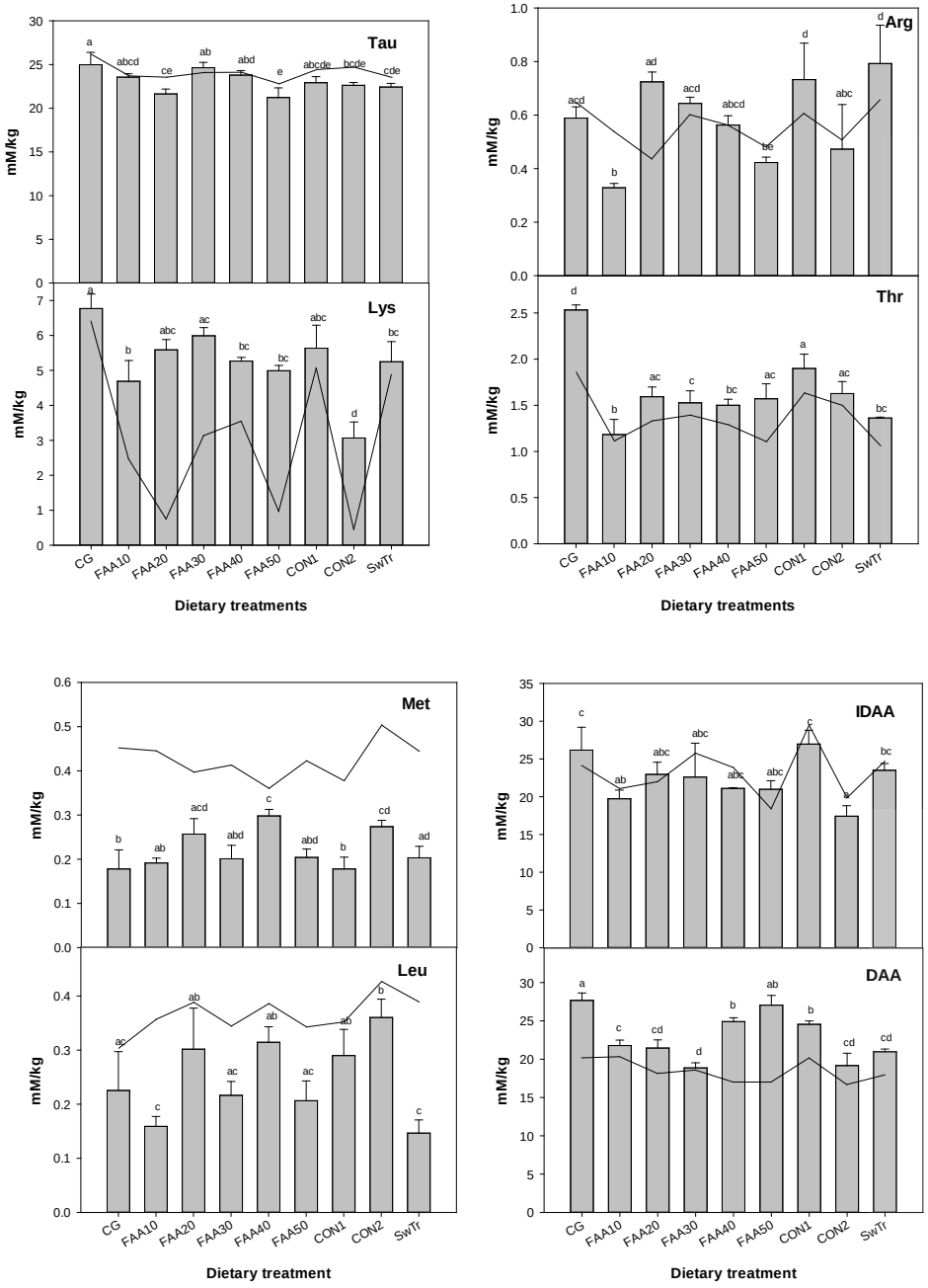

Figure 9 The free amino acid concentration of common carp muscle 6 hours after the last meal. The baseline indicates the level of free amino acid in common carp muscle 48 hours after the last meal. Different letters indicate statistical difference at $P<0.05$.

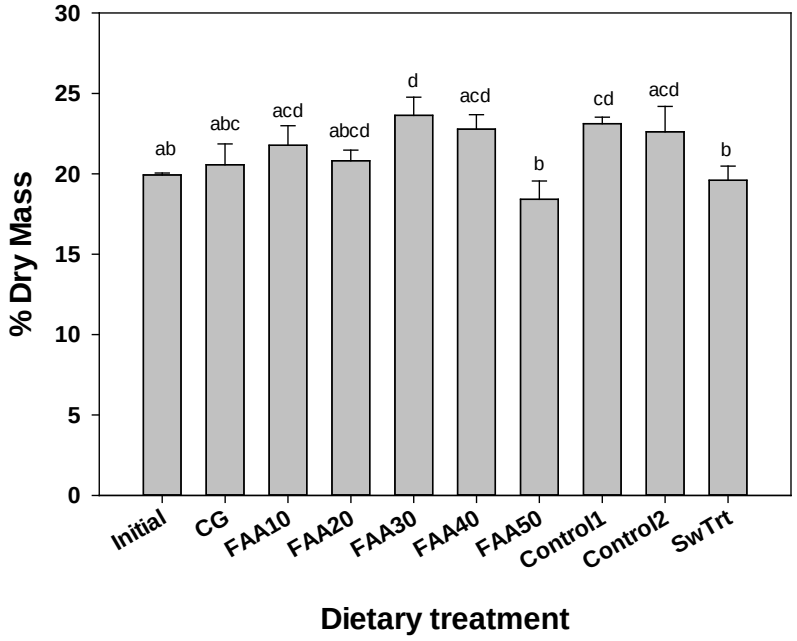

Figure 10 The concentration of lipids in common carp whole body (n=3). Different letters indicate statistical difference at $P<0.05$.

CHAPTER 5: INDISPENSABLE FREE AMINO ACIDS PROFILE OF MUSCLE TISSUES AND METHIONINE REQUIREMENTS IN JUVENILE COMMON CARP (CYPRINUS CARPIO) AT THE NEAR-MAXIMAL GROWTH RATE

Summary

High level substitution of fishmeal with plant protein sources in fish feeds may lead to dietary methionine deficiency, which must be supplemented in order to maintain diet nutritive value. However, it has been reported that both excess and deficiency of dietary methionine may affect growth performance, feed intake and cause some pathological changes in fish. Therefore, the main goals of this experiment were to evaluate common carp Met requirement at the near-maximal growth rate and evaluate postprandial free methionine concentration in muscle tissues, indicating its bioavailability.

In this study we used a reference diet based on casein and gelatin (CG) and five diets in with 20% of dietary protein was replaced with a mixture of CAA with identical AA profile except methionine, which was added to the diets in form of DL-methionine at graded level: 0% (Met0), 0.2% (Met0.2), 0.4% (Met0.4), 0.8% (Met0.8), 1.6% (Met1.6), respectively giving 0.9, 1.1, 1.3, 1.7, 2.5% DL-Met in the diets. The protein-bond Met level in CG diet was at the level of 1.14%. This experiment showed that the highest

weight gains and the lowest feeding coefficient presented fish fed the CG diet compared to all other treatment groups. The concentration of free Met in carp muscle tissues 6 hours after the last meal was the highest in Met 1.6 group compared to all other groups.

Introduction

High level substitution of fishmeal with plant protein sources in fish feeds may lead to dietary amino acid imbalances (Alexis et al., 1985, Poston, 1986) and reduction of a diet nutritional value (Friedman and Gumbmann, 1988). All essential amino acids must be supplied to the tissues simultaneously in an appropriate balance for optimal protein synthesis. Methionine is often the first limiting amino acid required for protein synthesis in alternative plant protein sources. Met is also required for many other key metabolic components such as cysteine and taurine and it is a methyl group donor for cellular metabolism (NRC, 2011). It has been reported that both excess (Jackson and Capper, 1982) and deficiency (Walton et al., 1982, Cowey et al., 1992) of dietary methionine may affect growth performance and feed intake in fish. Met deficiency causes lenticular cataracts development in salmonids (Rumsey et al., 1983, Kim et al., 1992c) and striped bass (Li et al., 2009). Furthermore, methionine has been shown to be one of the most toxic amino acids in mammals (Rees et al., 2006, Sauberlich, 1961, Klavins et al., 1963, Shinozuka et al., 1971, Fau et al., 1980, Fau et al., 1987) and birds (Katz and Baker, 1975) when given in amounts significantly exceeding the requirement. Therefore, the use of alternative protein sources requires evaluation of the exact amino acid requirements of cultured fish in order to avoid nutritional inadequacies (Simmons et al., 1997). Amino acids metabolism in fish is influenced by digestibility of the dietary protein, amino acids

composition, diet energy concentration, age of fish, and environmental conditions (Kim et al., 1992a, Wilson, 2002, Meyer and Fracalossi, 2004). An evaluation of indispensable amino acid (IDAA) requirement involves several dose-response experiments with graded levels of each IDAA that results in the optimal fish performance. The conclusive data on optimal dietary level of IDAA is important to develop feed formulation and achieve maximum feed efficiency and minimum nitrogen waste output. Moreover, dietary amino acids are required for growth and maintenance and in case of young, rapidly growing fish, the muscle growth is the major deposition site of required AA (Cowey, 1994). IDAA requirement for common carp was established by Nose (1979), however, the experimental diets based on free AA supported satisfactory growth of young fish. The IDAA requirement determined by the latter author may not represent values for healthy, fast growing fish. Therefore, the objectives of the present study were: (i) to test how stomachless fish - common carp will respond to diets supplemented with different levels of methionine at the near-maximal growth rate, (ii) to evaluate postprandial free amino acids concentration in muscle tissues, specifically methionine as an indicator of its availability, and (iii) to examine if different methionine concentrations may affect carp body proximate composition.

Materials and methods

Diet formulations and feeding strategy

Six diets, formulated to be isonitrogenous and isolipidic, were used in this 40 days long feeding trial (Table 13). A reference diet was based on casein and gelatin (CG) and five diets were used with 20% of dietary protein replaced with a mixture of FAA with

identical AA profile except methionine. Met was added to the diets in form of DL-methionine at graded level: 0% (Met0), 0.2% (Met0.2), 0.4% (Met0.4), 0.8% (Met0.8), 1.6% (Met1.6), respectively. These supplements resulted in total Met: 0.9, 1.1, 1.3, 1.7, 2.5% in the diets. The protein-bond Met level in CG diet was at the level of 1.14% based on information provided by manufacturer and data presented by Li et al. (2011). The diets were prepared according to the earlier experience with rainbow trout alevins (Oncorhynchus mykiss) (Terjesen et al., 2006) and common carp larvae (Cyprinus carpio) (Kwasek et al., 2010) with minor modifications. Casein and gelatin (5:1) were used as main protein source and dextrin was replaced with starch in comparison to the original formulation. All the experimental diets were designed to meet the requirements for indispensable amino acids by common carp (NRC, 1993). The pH of all of the diets was adjusted to 7 (± 0.1 unit) with 6 N NaOH during the mixing of dietary ingredients with FAA mixtures as recommended by Wilson et al., 1977 and Murai et al. (1983). In addition, all diets were supplemented with 5% of soluble fish protein concentrate (CPSP 90, Sopropeche S.A., Boulogne Sur Mer, France) to improve palatability. Dry ingredients were combined and mixed for extended time prior to addition of oil and water and mixed again. The complete mixture was pressed through 2 mm die using the Hobart feed mixer (Troy, Ohio) with a meat grinder attachment and subsequently freeze dried. Dry strands were then ground into appropriate particle size.

Fish maintenance and feeding experiment

Common carp broodstock of European descent originated from Auburn University Experimental Station and was housed for two generations in the Aquaculture

Laboratory at the Ohio State University, Columbus, Ohio, USA. Fish were bred following standard carp pituitary extract (Stroller Fisheries, Spirit Lake, IA) injection. The initial feeding included live brine shrimp naupli (Artemia sp.) and subsequent weaning to commercial starter diet (A, B1, B2, C1, Otohime, Japan).

The experiment was conducted in a 4,000L semi-recirculation system that consisted of 28 fiberglass conical tanks (50 L), overhead tank with temperature controlling device (26 ± 0.2 ºC), and a biofilter. The freshwater replacement rate was up to 10 L/min for entire system.

Full sibling fish at size 3.8±0.12g were randomly distributed into the tanks, 27 fish per tank in triplicate for each treatment. Each fiberglass tank was considered as an independent experimental unit with constant water replacement at a rate of minimum 1.5 L min-1. Supplemental aeration was provided to maintain dissolved oxygen level near saturation. Fish were fed at restricted feeding rates. Three meals per day were offered at 9:00, 13:00, and 17:00 seven days per week for 40 days, equal across all treatments. Restricted rate was adjusted at 5% of the biomass, with assumption that daily wet weight increase equals dry food consumed. Food particles size was gradually increased during the feeding trial from 0.8 to 1.7 mm. Every ten days total fish biomass of each tank was measured and feeding rate was re-adjusted. Feeding was stopped 24 h prior to each weighing; tanks were siphoned and brushed on regular basis in order to remove fecal material and algae from the tanks. All procedures and handling of animals were conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University.

The amount of food consumed by fish in one meal was evaluated in a satiation test, where fish were fed until clear food rejection. The amount of food consumed across all the experimental units was expressed as a percent of fish biomass for each treatment. All procedures and handling of animals was conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University. For details related to muscle free amino acid, diet and whole body proximate and mineral analyses the reader is referred to Chapters 2 and 3.

Statistical analysis

The experimental design was completely randomized for fish distribution and experimental diets assignment. The differences among dietary treatments were tested by one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at the value of $p < 0.05$.

Results

This experiment showed that the highest weight gains had fish fed the CG diet compared to all other treatment groups. The feeding coefficient was the lowest in the CG group compared to all other groups. The satiation test showed dissimilar trend (Figure 11). The group fed diet unsupplemented with Met had significantly higher intake than other treatments.

The concentration of free Met in carp muscle 6 hours after the last meal was the highest in Met 1.6 group compared to all other groups (Figure 12). The concentration of Tau, the derivative of Met metabolism, was the same among all treatment groups. The level of free Arg was lower in Met 0.2 compared to the Met 1.6 group but not different than in

other groups. The level of free Trp in Met 0 fed fish was lower compared to CG and Met 1.6 groups. The concentration of free Lys in Met 0 group was higher compared to Met 0.8 and Met 1.6 groups but not different with other treatments. No differences were found in the levels of free His, Glu, Asn, Gly, Gln, Val, Ile, Leu, Orn, and total DAA. The concentration of total IDAA was lower in Met 0.8 compared to Met 1.6 but not different than in other groups. All other free amino acid levels in carp muscle 3 hours and 48 hours after a meal are listed in Tables 14 and 15, respectively.

Table 16 presents proximate whole body composition at the termination of the feeding study. The lipid concentration was the highest in Met 0.4 group compared to all other treatments. No differences were found in mineral analysis of carp whole body except sulfur level which was higher in the Met 1.6 group compared to CG and Met 0 groups.

Discussion

Dietary amino acids are required in fish diet for two major purposes: growth, which mainly consists of protein deposition, and for a number of biochemical processes that are conventionally termed as maintenance (Cowey, 1994). The estimate of amino acids requirement is essential to achieve feed formulation that will result in maximal fish growth and feed utilization efficiency, minimal environmental impact (waste discharge) and cost effectiveness (overall farming operation) (NRC, 2011). However, substantial variations in IDAA requirements estimated for particular systematic group of fish still exist in the literature (Cowey 1979). For example, lysine requirement estimated for different salmonid species varies greatly from 1.3 to 2.9% of the diet (Bureau and

Encarnação, 2006). Probable causes of the differences may be related to factors such as means of expression of : units in which the requirement may be expressed, diet composition, feeding strategy (ad libitum or restricted rate) (Hauler and Carter, 2001b, Hauler and Carter, 2001a), laboratory variance (Kim et al., 1992b, Cowey, 1994), response factors measured (Bureau and Encarnação, 2006), experimental design, and statistical analysis (Shearer, 2000). Furthermore, the differences may be even larger when a researcher decides to establish IDAA requirements based on fish maximum growth performance, environmental impact reduction, cost effectiveness, or diet development for particular life stage of fish (Bodin et al., 2012). Nose (1978) used daily specific growth rate as a criterion to evaluate IDAA requirement in carp. He established methionine requirement (with excess of cysteine) at the level of 2.1 and 0.83% when expressed as % of dietary protein or diet, respectively. However, the 48 days feeding trial with diet based on free amino acids mixture, provided to apparent satiation of fish, resulted in poor fish growth performance (57% body weight increase). In the present study we obtained carp growth of approximately the same size of 340-480% during 40 days. The IDAA requirement for carp estimated by Ogino (1980) was based on different approach. He assumed that fish IDAA requirement corresponds approximately to daily increase of each IDAA in fish body tissues fed with high quality intact protein. The requirement of methionine and cysteine established using this method was 1.6 and 0.8% in dietary protein, respectively. However, Ogino (1980) in his assumption did not include IDAA required by fish for maintenance of other vital functions beside protein synthesis.

Our experiment showed that the highest weight gains and the lowest FCR was in the CG group compared to all other groups. The supplementation with DL-Met did not have significant influence on fish growth, although free Met level in muscles was impacted. Rumsey et al. (1983) observed that increase in growth performance of juvenile rainbow trout was associated with increasing methionine concentration in a diet deficient in sulfur AA. The authors established that methionine requirement was between 0.85 - 1.05% and 2.5 - 3.0% of the diet and dietary protein, respectively. Cysteine requirement was approximately 0.3%. Moreover, these authors noticed that methionine excess could easily satisfy the requirement for cysteine, however, excess of cysteine in diets marginally deficient in methionine was unable to satisfy requirement for this AA. Consequently, the study concluded that cysteine does not satisfy the requirement for methionine, but methionine satisfies the requirement for cysteine when supplied above the minimum requirement. Kim et al. (1992c) also observed the dependence of cysteine requirements for rainbow trout to be related to the supplementation level of methionine. Furthermore, fish weight gain and nitrogen retention increased with rising supplementation level of L-Met up to 0.5% with excess of L-Cys. The positive correlation of growth and feed conversion ratio with increasing dietary level of Met was also observed in juvenile cobia (Rachycentron canadum) (Zhou et al., 2006), arctic charr (Salvelinus alpines) (Simmons et al., 1999), common carp (Deng et al., 2011) and Indian carp juveniles for which dietary methionine was recommended at the level of 1.6-1.69% of the diet or 4.1-4.22% of protein with fixed level of cysteine 0.85% (Khan and Abidi, 2013). Additionally, diet supplementation with methionine significantly improved growth performance and feed

efficiency in rats fed diets deficient in Met (Chiji et al., 1990, Hara et al., 1997). Li et al. (2009) noticed that juvenile hybrid striped bass (Morone chrysops x M. saxatilis) fed sulfur AA deficient diets supplemented with three graded levels of DL-methionine increased linearly weight gain, survival, feed efficiency and protein efficiency ratio. Nevertheless, high mortality occurred in fish fed with sulfur AA deficient diets and this experiment was stopped earlier than intended. Similar results were observed by Keembiyehetty and Gatlin III (1993) during the experiment conducted with juvenile hybrid striped bass (*Morone chrysops x M. saxatilis*). These authors noticed that fish from Met deficient groups were more sensitive to fungal infection and all fish died after one week. However, these authors argue that increasing level of methionine elevated resistance to fungus infection and growth performance was improved. The best growth rate was obtained with 0.85% methionine in the diet, and higher levels of methionine did not improve growth performance. Overall, the results obtained in groups treated with high level of sulfur AA were comparable with control group fed diet based on fishmeal. The total sulfur amino acids requirement established for juvenile striped bass (2.9% of dietary protein) was consistent with available data for other species such as: carp 3.1% (Nose, 1978), tilapia 3.2% (Santiago and Lovell, 1988), red drum 3.0% (Moon and Gatlin III, 1991) and rainbow trout 3.0% (Kim et al., 1992c, Rumsey et al., 1983) and juvenile grouper 2.73% (Luo et al. 2005). The latter study is also important because no growth depression was observed in grouper fed a diet supplemented with 1.25% of Met. Besides the mortality, dietary methionine deficiency caused cataract in arctic charr (Simmons et

al., 1999), rainbow trout (Rumsey et al., 1983, Kim et al., 1992c, Cowey et al., 1992) and hybrid striped bass (Li et al., 2009).

Methionine concentration in the diet may influence feed intake even though we did not observe such phenomenon. De la Higuera et al. (1998) observed that dietary methionine supplementation improved feed intake of carp. However, hybrid striped bass fed with diets deficient in methionine expressed slow growth, reluctance to feed, and mortality (Li et al., 2009). Furthermore, kittens fed high methionine diets decreased their food intake and lost weight in proportion to dietary methionine level (Fau et al., 1987). In order to evaluate AAs requirement growth data may be supported by FAA concentration in blood plasma, liver or muscles tissues. The assumption of this indicator is based on the changes in concentration of FAA pool in tissues, which is very low when dietary AA level is below requirement, and increase when dietary AA exceeds the required level. A quick change in concentration of particular AA in animal tissues is recognized as the required level (Cowey, 1994).

Increase in dietary methionine supplementation resulted in elevated level of this AA in blood plasma in juvenile arctic charr (Simmons et al., 1999), cobia (Zhou et al., 2006), and rat (Chiji et al., 1990). These changes were positively related to the dietary supply of Met. In rat Met supplementation caused a decrease in threonine and serine concentration in blood plasma (Chiji et al., 1990). Interestingly, taurine, one of the methionine products and one of the most abundant FAA in muscle tissues (El Idrissi et al., 2009), was not affected by Met supplementation in our experiment. His (after

feeding) and Gly (after fasting) had the highest concentration in the present study in carp muscle (Table 14; Figure 12)

We did not observe any differences in proximate analysis of carp whole body except sulfur level which was higher in the Met 1.6 group compared to CG and Met 0 groups and higher lipid concentration in Met 0.4 group compared to all other treatments (Table 16). Increasing level of dietary methionine and cysteine had no significant effect on carcass composition of hybrid striped bass (Li et al., 2009), rainbow trout (Rumsey et al., 1983) and cobia (Zhou et al., 2006). Dietary methionine supplementation caused an increase of protein and decrease of moisture concentration in common carp (Deng et al., 2011), arctic charr (Simmons et al., 1999), and Indian carp whole body (Khan and Abidi, 2013). Reported changes regarding lipid content in carp in present study were in contradiction to Deng et al. (2011) who observed higher level of lipids in common carp body after Met supplementation. Khan and Abidi (2013) noticed decreased lipids levels after Met supplementation into Indian carp diet. Chiji et al. (1990) noticed that liver fat began to accumulate when supplemental Met reached certain level below the requirement in rats. All these authors did not observe any changes in ash content in the body.

Table 13 The composition of experimental diets.

Ingredients (%)	CG	Met 0	Met 0.2	Met 0.4	Met 0.8	Met 1.6
Casein[a]	40	32	32	32	32	32
Gelatin[b]	8	6.4	6.4	6.4	6.4	6.4
Free AA mix[c]		9.6	9.6	9.6	9.6	9.6
CPSP 90[d]	5	5	5	5	5	5
Starch[e]	30.2	29.7	29.7	29.7	29.7	29.7
Fish oil[f]	3	3	3	3	3	3
Soy oil[g]	3	3	3	3	3	3
Vitamin mix[h]	2	2	2	2	2	2
Mineral mix[i]	4	4	4	4	4	4
NaH2PO4[j]	2	2	2	2	2	2
Vitamin C[k]	0.06	0.06	0.06	0.06	0.06	0.06
Taurine[l]	1.5	1.5	1.5	1.5	1.5	1.5
Choline chloride[m]	0.1	0.1	0.1	0.1	0.1	0.1
Methionine[n]	0	0	0.2	0.4	0.8	1.6
Glutamic acid[o]	1.1	1.6	1.4	1.2	0.8	0
SUM	100	100	100	100	100	100

[a] Casein vitamin free (MP Biomedicals, Solon, OH, USA).
[b]Gelatin type A (MP Biomedicals, Solon, OH, USA).
[c]Composition (g/240g; all L-form AA otherwise indicated); Arg free base, 9.1; His free base, 3.7; Ile, 4.8; Leu, 7.5; Lys monohydrochloride, 9.6; DL-Met, 5.4; Phe, 9.6; Thr ali o free, 4.3; Trp, 1.1; Val, 6.4; Pro, 59.5; Ser, 59.5; DL-Ala, 59.5.
[d]Soluble fish protein hydrolyzate (Sopropeche S.A., Boulogne Sur Mer, France).
[f]Cod liver oil (MP Biomedicals, Solon, OH, USA).
[g]Soybean oil (ICN Biomedicals, Aurora, OH, USA).
[h]Vitamin mixture (mg/kg diet) sources were Rovimix series: retinyl acetate, 2.00; cholecalciferol, 0.10; DL-α-tocopheryl acetate, 125.00; menadione niacinamide bisulfite, 5.00; nicotinic acid, 25.00; riboflavin, 20.00; pyridoxine hydrochloride, 15.00; D-calcium pantothenate, 50.00; biotin, 1.00; folic acid, 5.00; cyanocobalamin, 0.05; *myo*-inositol, 500.00; thiamine mononitrate, 10.00 (Aquaculture Research Group, DSM Nutritional Products France, Animal Nutrition & Health Research, Saint-Louis, France).
[i]Five milligram Se in the form of sodium selenite per kg Bernhart Tomarelli salt mixture (ICN Pharmaceuticals, Costa Mesa, CA, USA).
[j]Monosodium phosphate (Sigma-Aldrich , St. Louis, MO)
[k]Mg-L-ascorbyl-2-phosphate (ShowaDenko K. K., Tokyo, Japan).
[l]Taurine (Evonik Industries, Essen, Germany)
[n]Carboxymethylcellulose (ICN Biomedicals, Solon, OH, USA).
[m]Choline chloride (MP Biomedicals, Solon, OH, USA)
[n]DL-Methionine (Evonik Industries, Essen, Germany)
[o]Glutamate (Sigma-Aldrich , St. Louis, MO)

Table 14 Free amino acid concentration in carp muscle six hours after a meal. Different letters indicate statistical difference at $P<0.05$.

mmol/kg	CG	Met 0	Met 0.2	Met 0.4	Met 0.8	Met 1.6
Ala	1.74±0.45^{ab}	1.70±0.23^{ab}	2.62±0.30^{a}	1.74±0.17^{ab}	1.55±0.34^{b}	2.06±0.47^{ab}
Asn	1.94±0.24	1.92±0.13	2.03±0.18	2.05±0.08	2.09±0.23	2.23±0.10
Asp	0.85±0.08^{abc}	0.90±0.14^{ab}	1.02±0.07^{a}	0.89±0.09^{ab}	0.77±0.05^{bc}	0.64±0.08^{b}
Cys	0.03±0.01^{ab}	0.03±0.00^{ab}	0.01±0.01^{a}	0.04±0.01^{abc}	0.06±0.01^{c}	0.05±0.02^{bc}
Gln	2.53±0.37	2.54±0.10	2.68±0.07	2.94±0.35	2.79±0.18	2.77±0.12
Glu	2.18±0.38	2.44±0.22	2.83±0.24	2.29±0.22	2.68±0.47	2.01±0.29
Gly	3.08±0.75	3.55±0.23	4.36±0.95	3.78±0.39	4.03±0.66	3.36±0.62
H-pro	1.78±0.33^{ac}	1.87±0.20^{bd}	2.21±0.17^{ab}	1.62±0.15^{cd}	1.55±0.21^{cd}	1.87±0.18^{abcd}
Ile	0.17±0.03	0.17±0.04	0.21±0.03	0.20±0.02	0.24±0.04	0.22±0.03
Leu	0.36±0.06	0.33±0.05	0.42±0.08	0.36±0.08	0.45±0.12	0.42±0.07
Orn	0.12±0.02	0.08±0.03	0.09±0.00	0.11±0.01	0.11±0.03	0.11±0.04
Phe/h-cys	0.11±0.03^{ab}	0.06±0.00^{a}	0.08±0.01^{ab}	0.11±0.01^{ab}	0.09±0.02^{ab}	0.12±0.02^{b}
Pro	7.82±1.54	7.33±0.20	8.47±1.79	6.82±0.82	6.71±0.55	7.39±0.31
Ser	1.99±0.25^{ab}	2.75±0.35^{c}	2.62±0.16^{bc}	2.13±0.23^{abc}	1.91±0.17^{a}	2.64±0.29^{bc}
Thr	1.10±0.13^{a}	1.39±0.17^{ab}	1.19±0.05^{ab}	1.28±0.10ab	1.30±0.20^{ab}	1.48±0.11^{b}
Tyr	0.31±0.06^{ab}	0.26±0.02^{a}	0.33±0.06^{ab}	0.40±0.05^{abc}	0.42±0.04^{bc}	0.51±0.07^{c}
Val	0.38±0.07	0.40±0.04	0.46±0.05	0.41±0.03	0.40±0.06	0.47±0.05
Indispensable	20.73±0.37^{ab}	22.89±2.49^{ab}	20.84±1.14^{ab}	21.70±0.35^{ab}	19.67±1.08^{a}	23.62±1.11^{b}
Dispensable	22.48±2.94	23.42±1.13	26.98±2.54	23.07±0.05	23.02±0.71	23.65±0.52

Table 15 Free amino acid concentration in carp muscle 48 hours after a meal.

mmol/kg	CG	Met 0	Met 0.2	Met 0.4	Met 0.8	Met 1.6
Ala	1.20	1.34	1.18	0.70	1.16	1.16
Arg	2.19	2.91	2.78	2.58	2.79	2.36
Asn	1.93	2.26	1.27	1.90	1.73	1.91
Asp	0.78	2.04	0.86	0.85	1.15	1.05
Cys	1.91	1.93	1.59	1.77	1.84	2.01
Gln	5.03	5.74	3.90	4.95	4.40	3.93
Glu	2.43	2.40	2.31	2.07	2.17	2.62
Gly	20.30	19.36	19.87	20.64	20.51	20.91
His	12.40	13.30	12.60	11.40	12.94	13.71
H-pro	1.17	1.52	1.18	1.05	1.36	1.06
Ile	2.17	2.38	1.82	1.69	1.66	2.33
Leu	0.51	0.64	0.33	0.51	0.51	0.50
Lys	5.45	4.04	2.63	5.03	3.79	5.91
Met	0.22	0.36	0.15	0.16	0.28	0.40
Orn	0.26	0.46	0.31	0.21	0.25	0.40
Phe/h-cys	0.36	0.50	0.49	0.41	0.42	0.72
Pro	0.04	0.05	0.02	0.01	0.04	0.01
Ser	0.16	0.31	0.20	0.14	0.16	0.28
Tau	0.31	0.55	0.39	0.29	0.31	0.54
Thr	0.08	0.13	0.09	0.08	0.07	0.12
Trp	0.05	0.05	0.05	0.06	0.05	0.08
Tyr	0.08	0.12	0.05	0.09	0.04	0.06
Val	4.26	5.05	3.22	3.24	3.64	3.32
Indispensable	19.56	22.53	18.87	17.40	19.71	20.73
Dispensable	21.41	23.21	17.24	19.81	19.26	21.80

Table 16 Proximate analysis of carp whole body (initial sample n=3 except dry matter n=1).

	Unit	Initial	CG	Met0	Met0.2	Met0.4	Met0.8	Met1.6
Dry Matter			20.4±1.8	18.4±0.2	18.8±0.4	19.0±0.7	19.0±1.2	18.7±0.3
Lipids	%	17.3[a]	18.5±2.1[a]	21.8±2.5[a]	20.1±2.8[a]	22.7±1.7[b]	18.5±0.3[a]	18.9±0.5[a]
Crude Protein		71.2	69.3±1.8	68.7±0.6	69.4±2.8	68.2±1.0	72.4±1.8	70.6±2.1
Ash		16.2	15.7±2.2	15.5±2.1	15.0±0.5	16.5±0.4	17.0±1.0	16.1±0.2
P		24837	20221±1211	20065±1833	20473±322	20077±791	20303±2004	21201±2791
K		11642	11311±763	11241±335	11365±323	11538±204	11445±356	11584±319
Ca		33646	31731±3600	31382±4880	30214±2857	30358±3361	30397±4579	32251±3601
Mg		1352	881±92	854±20	865±32	940±63	934±121	886±44
S		7578	8270±518[a]	8183±130[a]	8492±119[ab]	8486±188[ab]	8652±235[ab]	9186±462[b]
Cu	µg	68.2	39.9±51.1	6.9±8.0	129±197	8.6±12.6	47.9±81.3	283±488
Fe		92.2	45.9±8.2	58.0±5.9	75.4±28.8	62.9±12.6	48.4±7.7	59.8±4.2
Mn		3.6	9.0±2.0	7.4±1.4	8.0±1.5	7.1±1.0	7.0±0.9	7.8±1.4
Mo		0.4	0.4±0.0	0.5±0.1	0.4±0.1	0.5±0.1	0.7±0.3	0.6±0.2
Na		4888	6144±500	6366±728	6530±563	6265±595	6564±426	6555±571
Zn		396	291±53	294.1±3.4	394±123	326±42	358±55	498±321

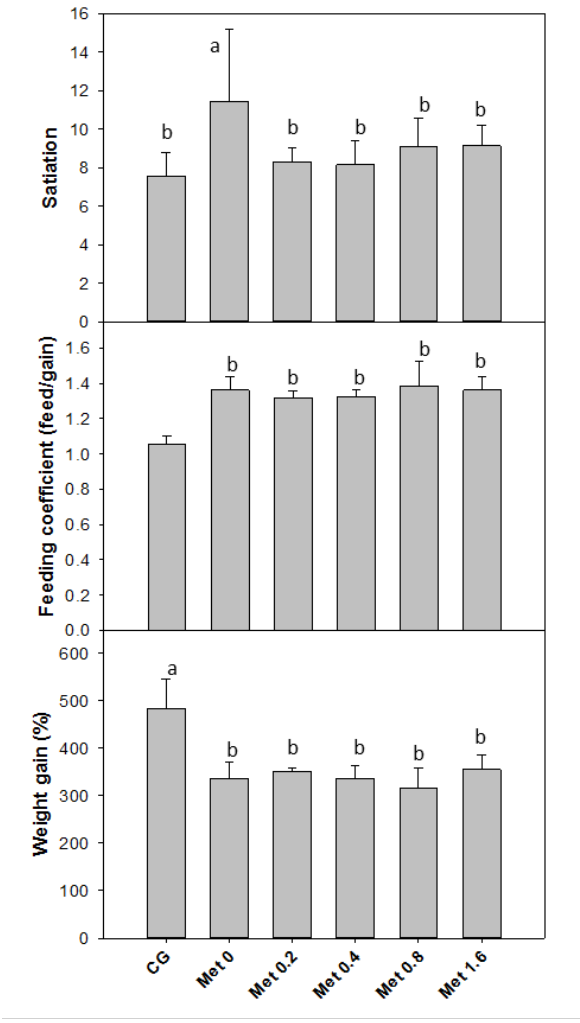

Figure 11 The weight gain, food coefficient and satiation (% body wet/day) of carp. Different letters indicate statistical difference at $P<0.05$.

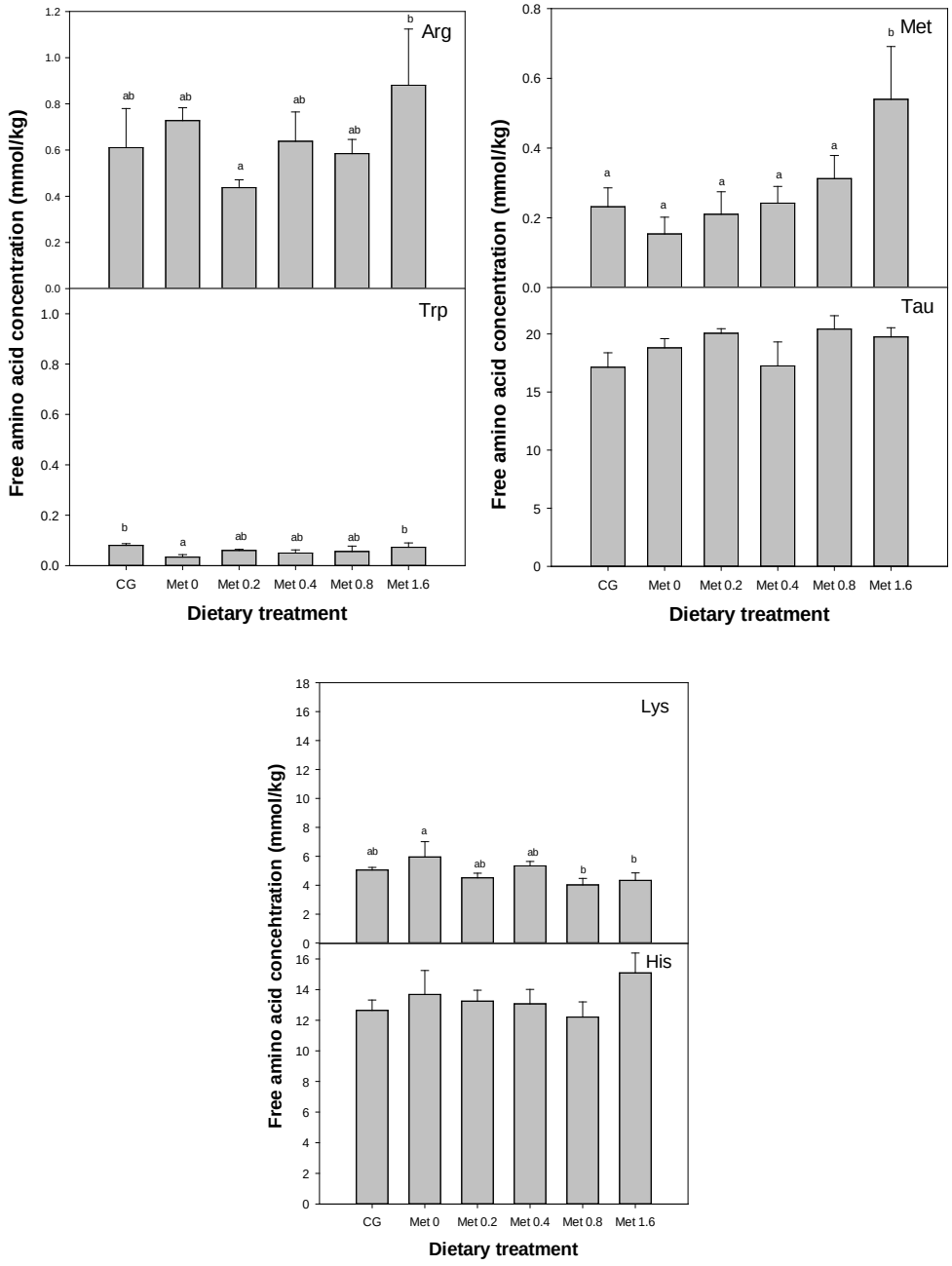

Figure 12 Free amino acid concentration of carp muscle six hours after a meal. Different letters indicate statistical difference at P<0.05.

CHAPTER 6: A NEW STRATEGY TO ENHANCE AMINO ACID UTILIZATION AND GROWTH IN FISH VIA DIETARY SUPPLEMENTATION OF BLACK PEPPER (PIPER NIGRUM) EXTRACTS

Summary

Long recognized physiological action of black pepper (*Piper nigrum*) and turmeric (*Curcuma longa*) or their active ingredients have been recently tested in nutritional and clinical experiments. Available data suggest that they may strongly influence food acceptance, metabolism, digestive physiology, and elevate bioavailability of drugs and nutrients. Consequently the main objectives of this study were to test how common carp will respond to diets supplemented with piperine, curcumine and black pepper extract. Moreover, to examine the spices influence on food intake fish and specifically FAA utilization.

In this experiment we used a reference diet that was based on casein and gelatin (CG), diet in which fraction of CG dietary protein was replaced with 20% of free amino acids (FAA) and supplemented with 0.4% DL-methionine (Met04), and three other diets identical to Met04 supplemented with 0.02% piperine (Pip), curcumine (Curc) and 0.2% black pepper extract (BP). The addition of spices and its active ingredients impacted fish growth (BP depressed growth by 30%), however, did not show any significant effect on

food intake across the treatments. Piperine increased total body lipids in carp body. We conclude that further study is warranted with different levels of spices in fish diets.

Introduction

The food quality enhancement and conservation functions of several species of medicinal plants (Govindarajan and Stahl, 1977, Srinivasan, 2005) have been recognized for centuries as well as was their health promoting properties in traditional medicine (Warrier, 1981, Griffiths et al., 2002, Wilson and Demmig-Adams, 2007). The features of black pepper (*Piper nigrum*) and turmeric (*Curcuma longa*), two the most common spices, are linked to their active ingredients piperine and curcumin, respectively (Srinivasan, 2005). Although, both spices are well known in alternative medicine, the physiological and pharmacological effects of their action have been explored only recently (Wilson and Demmig-Adams, 2007). Available data, mostly based on work with rodents, indicate its anti-inflammatory (Lee et al., 1984, Srinivasan, 2007), antioxidant (Sharma, 1976, Kunchandy and Rao, 1990, Pulla Reddy and Lokesh, 1994, Srinivasan, 2007), and anti-carcinogenic action (Ruby et al., 1995, Kelloff et al., 1996). Moreover, they have been recognized as factors with strong influence on metabolism and digestive physiology, elevating bioavailability of nutrients and drugs (Lambert et al., 2004; Johnson et al., 2011), and decreasing evacuation rate in digestive tract (Bajad et al., 2001). Westerterp-Plantenga et al. (2006) suggested that these spices can be considered as functional agents which lead to greater thermogenesis and in some cases greater satiety. In human subject they enhance secretion of saliva and activity of salivary amylase

(Glatzel, 1966). In rodents, piperine and black pepper positively influence bile synthesis (taurocholic acid) and its secretion (Bhat and Chandrasekhara, 1987). It also shows suppressive effect on body fat accumulation (Okumura et al., 2010). Furthermore, it has been observed that spices elevate digestive enzymes (trypsin, chymotrypsin) activity after its dietary intake (Platel and Srinivasan, 1996; Platel and Srinivasan, 2000, 2012).

There is limited information regarding the effect of spices in fish. For instance, intraperitoneal injection with curcumin solution caused reduction in food intake in goldfish (Kang et al., 2011). Manju et al. (2009, 2002) performed *in vitro* and *in vivo* studies with curcumin in teleost fish Anubas testudineus and provided evidence of impact on lipid peroxidation value of body lipids and increased level of superoxide dismutase activity when fed diet containing 1% of curcumin. Pezeshk et al. (2011) showed that freshness of refrigerated rainbow trout (Oncorhynchus mykiss) meat may be prolonged by dipping it in the turmeric extract solution before vacuum packing. Piperine has been successfully used as anti-parasitic agent against Argulus spp. in goldfish (Carassius auratus) (Kumar et al., 2012).

Consequently the objectives of this study were: (i) to test acceptance of diets supplemented with piperine, curcumine or black pepper extract by stomachless fish - common carp, (ii) to examine the influence of spices on appetite/satiation, (iii) to evaluate the influence of spices on dietary free amino acids (FAA) utilization for growth, (iv) to evaluate postprandial FAA concentration in muscle tissues, specifically methionine as an indicator of its availability, and (v) to examine if different spices may affect carp body proximate composition. These results may contribute to further

development of plant based, sustainable diets for omnivorous fish in intensive aquaculture production.

Materials and methods

Diet formulations and feeding strategy

Five isonitrogenous and isolipidic diets were used in this 40 days feeding trial (Table 17). A reference diet based on casein and gelatin (CG), diet in which fraction of CG dietary protein was replaced with 20% of free amino acids (FAA) and supplemented with 0.4% DL-methionine (Met04), and three other diets identical to Met04 supplemented with 0.02% piperine (Pip), curcumine (Cur) and 0.2% black pepper extract (BP). Level of spices supplementation was chosen based on experimentation with rats (Prakash and Srinivasan, 2012). The diets were prepared according to the earlier experience with rainbow trout alevins (*Oncorhynchus mykiss*) (Terjesen et al., 2006) and common carp larvae (*Cyprinus carpio*) (Kwasek et al., 2010) with minor modifications. Casein and gelatin (5:1) mixture were used as the main protein source and dextrin was replaced with starch in comparison to the original formulation. All the experimental diets were designed to meet the requirements for indispensable amino acids by common carp (NRC, 1993). The pH of all of the diets was adjusted to 7 (± 0.1 unit) with 6 N NaOH during the mixing of dietary ingredients with FAA mixtures (Wilson et al., 1977, Murai et al., 1983). In addition, all diets were supplemented with 5% of soluble fish protein concentrate (CPSP 90, Sopropeche S.A., Boulogne Sur Mer, France) to improve palatability. Dry ingredients were added together and mixed for extended time prior to addition of oil and water and mixed again. The complete mixture was processed through

2 mm die using the Hobart feed mixer (Troy, Ohio) with meat grinder attachment and subsequently freeze dried. Dry strands were then ground into appropriate particle size.

Fish maintenance and feeding experiment

Common carp broodstock of European descent originated from Auburn University Experimental Station (courtesy of Dr. R. Dunham) were housed for two generations in the Aquaculture Laboratory at the Ohio State University, Columbus, Ohio, USA. Fish were induced to spawn following standard carp pituitary extract (Stroller Fisheries, IA) injection. The initial feeding included live brine shrimp (Artemia sp.) naupli and subsequent weaning to commercial starter diet (A, B1, B2, C1, Otohime, Japan).

The experiment was conducted in a 4,000L semi-recirculation system that consisted of 15 fiberglass conical tanks (50 L), overhead tank with temperature controlling device (26 ± 0.2 ºC), and a biofilter. The freshwater replacement rate was up to 10 L/min for entire system.

Full sibling fish at size 3.8±0.12g were randomly distributed into the tanks, 27 fish per tank in triplicate for each treatment. Each fiberglass tank was considered as an independent experimental unit for statistical purposes. Constant water replacement at a rate of minimum 1.5 L min-1 was provided to each tank. Supplemental aeration was provided to maintain dissolved oxygen level near saturation. Fish were fed restricted feeding rates in three meals per day at 9:00, 13:00, and 17:00, seven days per week for 40 days. The ration was equal across all treatments at restricted rates of 5% of the biomass with assumption that daily weight increase equals to food consumed. Food particles size

was gradually increased during the feeding trial from 0.8 to 1.7 mm. Every ten days total fish biomass was measured in each tank and feeding rate was re-adjusted. Feeding was stopped 24 h prior to each weighing; tanks were siphoned and scraped on regular basis in order to remove fecal material and algae from the tanks.

The food attractiveness test (satiation rate) of experimental diets was conducted with seven groups of 25 fish (mean weight ± standard deviation; 1.32 ± 0.4 g), placed in separate tanks (28L volume) in a recirculated system (6,000 L) with constant water flow, 350 $ml \cdot min^{-1}$, at water temperature 27.5 ± 1.0^{o}C, and dissolved oxygen level near to saturation. During the feeding trial which lasted four days, each group of fish was assigned in rotating manner to different diet each day. Fish were fed until clear food rejection and the consumed diet was calculated as the mean (± standard deviation) of four replicate runs.

Total lipids were extracted with chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene as an antioxidant (Folch et al., 1957). Lipids were extracted from the whole fish and then Sep-pack silica cartridges (Waters Corp., Milford, MA) were used to separate neutral lipids (NL) from phospholipids (PL). Chloroform and methanol were used as mobile phases for NL and PL, respectively (Rinchard et al. 2007).

All procedures and handling of animals was conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University. For details related to muscle free amino acid, diet and whole body proximate and mineral analyses the reader is referred to experiment 3 (Chapter 5).

Statistical analysis

The experimental design was arranged in completely randomized manner that included fish distribution and experimental diets assignment. The differences among dietary treatments were tested by one-way ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at the value of $p < 0.05$.

Results

The experiment showed that fish fed the CG diet had the highest weight gains compared to all other groups. The lowest weight gain had fish fed black pepper extract (BP) diet compared to Met 0.4 and curcumine (Curc) diet but not different than piperine (Pip) diet fed fish (Figure 13A). Similarly, the lowest feeding coefficient had fish fed the CG diet compared to all other treatment groups (Figure 13B). In addition, fish fed Met 0.4 diet had lower feeding coefficient compared to Pip but not different than BP and Curc diets fed fish. No significant differences were found in the food intake between groups (Figure 14).

The concentration of free Met showed numerical trend with supplementation of BP and Curc, however, despite 30-33% numerical differences, the means were not statistically different. The level of free Tau, Cys, Asp, Glu, Hpro, Ile, Leu, Phe, Trp, Asn, Gly, His, Thr, Ala, and Val in carp muscle six hours after feeding was not different between groups (Figure 13, Table 18). The level of free Lys was lower in fish fed the CG diet compared to Curc diet fed fish but not different than other treatment groups (Figure 13). This trend of higher concentration of IDAA (His, Thr) in Curc diet fed group appears to be consistent although differences with other groups were not significant. The free Ser

level was higher in Curc groups compared to CG, Met 0.4, and BP group but not different than Pip group (Table 18). The concentration of free Arg was higher in Pip group compared to CG and BP groups but not different than Met 0.4 and Cur groups. The free Gln level was higher in Cur group compared to BP group but not different with the other groups. The level of free Pro was the highest in the BP group compared to all other treatments. The concentration of Tyr was higher in the Pip group than in the CG group but not different than other groups. The total IDAA level was higher in the Curc group compared to the CG group only. The total DAA level was higher in BP group compared to CG and Met 0.4 groups but not different than Pip and Curc group. All other free amino acid levels 6 and 48 hours after feeding are listed in Tables 18 and 19, respectively.

The lowest lipid concentration was found in the CG group compared to all other treatments. The highest level of lipids had fish from the Pip group compared to all other treatment groups. Similarly the neutral lipids, storage type of lipids such as triglycerides, were the highest in the Pip group compared to all other groups except the Cur group. Conversely, the phospholipid level was the lowest in the Pip group compared to CG, Met 0.4, and BP groups but not different than Cur group. The results of proximate analysis of carp whole body are listed in Table 2.

Discussion

It was evident that 0.2% BP diet resulted in food rejection in common carp juveniles. The decrease of BP level to 0.02% significantly improved diet acceptance. However, overall growth performance for 40 days was compromised. These preliminary

data clearly suggest that further studies are warranted with several levels of supplementation of spices.

Black pepper and turmeric as well as their structurally similar and physiologically active compounds, piperine and curcumin, respectively, have been recognized for their effects on animal digestive physiology (Srinivasan, 2005, Suresh and Srinivasan, 2007). Prakash and Srinivasan (2012) observed that orally administrated spices to rats may increase bile secretion and enhance activity of pancreatic lipase, amylase, trypsin, and chymotrypsin up to 80%. Moreover, piperine may inhibit gastric empting and gastrointestinal transit in rats and mice, and prolong digestion and absorption of nutrients (Bajad et al., 2001). Recently, piperine has been used in clinical studies as an enhancer of nutrients absorption and drug efficacy (Szallasi, 2005). It has been reported that piperine improved the bioavailability of several nutrients and drugs such as beta-carotene (Badmaev et al., 1999), coenzyme Q10 (Badmaev et al., 2000) or tea polyphenol-epigallocetechin-3-gallate (Lambert et al., 2004) in human or rodents. Furthermore, Johri et al. (1992) noticed that piperine (25-100μM) significantly stimulated γ-glutamyl transpeptidase activity, enhanced an uptake of radiolabeled L-leucine, L-isoleucine and L-valine, in freshly isolated epithelial cells of rat jejunum. Our results in carp corroborate in part those *in vitro* findings as the postabsorptive level of FAA in respect to several amino acids in fish muscle was numerically elevated (Figure 15).

Kang et al. (2011) showed that intraperitoneal injection of 100 nMol curcumine or piperine to juvenile goldfish significantly decreased food intake, whereas piperine did not effect on fish appetite. However, the results obtained in our experiment did not show

significant influence of piperine, curcumine or black pepper extract on food intake by carp. Piperine has been shown to influence thermogenesis and energy expenditure trough adrenal sympathetic nerves activation, involved in thermogenesis regulation in rats (Kawada et al., 1988), or by stimulating bioenergetic functions of mitochondria (Reanmongkol et al., 1988). Okumura et al. (2010) investigated energy metabolism enhancement expressed as suppression of body fat accumulation in mice due to piperine and black pepper. These authors observed that experimental animals with induced obesity decreased significantly their visceral fat weight after treating them with diets supplemented with 0.03% and 0.05% of piperine. The higher level of supplementation of piperine and black pepper significantly suppressed rats body and adipose tissues weight compared to control group. These authors concluded that black pepper administrated orally reduces adiposity as effectively as piperine. To the contrary, Prakash and Srinivasan (2012) observed positive effect of piperine (0.02%) and curcumine (0.5%) on fat digestion and absorption in obese rats. They noticed increased pancreatic lipase activity, bile secretion and consequently fat absorption in treated animals compared to control group. Moreover, dietary intake of piperine and curcumine decreased activity of hepatic enzymes involved in lipogenesis in high fat fed animals and increased bile secretion. Animals fed with low lipid diets showed decrease in body weight after treatment with curcumine and piperine, whereas adipose tissue was unaffected.

The results from our experiment showed that carp in the control treatment (CG) had the highest weight gains and the lowest feeding coefficient compared to all other groups. Fish fed diet supplemented with BP extract had the lowest body weight gain

compared to other treatments, except Pip group. Supplementation with piperine, curcumin or black pepper extract did have significant effect on lipids concentration (Figure 16). Jin et al. (2011) found out that zebrafish fed diet containing 10% of turmeric powder (curcumin) had depressed body weight gain and blood triglycerides in comparison to high cholesterol containing diet fed fish. Piperine group was characterized by the highest body fat level compared to other treatments; BP was comparable to Met 0.4, and some fat reduction properties were associated with curcumine, which was lower compared to Met 0.4, BP and Pip treatments. Phospholipids level was significantly lower in Pip and Curc groups compared to CG and Met 0.4. Pip treatment was significantly lower than BP, which was similar to Cur. Neutral lipids presented opposite relationship.

In Figure 17 we attempt to summarize the current state of knowledge on the effect of piperine on nutrient flux and utilization and possible regulatory mechanism(s) involved in the action of spices in fish growth. Dietary protein is the source of methionine, however Met is the first limiting amino acid in the case of many proteins, both in natural diet of fish (Yurkowski and Tabachek, 1979) and of plant origin. Data gathered by Hawkins et al. (1949) are still unique as they demonstrate 5-6 fold increase in taurocholic acid excretion following inclusion of Met in the diet of a dog. Withdrawal of Met resulted in a decrease of taurocholate in the bile to the level prior to Met supplementation. It clearly indicated that taurocholate synthesis is under strict dietary Met control. This phenomenon warrants further studies in fish, in particular in the case of plant protein based diets where dietary taurine is limited (Kim et al. 2007). It has been demonstrated in our laboratory that methionine deficiency leads to severe depression of

pancreatic enzymes activity in Atlantic salmon (Lee, 2013). Piperine was shown to increase pancreatic enzymes activity (Prakash and Srinivasan, 2012) and may counteract the Met deficiency (Figure 17). Bile acids are synthesized by conjugation of taurine and cholic acid. Inhibition of taurocholic acid synthesis was shown when taurine analogue (inhibitor) was present in liver microsomal preparation (Lombardini 1977). Johri et al. (1992) suggested an increased absorption of several IDAA, leucine, valine, isoleucine by epithelial intestine cells at 50 µM piperine concentration.

It appears that there are many metabolic similarities in respect to function of piperine in mammals and stomachless fish, common carp, in respect to handling piperine and curcumin and their effect on metabolism (Figure 17). For the first time we addressed the possible regulatory mechanism and interaction between intake of methionine, transsulfuration, and synthesis of taurine, and direct effects of these two spices on lipid deposition in fish.

Table 17 The composition of experimental diets.

Ingredients (%)	CG	Met 0.4	Piperine	Black Peper extract	Curcumin
Casein[a]	40	32	32	32	32
Gelatin[b]	8	6.4	6.4	6.4	6.4
Free AA mix[c]	-	9.6	9.6	9.6	9.6
CPSP 90[d]	5	5	5	5	5
Starch[e]	30.2	29.7	29.7	29.5	29.7
Fish oil[f]	3	3	3	3	3
Soy oil[g]	3	3	3	3	3
Vitamin mix[h]	2	2	2	2	2
Mineral mix[i]	4	4	4	4	4
NaH2PO4[j]	2	2	2	2	2
Vitamin C[k]	0.06	0.06	0.06	0.06	0.06
Taurine[l]	1.5	1.5	1.5	1.5	1.5
Choline chloride[m]	0.1	0.1	0.1	0.1	0.1
Methionine[n]		0.4	0.4	0.4	0.4
Glutamic acid[o]	1.1	1.2	1.2	1.2	1.2
Piperine[p]			0.02		
B. pepper extract[r]				0.2	
Curcumine[s]					0.02
SUM	100	100	100	100	100

[a] Casein vitamin free (MP Biomedicals, Solon, OH, USA).
[b]Gelatin type A (MP Biomedicals, Solon, OH, USA).
[c]Composition (g/240g; all L-form AA otherwise indicated); Arg free base, 9.1; His free base, 3.7; Ile, 4.8; Leu, 7.5; Lys monohydrochloride, 9.6; DL-Met, 5.4; Phe, 9.6; Thr ali o free, 4.3; Trp, 1.1; Val, 6.4; Pro, 59.5; Ser, 59.5; DL-Ala, 59.5.
[d]Soluble fish protein hydrolyzate (Sopropeche S.A., Boulogne Sur Mer, France).
[f]Cod liver oil (MP Biomedicals, Solon, OH, USA).
[g]Soybean oil (ICN Biomedicals, Aurora, OH, USA).
[h]Vitamin mixture (mg/kg diet) sources were Rovimix series: retinyl acetate, 2.00; cholecalciferol, 0.10; DL-α-tocopheryl acetate, 125.00; menadione niacinamide bisulfite, 5.00; nicotinic acid, 25.00; riboflavin, 20.00; pyridoxine hydrochloride, 15.00; D-calcium pantothenate, 50.00; biotin, 1.00; folic acid, 5.00; cyanocobalamin, 0.05; *myo*-inositol, 500.00; thiamine mononitrate, 10.00 (Aquaculture Research Group, DSM Nutritional Products France, Animal Nutrition & Health Research, Saint-Louis, France).
[i]Five milligram Se in the form of sodium selenite per kg Bernhart Tomarelli salt mixture (ICN Pharmaceuticals, Costa Mesa, CA, USA).
[j]Monosodium phosphate (Sigma-Aldrich , St. Louis, MO)
[k]Mg-L-ascorbyl-2-phosphate (ShowaDenko K. K., Tokyo, Japan).
[l]Taurine (Evonik Industries, Essen, Germany)
[n]Carboxymethylcellulose (ICN Biomedicals, Solon, OH, USA).
[m]Choline chloride (MP Biomedicals, Solon, OH, USA)
[n]DL-Methionine (Evonik Industries, Essen, Germany)
[o]Glutamate (Sigma-Aldrich , St. Louis, MO)
[p]Piperine, Kosher (Sigma-Aldrich , St. Louis, MO)
[r]Black Pepper (Local vendor; extracted according to Rao et al. (2012))
[s]*Curcuma longa* (Verdure Sciences, Noblesville, IN)

Table 18 The free amino acid concentration of carp muscle (n=3) six hours after the last meal. Different letters indicate statistical difference at $P<0.05$.

mmol/kg	CG	Met 0.4	Piperine	Black pepper extract	Curcumine
Asp	0.85±0.08	0.89±0.09	0.87±0.07	0.92±0.04	1.12±0.23
Ala	1.74±0.45	1.74±0.17	1.95±0.13	2.19±0.19	2.08±0.10
Arg	0.61±0.17^{b}	0.64±0.17^{ab}	0.93±0.07^{a}	0.59±0.10^{b}	0.75±0.06^{ab}
Asn	1.94±0.24	2.05±0.08	2.28±0.16	2.10±0.18	2.12±0.19
Gln	2.53±0.37^{ab}	2.94±0.35^{ab}	2.45±0.33^{ab}	2.17±0.21^{a}	3.05±0.13^{b}
Glu	2.18±0.38	2.29±0.22	2.32±0.52	1.85±0.17	2.68±0.34
Gly	3.08±0.75	3.78±0.39	4.14±0.85	4.35±0.47	3.31±0.33
H-pro	1.78±0.33	1.62±0.15	1.94±0.18	2.20±0.31	1.95±0.36
Ile	0.17±0.03	0.20±0.02	0.21±0.05	0.18±0.04	0.25±0.09
Leu	0.36±0.06	0.36±0.08	0.35±0.10	0.31±0.07	0.47±0.15
Orn	0.12±0.02	0.11±0.01	0.12±0.05	0.15±0.01	0.15±0.03
Pro	7.82±1.54^{a}	6.82±0.82^{a}	8.39±1.64^{a}	13.69±1.51^{b}	8.12±0.55^{a}
Ser	1.99±0.25^{a}	2.13±0.23^{a}	2.26±0.19^{ab}	1.86±0.21^{a}	2.78±0.21^{b}
Trp	0.08±0.01	0.05±0.01	0.06±0.01	0.07±0.01	0.06±0.02
Tyr	0.31±0.06^{a}	0.40±0.05^{ab}	0.49±0.09^{b}	0.48±0.06^{ab}	0.42±0.05^{ab}
Val	0.38±0.07	0.41±0.03	0.45±0.06	0.38±0.07	0.50±0.10
Indispensable	20.73±0.37^{a}	21.70±0.35^{ab}	22.55±2.90^{ab}	21.85±2.68^{ab}	26.59±1.02^{b}
Dispensable	22.48±2.94^{a}	23.07±0.05^{a}	25.21±2.09^{ab}	29.65±2.18^{b}	25.71±0.79^{ab}

Table 19 The free amino acid concentration of carp muscle 48 hours after the last meal.

mmol/kg	CG	Met 0.4	Piperine	Black Pepper Extract	Curcumine
Asp	1.20	0.70	0.99	0.91	0.96
Glu	2.19	2.58	2.36	1.51	3.18
H-pro	1.93	1.90	1.78	1.94	1.31
Ser	0.78	0.85	1.13	0.65	1.06
Asn	1.91	1.77	2.04	1.75	2.01
Gly	5.03	4.95	6.35	4.70	4.54
Gln	2.43	2.07	2.68	2.04	2.61
Tau	20.30	20.64	22.16	21.39	19.30
His	12.40	11.40	16.12	13.64	14.98
Thr	1.17	1.05	1.27	1.18	1.17
Ala	2.17	1.69	2.02	1.95	1.91
Arg	0.51	0.51	0.49	0.39	0.47
Pro	5.45	5.03	3.28	8.43	2.78
Tyr	0.22	0.16	0.11	0.17	0.15
Val	0.26	0.21	0.25	0.33	0.32
Met	0.36	0.41	0.36	0.45	0.48
Cys	0.04	0.01	0.04	0.04	0.05
Ile	0.16	0.14	0.15	0.12	0.21
Leu	0.31	0.29	0.30	0.23	0.42
Phe/h-cys	0.08	0.08	0.07	0.07	0.09
Trp	0.05	0.06	0.05	0.05	0.06
Orn	0.08	0.09	0.04	0.07	0.07
Lys	4.26	3.24	3.44	4.21	3.50
Indispensable	19.56	17.40	22.51	20.68	21.71
Dispensable	21.41	19.81	20.99	22.16	19.26

Table 20 The proximate whole body analysis of carp.

In Crude Protein	Unit	Initial	CG	Met 0.4	Piperine	Black Pepper Extract	Curcumin
Dry Matter			20.4±1.8	19.0±0.7	18.6±0.4	18.7±0.8	19.1±0.6
Crude Protein	%	71.2	69.3±1.8	68.2±1.0	71.8±0.5	69.3±2.3	70.7±0.6
Ash		16.2	15.7±2.2	16.5±0.4	16.2±1.2	16.1±0.5	16.3±1.8
P		24837	20221±1211	20077±791	19020±1666	19030±797	20127±2092
K		11642	11311±763	11538±204	11462±534	11695±118	10972±306
Ca		33646	31731±3600	30358±3361	26963±1835	28349±2612	29660±4184
Mg		1352	881±92	940±63	960±133	947±54	855±73
S		7578	8270±518	8486±188	8429±484	8396±88	8286±185
Cu	µg	68.2	39.9±51.1	8.6±12.6	15.6±15.1	7.1±5.8	4.2±5.2
Fe		92.2	45.9±8.2	62.9±12.6	51.9±2.7	59.7±2.7	49.8±1.3
Mn		3.6	9.0±2.0	7.1±1.0	8.2±1.3	10.2±1.2	6.8±1.2
Mo		0.4	0.4±0.0	0.5±0.1	0.5±0.1	0.5±0.1	0.4±0.1
Na		4888	6144±500	6265±595	5714±210	5584±704	6324±478
Zn		396	291±53	326±42	361±67	304±18	322±38

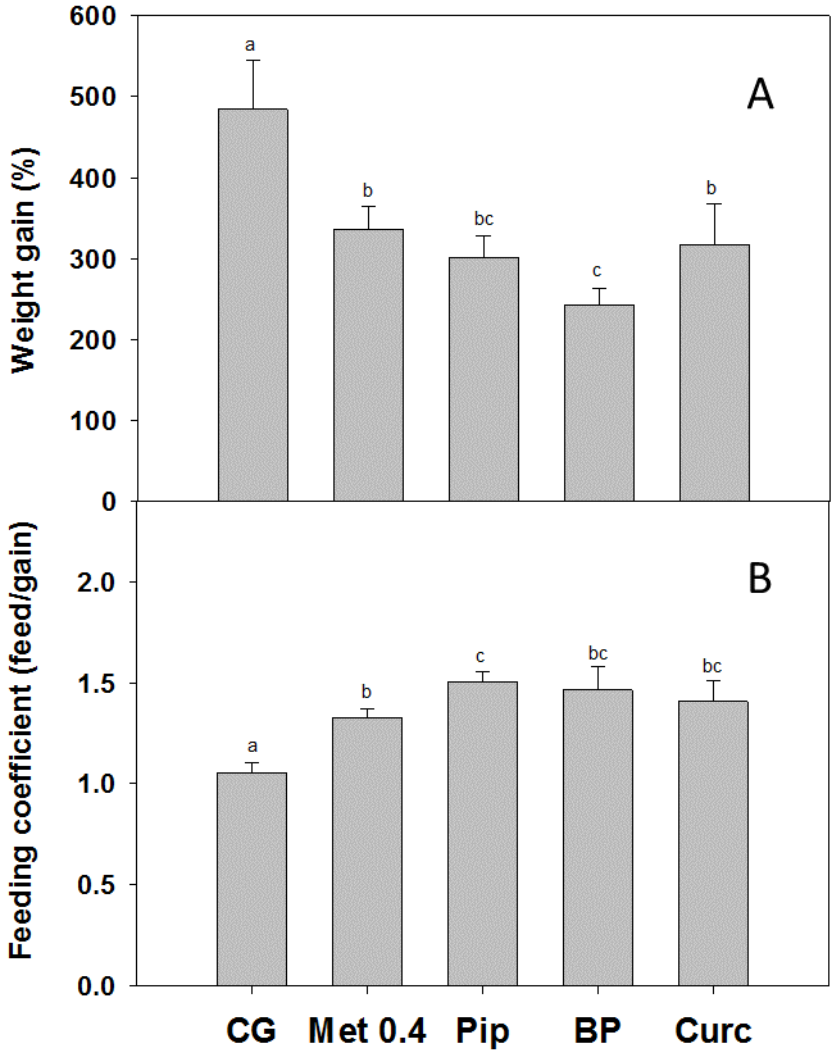

Figure 13 The weight gain (A) and feeding coefficient (B) of carp fed experimental diets. Different letters indicate statistical difference at $P<0.05$.

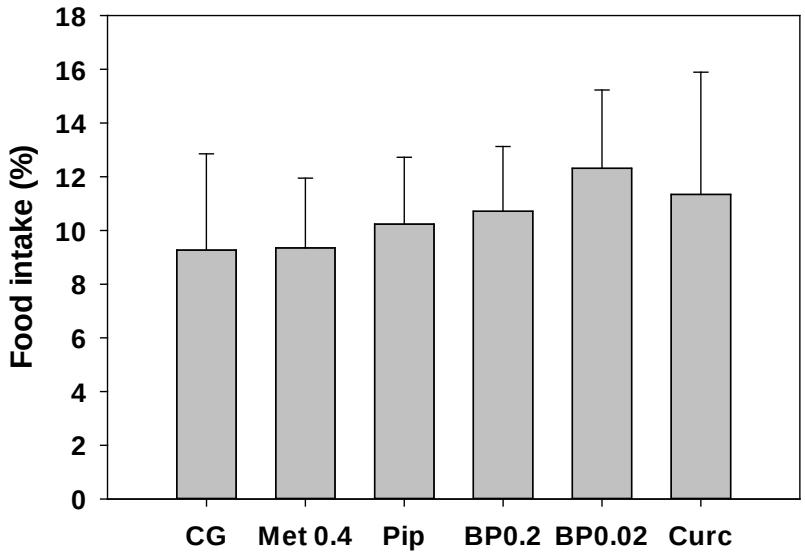

Figure 14 The daily food intake (satiation) of carp fed experimental diets.

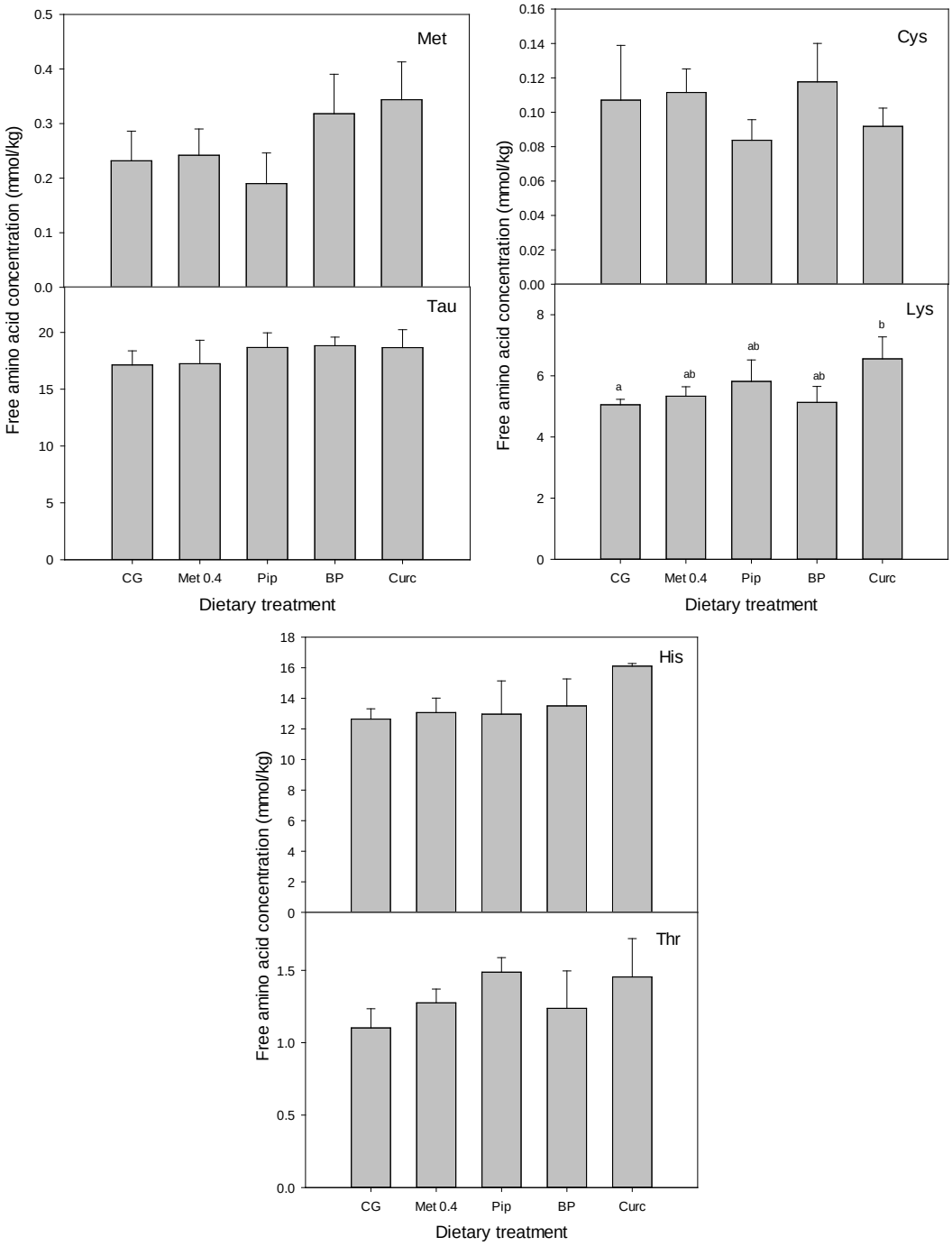

Figure 15 The concentration of free amino acids in carp muscle six hours after a meal. Different letters indicate statistical difference at $P<0.05$.

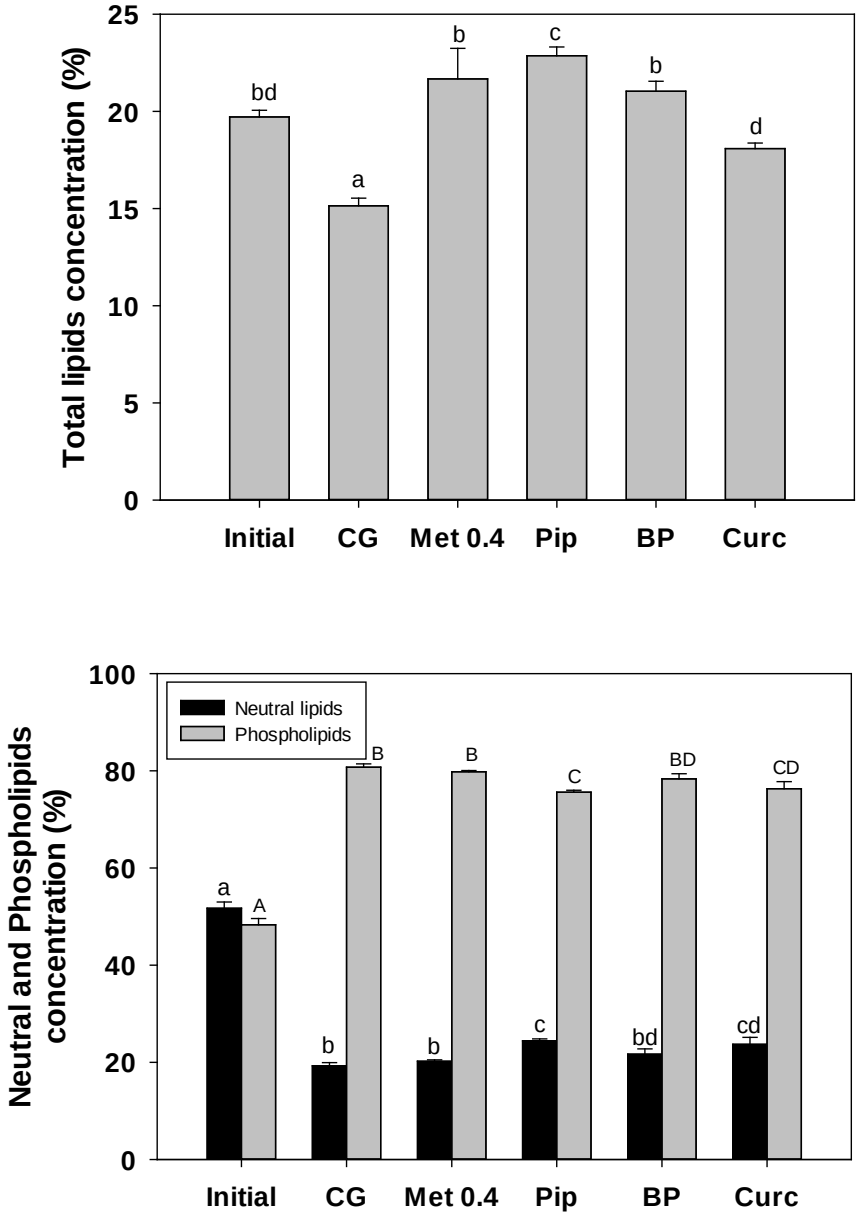

Figure 16 The concentration of lipids (% dry matter), neutral, and phospholipids (% total lipids) in carp whole body (n=3). Different letters indicate statistical difference at $P<0.05$.

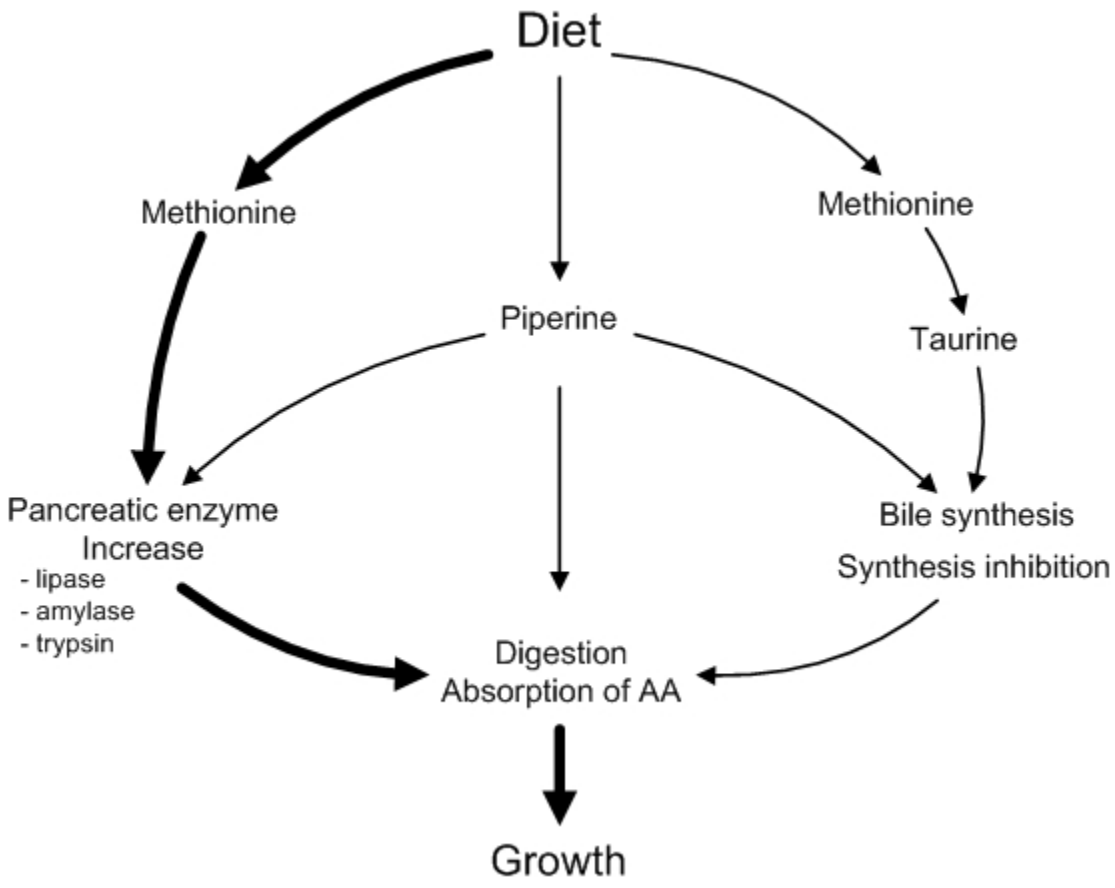

Figure 17 The effect of diet, dietary methionine, and piperine supplementation on animal metabolism.

CHAPTER 7: CONCLUSIONS

The decline of ocean caught fisheries production, together with growing demand for fishery products by increasing human population provided impetus for rapid growth in fish farming (Naylor *et al.*, 2000). Moreover, technical improvements in feeds production and development of nutritionally complete feeds enabled higher efficiency of fish culture (Hardy, 2010). Recently aquaculture provided nearly 50% of total fish production for human consumption (FAO, 2012). Although, it seems as aquaculture is a possible solution in conservation of natural fish stocks and improvement in food security in many regions of the globe, it may also be a factor contributing to the decrease of ocean fish stocks due to increasing demand for fish meal leading to environmental degradation (Naylor *et al.*, 2009).

One of the major issues in the aquaculture sustainability is the use of the already heavily harvested wild stocks for the production of fish meal and fish oil (Grigorakis and Rigos, 2011). These ingredients are used in manufacturing diets for farmed aquatic animals (Tacon and Metian, 2008). The other negative effect of aquaculture practices on environment, includes organic pollution and consequently eutrophication. This frequently can cause algal blooms, depletion of oxygen, and habitat destruction (Boesch *et al.*, 2001). Therefore, mitigating environmental impacts is a major task in order to preserve sustainability of the aquaculture industry. One of the ways to minimize waste outputs

from aquaculture operations and impact on wild stocks should be at the origin of the dilemma, that is a diet formulation (Bergheim *et al.*, 1984, Cho and Bureau, 2001). Use of alternative protein and oil sources may directly reduce use of fishmeal and fish oil in the diet formulations and eventually reduce the impact (overfishing) on wild fish populations used to produce fish meal and fish oil. However, feedstuffs of plant origin have characteristics that still make them inferior compared to fish meal in terms of suitability for use in aquatic animal feeds, protein content, indispensable amino acid profile, nutrient digestibility, antinutrients, and palatability (Hardy, 2010).

In any fish production system part of consumed feeds (25-30%) is excreted by fish as products of metabolic processes (solubles) or undigested feed components (particulates). Therefore, management of aquaculture wastes must be approached through diet formulation, improvement of feed utilization for specific farming conditions. The process begins with the selection of highly digestible ingredients which must be nutritionally well balanced in accordance with the requirements of particular fish species (Cho *et al.*, 1994, Grigorakis and Rigos, 2011).

Based on the results obtained in our project (Chapter 2) we demonstrate that plant protein based diets may support acceptable growth rate and feed utilization ratio in carp. Although the performance of fish fed with plant protein based diets was still lower compared to fish fed casein-gelatin- based diets (purified ingredients), the body weight increases around seven fold during 35 days of feeding clearly indicates high performance of carp in the present study compared to recent data in the literature (Omar et al. 2012). Furthermore, the present study showed that DL-methionine added to the diet may be lost

from fish body after diet digestion during metabolic processes. However, the DD/LL/DL/LD-methionylmethionine - a novel methionine derivative, had similar availability as DL-methionine in carp. It could have been due to less soluble and more efficient retention in fish tissues. This aspect, however, should be further investigated. Moreover, we proved that dietary protein replacement with AA mixture of the same AA profile may significantly improve carp growth performance and feed utilization ratio even in the case of high quality protein source such as casein and gelatin mixture. Furthermore, reported by many researchers, toxic effect of methionine overdose in fish (Khan et al. 2013) was not observed in our study (Chapter 5), which provides safety margin in terms of diet supplementation with this indispensable amino acid. We also found a decreased lipid deposition in carp body after diet supplementation with 0.02% curcumin suggesting possible use of this spice for improvement of diet utilization efficiency. We conclude that all the results may significantly contribute to the intensification of carp culture in order to improve growth, feed efficiency ratio, and maximize protein accretion and therefore, reduce nitrogen and phosphorus discharge to the surrounding waters.

REFERENCES

Alexis, M.N., Papaparaskeva-Papoutsoglou, E. & Theochari, V. (1985) Formulation of practical diets for rainbow trout (*Salmo gairdneri*) made by partial or complete substitution of fish meal by poultry by-products and certain plant by-products. Aquaculture, 50, 61-73.

AOAC (1995) Official methods of analysis of the Association of Official Analytical Chemists, Association of Official Analytical Chemists.

Aoe, H., Ikeda, K. & Saito, T. (1974) Nutrition of protein in young carp, 2: Nutritive value of protein hydrolyzates. Bulletin of the Japanese Society of Scientific Fisheries.

Aoe, H., Masuda, I., Abe, I., Saito, T., Toyoda, T. & Kitamura, S. (1970) Nutrition of protein in young carp. 1. Nutritive value of free amino acids. Bull. Jap. Soc. Scient. Fish., 36, 407-413.

Atal, C., Dubey, R. & Singh, J. (1985) Biochemical basis of enhanced drug bioavailability by piperine: evidence that piperine is a potent inhibitor of drug metabolism. Journal of Pharmacology and Experimental Therapeutics, 232, 258-262.

Badmaev, V., Majeed, M. & Norkus, E.P. (1999) Piperine, an alkaloid derived from black pepper increases serum response of beta-carotene during 14-days of oral beta-carotene supplementation. Nutrition research, 19, 381-388.

Badmaev, V., Majeed, M. & Prakash, L. (2000) Piperine derived from black pepper increases the plasma levels of coenzyme Q10 following oral supplementation. The Journal of nutritional biochemistry, 11, 109-113.

Bajad, S., Bedi, K., Singla, A. & Johri, R. (2001) Piperine inhibits gastric emptying and gastrointestinal transit in rats and mice. Planta medica, 67, 176-179.

Baker, D.H. (2006) Comparative species utilization and toxicity of sulfur amino acids. The Journal of nutrition, 136, 1670S-1675S.

Bergheim, A., Hustveit, H., Kittelsen, A. & Selmer-Olsen, A. (1984) Estimated pollution loadings from Norwegian fish farms. II. Investigations 1980–1981. Aquaculture, **36,** 157-168.

Bhat, B.G. & Chandrasekhara, N. (1987) Metabolic disposition of piperine in the rat. Toxicology, 44, 99-106.

Bodin, N., Delfosse, G., Nang Thu, T.T., Le Boulengé, E., Abboudi, T., Larondelle, Y. & Rollin, X. (2012) Effects of fish size and diet adaptation on growth performances and nitrogen utilization of rainbow trout (*Oncorhynchus mykiss*) juveniles given diets based on free and/or protein-bound amino acids. Aquaculture, 356, 105-115.

Boesch, D., Burroughs, R., Baker, J., Mason, R., Rowe, C. & Siefert, R. (2001) Marine pollution in the United States: Significant accomplishments, future challenges. Pew Oceans Commission, Arlington, Virginia.

Bond, D. & Duc, G. (1993) Plant breeding as a means of reducing antinutritional factors in grain legumes. Publication-European Association for Animal Production, 70, 379-379.

Bureau, D.P. & Encarnação, P.M. (2006) Adequately defining the amino acid requirements of fish: the case example of lysine. Simposium internacional de nutrición acuícola; avances en nutrición acuícola, 8, 29-54.

Burel, C., Boujard, T., Tulli, F. & Kaushik, S.J. (2000) Digestibility of extruded peas, extruded lupin, and rapeseed meal in rainbow trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima*). Aquaculture, 188, 285-298.

Castell, A., Guenter, W. & Igbasan, F. (1996) Nutritive value of peas for nonruminant diets. Animal feed science and technology, 60, 209-227.

Cheng, Z.J., Hardy, R.W. & Usry, J.L. (2003) Effects of lysine supplementation in plant protein-based diets on the performance of rainbow trout (*Oncorhynchus mykiss*) and apparent digestibility coefficients of nutrients. Aquaculture, 215, 255-265.

Chiji, H., Harayama, K. & Kiriyama, S. (1990) Effects of feeding rats low protein diets containing casein or soy protein isolate supplemented with methionine or oligo-L-methionine. The Journal of nutrition, 120, 166-171.

Cho, C. & Bureau, D. (2001) A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. Aquaculture Research, **32,** 349-360.

Cho, C., Hynes, J., Wood, K. & Yoshida, H. (1994) Development of high-nutrient-dense, low-pollution diets and prediction of aquaculture wastes using biological approaches. Aquaculture, **124,** 293-305.

Cohen, S.A., Meys, M. & Tarvin, T.L. (1989) The pico-tag method: a manual of advanced techniques for amino acid analysis, Millipore Corporation.

Commission Directive. (2000). Establishing community methods for the determination of vitamin A, vitamin E and tryptophan, annex part C. Determination of Tryptophan. Offic. J. L174, 45-50.

Commission Directive. 1998. Establishing community methods for 434 the determination of amino acids, crude oils and fats, and olan-quindox in feeding stuff and amending Directive 71/393/EEC, annex part A. Determination of Amino Acids. Offic. J. L257, 14-23.

Cowey, C. & Walton, M. (1988) Studies on the uptake of (^{14}C) amino acids derived from both dietary (^{14}C) protein and dietary (^{14}C) amino acids by rainbow trout, *Salmo gairdneri* Richardson. Journal of fish biology, 33, 293-305.

Cowey, C. (1994) Amino acid requirements of fish: a critical appraisal of present values. Aquaculture, 124, 1-11.

Cowey, C. (1995) Protein and amino acid requirements: a critique of methods. Journal of Applied Ichthyology, 11, 199-204.

Cowey, C.B., Cho, C.Y., Sivak, J.G., Weerheim, J.A. & Stuart, D.D. (1992) Methionine intake in rainbow trout (*Oncorhynchus mykiss*), relationship to cataract formation and the metabolism of methionine. The Journal of nutrition, 122, 1154-1163.

Cowey, C.B., Sargent, J.R. (1979) Nutrition, Fish Physiol., VIII, 1–69.

Dabrowski, K. (1983) Comparative aspects of protein digestion and amino acid absorption in fish and other animals. Comparative Biochemistry and Physiology Part A: Physiology, 74, 417-425.

Dabrowski, K., Zhang, Y.F., Kwasek, K., Hliwa, P. & Ostaszewska, T. (2010) Effects of protein-peptide-and free amino acid-based diets in fish nutrition. Aquaculture Research, 41, 668-683.

Davies, S. & Gouveia, A. (2010) Response of common carp fry fed diets containing a pea seed meal (*Pisum sativum*) subjected to different thermal processing methods. Aquaculture, 305, 117-123.

Davies, S., Morris, P. & Baker, R. (1997) Partial substitution of fish meal and full-fat soya bean meal with wheat gluten and influence of lysine supplementation in diets for rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture Research, 28, 317-328.

De la Higuera, M., Garzón, A., Hidalgo, M., Peragón, J., Cardenete, G. & Lupiáñez, J. (1998) Influence of temperature and dietary-protein supplementation either with free or coated lysine on the fractional protein-turnover rates in the white muscle of carp. Fish Physiology and Biochemistry, 18, 85-95.

Deng, J., Kong, L., An, Q., Bi, B., Tao, L. & Zhang, X. (2011) Effect of dietary pH adjustment on the utilization of supplemental methionine and lysine by juvenile common carp, *Cyprinus carpio*. Journal of the World Aquaculture Society, 42, 696-704.

Directive, C. (1998) Establishing community methods for 434 the determination of amino acids, crude oils and fats, and olan-quindox in feeding stuff and amending Directive 71/393/EEC, annex part A. Determination of Amino Acids. In Official Journal of the European Communities, 14-23.

Dunstan, J., Simmer, K., Dixon, J., & Prescott, S. (2008) Cognitive assessment of children at age 2½ years after maternal fish oil supplementation in pregnancy: a randomised controlled trial. Archives of Disease in Childhood Fetal and Neonatal Edition, 93, 45-50.

Dupree, H.K. & Halver, J.E. (1970) Amino acids essential for the growth of channel catfish, Ictalurus punctatus. Transactions of the American fisheries Society, 99, 90-92.

Eisenberg, D., Weiss, R.M., Terwilliger, T.C. & Wilcox, W. (1982) Hydrophobic moments and protein structure In Faraday Symposia of the Chemical Society, 17, 109-120. Royal Society of Chemistry.

El Idrissi, A., Boukarrou, L. & L'Amoreaux, W. (2009) Taurine supplementation and pancreatic remodeling In Taurine 7, pp. 353-358. Springer.

FAO, 2012: FAOSTAT. In: FAO (ed.). Rome, Italy.

Fau, D., Delhomme, B., Bourdon, D. & Rerat, A. (1980) Liver enzyme activities and muscle nucleic acids in pigs fed an excess methionine diet for 100 days. CR Acad. Sci. Paris, 291, 565-568.

Fau, D., Smalley, K.A., Morris, J.G. & Rogers, Q.R. (1987) Effect of excess dietary methionine on weight gain and plasma amino acids in kittens. The Journal of nutrition, 117, 1838-1843.

Folch, J., Lees, M. & Sloane-Stanley, G. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. biol. Chem, 226, 497-509.

Francis, G., Makkar, H.P. & Becker, K. (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture, 199, 197-227.

Friedman, M. & Gumbmann, M.R. (1988) Nutritional value and safety of methionine derivatives, isomeric dipeptides and hydroxy analogs in mice. The Journal of nutrition, 118, 388-397.

Friedman, M., 1996: Nutritional value of proteins from different food sources. A review. Journal of Agricultural and Food Chemistry, 44, 6-29.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å. & Nelson, R. (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquaculture Research, 38, 551-579.

Glatzel, H. (1966) Physiological aspects of flavour compounds. Bibliotheca nutritio et dieta, 9, 71-86.

Goff, J.B. & Gatlin III, D.M. (2004) Evaluation of different sulfur amino acid compounds in the diet of red drum, *Sciaenops ocellatus*, and sparing value of cystine for methionine. Aquaculture, 241, 465-477.

Gouveia, A. & Davies, S. (1998) Preliminary nutritional evaluation of pea seed meal (Pisum sativum) for juvenile European sea bass (*Dicentrarchus labrax*). Aquaculture, 166, 311-320.

Govindarajan, V. & Stahl, W.H. (1977) Pepper-chemistry, technology, and quality evaluation. Critical Reviews in Food Science and Nutrition, 9, 115-225.

Griffiths, G., Trueman, L., Crowther, T., Thomas, B. & Smith, B. (2002) Onions-a global benefit to health. Phytotherapy Research, 16, 603-615.

Grigorakis, K. & Rigos, G. (2011) Aquaculture effects on environmental and public welfare-the case of Mediterranean mariculture. Chemosphere, 85**,** 899-919.

Haeussner, T., Kobler, C. & Weckbecker, C. (2009) Preparation and use of methionylmethionine as feed additive for fish and crustaceans. Google Patents.

Haeussner, T., Kobler, C. & Weckbecker, C. (2011) Cyclic dipeptides as feed additives. Google Patents.

Halver, J.E., DeLong, D.C. & Mertz, E.T. (1957) Nutrition of Salmonoid Fishes V. Classification of Essential Amino Acids for Chinook Salmon. The Journal of nutrition, 63, 95-105.

Hara, H., Kiriyama, S. & Kasai, T. (1997) Supplementation of methionine to a low soybean protein diet strikingly increases pancreatic amylase activity in rats. Journal of nutritional science and vitaminology, 43, 161-166.

Hara, H., Kiriyama, S. & Kasai, T. (1997) Supplementation of methionine to a low soybean protein diet strikingly increases pancreatic amylase activity in rats. Journal of nutritional science and vitaminology, 43, 161-166.

Hardy, R. W., 2010: Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquaculture Research, 41, 770-776.

Hauler, R. & Carter, C. (2001a) Lysine deposition responds linearly to marginal lysine intake in Atlantic salmon (Salmo salar L.) parr. Aquaculture Research, 32, 147-156.

Hauler, R.C. & Carter, C.G. (2001b) Reevaluation of the quantitative dietary lysine requirements of fish. Reviews in Fisheries Science, 9, 133-163.

Hawkins, W., Hanson, P., Coon, R. & Terry, R. (1949) Bile salt metabolism as influenced by pure amino acids and casein digests. The Journal of experimental medicine, 90, 461.

Jackson, A. & Capper, B. (1982) Investigations into the requirements of the tilapia Sarotherodon mossambicus for dietary methionine, lysine and arginine in semi-synthetic diets. Aquaculture, 29, 289-297.

Johnson, J.J., Nihal, M., Siddiqui, I.A., Scarlett, C.O., Bailey, H.H., Mukhtar, H. & Ahmad, N. (2011) Enhancing the bioavailability of resveratrol by combining it with piperine. Molecular nutrition & food research, 55, 1169-1176.

Johri, R.K., Thusu, N., Khajuria, A. & Zutshi, U. (1992) Piperine-mediated changes in the permeability of rat intestinal epithelial cells: The status of γ-glutamyl transpeptidase activity, uptake of amino acids and lipid peroxidation. Biochemical pharmacology, 43, 1401-1407.

Kang, K.S., Yahashi, S., Azuma, M., Sakashita, A., Shioda, S. & Matsuda, K. (2011) Effect of intraperitoneal injection of curcumin on food intake in a goldfish model. Journal of Molecular Neuroscience, 45, 172-176.

Kasumyan, A. & Morsi, A.K. (1996) Taste sensitivity of common carp *Cyprinus carpio* to free amino acids and classical taste substances. Journal of Ichthyology, 36, 391-403.

Katz, R.S. & Baker, D.H. (1975) Methionine toxicity in the chick: nutritional and metabolic implications. The Journal of nutrition, 105, 1168-1175.

Kaushik, S.J. & Dabrowski, K. (1983) Nitrogen and energy-utilization in juvenile carp (*Cyprinus-carpio*) fed casein, amino-acids or a protein-free diet. Reproduction Nutrition Development, 23, 741-754.

Kawada, T., Sakabe, S.-I., Watanabe, T., Yamamoto, M. & Iwai, K. (1988) Some pungent principles of spices cause the adrenal medulla to secrete catecholamine in anesthetized rats. Experimental Biology and Medicine, 188, 229-233.

Keembiyehetty, C. & Gatlin III, D. (1995) Evaluation of different sulfur compounds in the diet of juvenile sunshine bass (*Morone chrysops*♀ × *M. saxatilis*♂). Comparative Biochemistry and Physiology Part A: Physiology, 112, 155-159.

Keembiyehetty, C.N. & Gatlin III, D.M. (1993) Total sulfur amino acid requirement of juvenile hybrid striped bass (*Morone chrysops*× *M. saxatilis*). Aquaculture, 110, 331-339.

Keembiyehetty, C.N. & Gatlin III, D.M. (1993) Total sulfur amino acid requirement of juvenile hybrid striped bass (*Morone chrysops*× *M. saxatilis*). Aquaculture, 110, 331-339.

Keembiyehetty, C.N. & Gatlin III, D.M. (1997) Performance of sunshine bass fed soybean-meal-based diets supplemented with different methionine compounds. The Progressive fish-culturist, 59, 25-30.

Keith, M., Youngs, C. & McLaughlan, J. (1977) The Supplementation of Pea Protein Concentrate with DL-Methionine or Methionine Hydroxy Analog. Canadian Institute of Food Science and Technology Journal, 10, 1-4.

Kelloff, G.J., Crowell, J.A., Hawk, E.T., Steele, V.E., Lubet, R.A., Boone, C.W., Covey, J.M., Doody, L.A., Omenn, G.S. & Greenwald, P. (1996) Strategy and planning for chemopreventive drug development: clinical development plans II. Journal of Cellular Biochemistry, 63, 54-71.

Khan, M.A. & Abidi, S.F. (2013) Dietary methionine requirement of Indian major carp fry, Cirrhinus mrigala (Hamilton) based on growth, feed conversion and nitrogen retention efficiency. Aquaculture Research, 44, 268-281.

Kim, B., Lindemann, M., Rademacher, M., Brennan, J. & Cromwell, G. (2006) Efficacy of dl-methionine hydroxy analog free acid and DL-methionine as methionine sources for pigs. Journal of animal science, 84, 104-111.

Kim, K.-I., Grimshaw, T.W., Kayes, T.B. & Amundson, C.H. (1992a) Effect of fasting or feeding diets containing different levels of protein or amino acids on the activities of the liver amino acid-degrading enzymes and amino acid oxidation in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 107, 89-105.

Kim, K.-I., Kayes, T.B. & Amundson, C.H. (1992b) Requirements for lysine and arginine by rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 106, 333-344.

Kim, K.-I., Kayes, T.B. & Amundson, C.H. (1992c) Requirements for sulfur amino acids and utilization of D-methionine by rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 101, 95-103.

Kim, S.-K., Matsunari, H., Takeuchi, T., Yokoyama, M., Murata, Y. & Ishihara, K. (2007) Effect of different dietary taurine levels on the conjugated bile acid composition and growth performance of juvenile and fingerling Japanese flounder *Paralichthys olivaceus*. Aquaculture, 273, 595-601.

Klavins, J., Kinney, T. & Kaufman, N. (1963) Histopathologic changes in methionine excess. Archives of pathology, 75, 661.

Knudsen, K.E.B. (1997) Carbohydrate and lignin contents of plant materials used in animal feeding. Animal feed science and technology, 67, 319-338.

Kris-Etherton, P. M., W. S. Harris and L. J. Appel, 2003: Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Arteriosclerosis, thrombosis, and vascular biology, 23, 20-30.

Kumar, A., Raman, R., Kumar, K., Pandey, P., Kumar, V., Mohanty, S. & Kumar, S. (2012) Antiparasitic efficacy of piperine against *Argulus* spp. on *Carassius auratus* (Linn. 1758): in vitro and in vivo study. Parasitology research, 111, 2071-2076.

Kunchandy, E. & Rao, M. (1990) Oxygen radical scavenging activity of curcumin. International Journal of Pharmaceutics, 58, 237-240.

Kwasek, K., Zhang, Y. & Dabrowski, K. (2010) Utilization of dipeptide/protein based diets in larval and juvenile Koi carp–post-prandial free amino acid levels. Journal of Animal Physiology and Animal Nutrition, 94, 35-43.

Lambert, J.D., Hong, J., Kim, D.H., Mishin, V.M. & Yang, C.S. (2004) Piperine enhances the bioavailability of the tea polyphenol (−)-epigallocatechin-3-gallate in mice. The Journal of nutrition, 134, 1948-1952.

Lee, E.B., Shin, K.H. & Woo, W.S. (1984) Pharmacological study on piperine. Archives of Pharmacal Research, 7, 127-132.

Li, P., Burr, G.S., Wen, Q., Goff, J.B., Murthy, H.S. & Gatlin III, D.M. (2009) Dietary sufficiency of sulfur amino acid compounds influences plasma ascorbic acid concentrations and liver peroxidation of juvenile hybrid striped bass (*Morone chrysops*× *M. saxatilis*). Aquaculture, 287, 414-418.

Li, X., Rezaei, R., Li, P. & Wu, G. (2011) Composition of amino acids in feed ingredients for animal diets. Amino acids, 40, 1159-1168.

Llames, C. R., and J. Fontaine. (1994) Determination of amino acids in feeds: Collaborative study. J. Assoc. Off. Anal. Chem. 77:1362–1402.

Marcouli, P., Alexis, M., Andriopoulou, A. & Iliopoulou-Georgudaki, J. (2004) Development of a reference diet for use in indispensable amino acid requirement studies of gilthead seabream Sparus aurata L. Aquaculture Nutrition, 10, 335-343.

Marui, T., Harada, S. & Kasahara, Y. (1983) Gustatory specificity for amino acids in the facial taste system of the carp, *Cyprinus carpio* L. Journal of comparative physiology, 153, 299-308.

Mazid, M., Tanaka, Y., Katayama, T., Simpson, K. & Chichester, C. (1978) Metabolism of amino acids in aquatic animals, 3: Indispensable amino acids for *Tilapia zillii*. Bulletin of the Japanese Society of Scientific Fisheries.

Melcion, J. & Van der Poel, A. (1993) Process technology and antinutritional factors: principles, adequacy and process optimization.

Mertz, E.T. (1972) The protein and amino acid needs. Fish Nutrition, 143.

Meyer, G. & Fracalossi, D.M. (2004) Protein requirement of jundia fingerlings, Rhamdia quelen, at two dietary energy concentrations. Aquaculture, 240, 331-343.

Moon, H.Y. & Gatlin III, D.M. (1991) Total sulfur amino acid requirement of juvenile red drum, Sciaenops ocellatus. Aquaculture, 95, 97-106.

Murai, T. & Ogata, H. (1990) Changes in free amino acid levels in various tissues of common carp in response to insulin injection followed by force-feeding an amino acid diet. The Journal of nutrition, 120, 711-718.

Murai, T., Akiyama, T. & Nose, T. (1980) Use of crystalline amino acids coated with casein in diets for carp. Bulletin of the Japanese Society of Scientific Fisheries, 47, 523-527.

Murai, T., Akiyama, T. & Nose, T. (1983) Effects of glucose chain length of various carbohydrates and frequency of feeding on their utilization by fingerling carp. Bulletin of the Japanese Society of Scientific Fisheries, 49.

Murai, T., Akiyama, T. & Nose, T. (1984a) Effect of amino-acid balance on efficiency in utilization of diet by fingerling carp. Bulletin of the Japanese Society of Scientific Fisheries, 50, 893-897.

Murai, T., Akiyama, T., Nose, T. & Hirasawa, Y. (1983) Effects of dietary pH and electrolyte concentration on utilization of crystalline amino acids by fingerling carp. Bulletin of the Japanese Society of Scientific Fisheries (Japan).

Murai, T., Akiyama, T., Ogata, H., Nose, T. & Hirasawa, Y. (1982) Effect of coating amino acids with casein supplemented to gelatin diet on plasma free amino acids of carp [Cyprinus carpio]. Bulletin of the Japanese Society of Scientific Fisheries (Japan).

Murai, T., Daozun, W. & Ogata, H. (1989) Supplementation of methionine to soy flour diets for fingerling carp, *Cyprinus carpio*. Aquaculture, 77, 373-385.

Murai, T., Ogata, H. & NosE, T. (1982) Methionine coated with various materials supplemented to soybean meal diet for fingerling carp *Cyprinus carpio* and channel catfish *Ictalurus punctatus*. Bulletin of the Japanese Society of Scientific Fisheries.

Murai, T., Ogata, H., Nose, T., Takeuchi, T. & Watanabe, T. (1984b) Composition of free amino acid in excretion of carp (*Cyprinus carpio*) fed amino acid diets and casein-gelatin diet. Bulletin of the Japanese Society of Scientific Fisheries, 50.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C., Clay, J., Folke, C., Lubchenco, J., Mooney, H. & Troell, M. (2000) Effect of aquaculture on world fish supplies. Nature, 405, 1017-1024.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J. & Hua, K. (2009) Feeding aquaculture in an era of finite resources. Proceedings of the National Academy of Sciences, 106, 15103-15110.

Ng, W.K., Hung, S.S. & Herold, M.A. (1996) Poor utilization of dietary free amino acids by white sturgeon. Fish Physiology and Biochemistry, 15, 131-142.

Nolles, J., Verreijen, A., Koopmanschap, R., Verstegen, M. & Schreurs, V. (2009) Postprandial oxidative losses of free and protein-bound amino acids in the diet: interactions and adaptation. Journal of Animal Physiology and Animal Nutrition, 93, 431-438.

Nose, T. (1979) Summary report on the requirements of essential amino acids for carp In Symposium on Finfish Nutrition and Feed Technology, Hamburg (Germany, FR), 20 Jun 1978.

Nose, T., Arai, S., Lee, D. & Hashimoto, Y. (1974) A note on amino acids essential for growth of young carp. Bulletin of the Japanese Society of Scientific Fisheries.

NRC (1993) National Research Council Nutrient Requirements of Fish, National Academies Press, Washington, DC.

NRC (2011) Nutrient Requirements of Fish and Shrimp, The National Academies Press.

Nwanna, L., Lemme, A., Metwally, A. & Schwarz, F. (2012) Response of common carp (*Cyprinus carpio* L.) to supplemental DL-Methionine and different feeding strategies. Aquaculture.

Ogata, H. (1986) Correlations of essential amino acid patterns between the dietary protein and the blood, hepatopancreas, or skeletal muscle in carp (*Cyprinus carpio*). Bulletin of the Japanese Society of Scientific Fisheries, 52.

Ogino, C. (1980) Requirements of carp and rainbow trout for essential amino acids. Bulletin of the Japanese Society of Scientific Fisheries, 46, 171-174.

Okumura, Y., M. Narukawa and T. Watanabe, 2010: Adiposity suppression effect in mice due to black pepper and its main pungent component, piperine. Bioscience, biotechnology, and biochemistry, 74, 1545-1549.

Omar, S.S., Merrifield, D.L., Kühlwein, H., Williams, P.E. & Davies, S.J. (2012) Biofuel derived yeast protein concentrate (YPC) as a novel feed ingredient in carp diets. Aquaculture, 330, 54-62.

Øverland, M., Sørensen, M., Storebakken, T., Penn, M., Krogdahl, Å. & Skrede, A. (2009) Pea protein concentrate substituting fish meal or soybean meal in diets for Atlantic salmon (*Salmo salar*)-Effect on growth performance, nutrient digestibility, carcass composition, gut health, and physical feed quality. Aquaculture, 288, 305-311.

Peres, H. & Oliva-Teles, A. (2005) The effect of dietary protein replacement by crystalline amino acid on growth and nitrogen utilization of turbot Scophthalmus maximus juveniles. Aquaculture, 250, 755-764.

Pérez-Jiménez, A., Peres, H. & Oliva-Teles, A. (2014) Effective replacement of protein-bound amino acids by crystalline amino acids in Senegalese sole (Solea senegalensis) juveniles. Aquaculture Nutrition, 20, 60-68.

Pezeshk, S., Rezaei, M. & Hosseini, H. (2011) Effects of Turmeric, Shallot Extracts, and Their Combination on Quality Characteristics of Vacuum-Packaged Rainbow Trout Stored at 4±1° C. Journal of food science, 76, M387-M391.

Plakas, S.M., Katayama, T., Tanaka, Y. & Deshimaru, O. (1980) Changes in the levels of circulating plasma free amino acids of carp (Cyprinus carpio) after feeding a protein and an amino acid diet of similar composition. Aquaculture, 21, 307-322.

Platel, K. & Srinivasan, K. (1996) Influence of dietary spices or their active principles on digestive enzymes of small intestinal mucosa in rats. International journal of food sciences and nutrition, 47, 55-59.

Platel, K. & Srinivasan, K. (2000) Influence of dietary spices and their active principles on pancreatic digestive enzymes in albino rats. Food Nahrung, 44, 42-46.

Poston, H.A. (1986) Response of rainbow trout to source and level of supplemental dietary methionine. Comparative Biochemistry and Physiology Part A: Physiology, 83, 739-744.

Prakash, U.N. & Srinivasan, K. (2012) Fat digestion and absorption in spice-pretreated rats. Journal of the Science of Food and Agriculture, 92, 503-510.

Pulla Reddy, A.C. & Lokesh, B. (1994) Effect of dietary turmeric (*Curcuma longa*) on iron-induced lipid peroxidation in the rat liver. Food and chemical Toxicology, 32, 279-283.

Reanmongkol, W., Janthasoot, W., Wattanatorn, W., Dhumma-Upakorn, P. & Chudapongse, P. (1988) Effects of piperine on bioenergetic functions of isolated rat liver mitochondria. Biochemical pharmacology, 37, 753-757.

Rees, W.D., Wilson, F.A. & Maloney, C.A. (2006) Sulfur amino acid metabolism in pregnancy: the impact of methionine in the maternal diet. The Journal of nutrition, 136, 1701-1705.

Regost, C., Arzel, J. & Kaushik, S. (1999) Partial or total replacement of fish meal by corn gluten meal in diet for turbot (*Psetta maxima*). Aquaculture, 180, 99-117.

Robinson, E.H., Allen Jr, O.W., Poe, W.E. & Wilson, R.P. (1978) Utilization of dietary sulfur compounds by fingerling channel catfish: L-methionine, DL-methionine, methionine hydroxy analogue, taurine and inorganic sulfate. The Journal of nutrition, 108, 1932-1936.

Rodehutscord, M., Mandel, S., Pack, M., Jacobs, S. & Pfeffer, E. (1995) Free amino acids can replace protein-bound amino acids in test diets for studies in rainbow trout (*Oncorhynchus mykiss*). The Journal of nutrition, 125, 956-963.

Ruby, A., Kuttan, G., Dinesh Babu, K., Rajasekharan, K. & Kuttan, R. (1995) Anti-tumour and antioxidant activity of natural curcuminoids. Cancer letters, 94, 79-83.

Rumsey, G.L., Page, J.W. & Scott, M.L. (1983) Methionine and cystine requirements of rainbow trout. The Progressive fish-culturist, 45, 139-143.

Santiago, C.B. & Lovell, R.T. (1988) Amino acid requirements for growth of Nile tilapia. The Journal of nutrition, 118, 1540-1546.

Sargent, J., 1997: Fish oils and human diet. British Journal of Nutrition, 78, S5-S13.

Sauberlich, H. (1961) Studies on the toxicity and antagonism of amino acids for weanling rats. The Journal of nutrition, 75, 61-72.

Savolainen, L.C. & Gatlin III, D.M. (2010) Evaluation of sulfur amino acid and zinc supplements to soybean meal based diets for hybrid striped bass. Aquaculture, 307, 260-265.

Schulz, C., Wickert, M., Kijora, C., Ogunji, J. & Rennert, B. (2007) Evaluation of pea protein isolate as alternative protein source in diets for juvenile tilapia (*Oreochromis niloticus*). Aquaculture Research, 38, 537-545.

Schwarz, F.J., Kirchgessner, M. & Deuringer, U. (1998) Studies on the methionine requirement of carp (*Cyprinus carpio* L.). Aquaculture, 161, 121-129.

Sharma, O. (1976) Antioxidant activity of curcumin and related compounds. Biochemical pharmacology, 25, 1811-1812.

Shearer, K. (2000) Experimental design, statistical analysis and modelling of dietary nutrient requirement studies for fish: a critical review. Aquaculture Nutrition, 6, 91-102.

Shinozuka, H., Estes, L.W. & Farber, E. (1971) Studies on Acute Methionine Toxicity: I. Nucleolar Disaggregation in Guinea Pig Hepatic Cells with Methionine or Ethionine and Its Reversal with Adenine. The American journal of pathology, 64, 241.

Shoveller, A., Moehn, S., Rademacher, M., Htoo, J. & Ball, R. (2010) Methionine-hydroxy analogue was found to be significantly less bioavailable compared to DL-methionine for protein deposition in growing pigs. Animal, 4, 61-66.

Simmons, L., Moccia, R., Bureau, D., Sivak, J. & Herbert, K. (1999) Dietary methionine requirement of juvenile Arctic charr *Salvelinus alpinus* (L.). Aquaculture Nutrition, 5, 93-100.

Simmons, L., Moccia, R., Waddy, S. & Fréchette, M. (1997) Dietary methionine requirement of juvenile Arctic charr (*Salvelinus alpinus*). Bulletin of the Aquaculture Association of Canada, 36-38.

Srinivasan, K. (2005) Role of spices beyond food flavoring: Nutraceuticals with multiple health effects. Food Reviews International, 21, 167-188.

Srinivasan, K. (2007) Black pepper and its pungent principle-piperine: a review of diverse physiological effects. Critical reviews in food science and nutrition, 47, 735-748.

Stickney, R.R. (1994) Principles of aquaculture, John Wiley and Sons, Inc.

Suresh, D. & Srinivasan, K. (2007) Studies on the in vitro absorption of spice principles–Curcumin, capsaicin and piperine in rat intestines. Food and chemical Toxicology, 45, 1437-1442.

Sveier, H., Nordas, H., Berge, G. & Lied, E. (2001) Dietary inclusion of crystalline D- and L-methionine: effects on growth, feed and protein utilization, and digestibility in small and large Atlantic salmon (*Salmon salar* L.). Aquaculture Nutrition, 7, 169-181.

Szallasi, A. (2005) Piperine: researchers discover new flavor in an ancient spice. Trends in pharmacological sciences, 26, 437-439.

Tacon, A.G. & Metian, M. (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture,* **285,** 146-158.

Terjesen, B. F., K.-J. Lee, Y. Zhang, M. Failla and K. Dabrowski, 2006: Optimization of dipeptide–protein mixtures in experimental diet formulations for rainbow trout (*Oncorhynchus mykiss*) alevins. Aquaculture, 254**,** 517-525.

Terjesen, B.F., Lee, K.-J., Zhang, Y., Failla, M. & Dabrowski, K. (2006) Optimization of dipeptide–protein mixtures in experimental diet formulations for rainbow trout (*Oncorhynchus mykiss*) alevins. Aquaculture, 254, 517-525.

Terjesen, B.F., Park, K., Tesser, M.B., Portella, M.C., Zhang, Y. & Dabrowski, K. (2004) Lipoic acid and ascorbic acid affect plasma free amino acids selectively in the teleost fish pacu (Piaractus mesopotamicus). The Journal of nutrition, 134, 2930-2934.

Todd, W., Laastuen, L. & Thomas, A. (1967) Effect of amino acid imbalance on liver glycogen levels in young salmon. Comparative biochemistry and physiology, 23, 431-435.

Ueki, I., Roman, H.B., Hirschberger, L.L., Junior, C. & Stipanuk, M.H. (2012) Extrahepatic tissues compensate for loss of hepatic taurine synthesis in mice with liver-specific knockout of cysteine dioxygenase. American Journal of Physiology-Endocrinology And Metabolism, 302, 1292.

Valencia, D., Serrano, M., Jiménez-Moreno, E., Lázaro, R. & Mateos, G. (2009) Ileal digestibility of amino acids of pea protein concentrate and soya protein sources in broiler chicks. Livestock science, 121, 21-27.

Walton, M.J., Cowey, C.B. & Adron, J.W. (1982) Methionine metabolism in rainbow trout fed diets of differing methionine and cystine content. The Journal of nutrition, 112, 1525-1535.

Warrier, P. (1981) The importance of black pepper in Ayurveda. Indian Spices, 18.

Watson, M., Isaac, R. & Westerman, R. (1990) Analytical instruments for soil and plant analysis. Soil testing and plant analysis., 691-740.

Wilson, E.A. & Demmig-Adams, B. (2007) Antioxidant, anti-inflammatory, and antimicrobial properties of garlic and onions. Nutrition and food science, 37, 178-183.

Wilson, R. & Cowey, C. (1985) Amino acid composition of whole body tissue of rainbow trout and atlantic salmon. Aquaculture, 48, 373-376.

Wilson, R.P. (2002) Amino acids and proteins. Fish Nutrition, 143.

Wilson, R.P., Harding, D.E. & Garling Jr, D.L. (1977) Effect of dietary pH on amino acid utilization and the lysine requirement of fingerling channel catfish. The Journal of nutrition, 107, 166.

Yamada, S. & Yone, Y. (1986) Loss of dietary amino acids during mastication by carp (*Cyprinus carpio*). Bulletin of the Japanese Society of Scientific Fisheries (Japan).

Yamada, S., Tanaka, Y. & Katayama, T. (1981a) Feeding experiments with carp fry fed an amino acid diet by increasing the number of feedings per day. Bulletin of the Japanese Society of Scientific Fisheries.

Yamada, S., Tanaka, Y., Katayama, T., Sameshima, M. & Simpson, K.L. (1982) Plasma amino acid changes in *Tilapia nilotica* fed a casein and a corresponding free amino aicd diet. Bulletin of the Japanese Society of Scientific Fisheries (Japan).

Yúfera, M., Kolkovski, S., Fernández-Dıaz, C. & Dabrowski, K. (2002) Free amino acid leaching from a protein-walled microencapsulated diet for fish larvae. Aquaculture, 214, 273-287.

Yurkowski, M. & Tabachek, J. (1979) Proximate and amino acid composition of some natural fish foods In Proceedings of the World Symposium on Finfish Nutrition and Fishfeed Technology, Vol. 1, pp. 435-448.

Zhang, Y., Dabrowski, K., Hliwa, P. & Gomulka, P. (2006) Indispensable amino acid concentrations decrease in tissues of stomachless fish, common carp in response to free amino acid-or peptide-based diets. Amino acids, 31, 165-172.

Zhou, Q.-C., Wu, Z.-H., Tan, B.-P., Chi, S.-Y. & Yang, Q.-H. (2006) Optimal dietary methionine requirement for Juvenile Cobia (*Rachycentron canadum*). Aquaculture, 258, 551-557.