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Indispensable amino acid concentrations decrease in tissues of stomachless fish, common carp in response to free amino acid- or peptide-based diets

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Summary. The premise that free amino acid or dipeptide based diets will resolve the nutritional inadequacy of formulated feeds for larval and juvenile fish and improve utilization of nitrogen in comparison to protein-based diets was tested in stomachless fish, common carp (*Cyprinus carpio* L.) larvae. We examined the postprandial whole body free amino acid (FAA) pool in fish that were offered a FAA mixture based diet for the duration of 2 or 4 weeks. We found that the total amount and all indispensable amino acids concentrations in the whole body decreased after a meal. We then fed juvenile carp with dietary amino acids provided in the FAA, dipeptide (PP), or protein (live feed organisms; brine shrimp *Artemia salina* nauplii, AS) forms. Histidine concentrations in the whole fish body increased in all dietary groups after feeding whereas all other indispensable amino acids decreased in FAA and PP groups in comparison to the AS group. Taurine appears to be the major osmotic pressure balancing free amino acid in larval freshwater fish which may indicate a conditional requirement. We present the first evidence in larval fish that in response to synthetic FAA and PP diets, the whole body indispensable free AA concentrations decreased after feeding. This study shows that amino acids given entirely as FAA or PP cannot sustain stomachless larval fish growth, and may result in depletion of body indispensable AA and most of dispensable AA. The understanding of these responses will determine necessary changes in diet formulations that prevent accelerated excretion of amino acids without protein synthesis.

Keywords: Protein – Dipeptide – Amino acids – Stomachless fish – Teleost

Abbreviations: DAA, dispensable amino acids; FAA, free amino acids; IDAA, indispensable amino acids; PP, dipeptide

Introduction

The early life stages of fish (larval and juvenile) have high protein turnover rates and consequently high dietary amino acids requirements for protein synthesis and accretion (Houlihan et al., 1995). However, there is no evidence that due to a high free amino acid pool in larvae, protein turnover is ''wasteful'' or that their rapid growth rate is compromised (Terjesen et al., 2000). Most frequently live food (protein bound amino acids) has been offered to stomachless fish larva during early stages, or when artificial, semi-purified diets based on casein or free amino acid mixture diets have been provided from first feeding, unexplained losses of nitrogen were encountered that could not be traced to excretion as ammonia or feces (Kaushik and Dabrowski, 1983). Although dietary AA imbalance of live food was suggested in the rearing of flatfishes, it is difficult to manipulate the nutrient composition of live food except for lipids (Aragão et al., 2004). Utilization of formulated diets is a promising alternative to rear larval fish. However, in respect to many species it has not yet been achieved. AA of the formulated diets can be provided in the form of protein-bound, free amino acids or peptides. Currently, absorptions of peptides (protein hydrolysates) and free amino acids (FAA) in the intestine are considered as major transport routes for protein utilization in mammals (Abidi, 1997; Ganapathy et al., 1994). When peptides or FAA are the major amino acid sources in the diets, absorption can be completed in the intestine and bypass the digestion by proteases secreted by the stomach and pancreas. Faster absorption of small peptides and FAA compared with protein were observed in studies with several fish species if injected into the digestive tract prior to their metamorphosis (Rust et al., 1993; Ronnestad et al., 2003). This was most of all due to the use of methylated protein sources with altered digestibility (Keil and Kirchman, 1992). However, the

effects of dietary FAA and peptides on larval fish growth and metabolism are still controversial.

Present studies focus on distinguishing the dietary effects of protein, peptides, and FAA on transport and/or or metabolism in the body free amino acid pool in relation to fish growth, i.e. utilization for protein synthesis. These results are applicable to understanding larval fish amino acid accretion for protein synthesis, helping to improve the formulated diets for early life stages of stomachless fish. The present study aimed to determine how a FAA-based diet (free amino acid as the exclusive amino acids source) or a dipeptide-based diet (dipeptides as the exclusive amino acid source) will affect common carp (*Cyprinus carpio*) larval and juvenile whole body FAA concentrations and profiles. We hypothesized that fish would respond to the ingestion of large amounts of free amino acids or dipeptides (1 h postprandial levels) by changing their whole body FAA concentrations and profiles, corresponding to the food evacuation rate and the profile of ammonia excretion rate in common carp larvae/juveniles (Kaushik and Dabrowski, 1983). This in turn would be an integrated measure of availability of dietary amino acids for body protein metabolism (synthesis or catabolism).

Materials and methods

Feeding trials

The design of experiments was, in part, dictated by earlier experiences with stomach possessing rainbow trout alevins, where diets based solely on peptides proved to be acceptable and resulted in significant weight gains (Dabrowski et al., 2003). Diet formulations used in the present study were identical as used previously (Dabrowski et al., 2003).

In Experiment 1, common carp larva were hatched and at the first-feeding stage (1.4 ± 0.16 mg wet weight ind^{-1}) distributed randomly into triplicate tanks per dietary treatment. These fish were offered a formulated free amino acid mixture diet (FAA diet) for the subsequent 4 weeks (group AA-4W). Another group of carp was fed initially live brine shrimp, *Artemia salina* nauplii for two weeks and then transferred to the formulated FAA diet for 2 additional weeks (group AA-2W). At the completion of feeding experiments at 4 weeks, fish were fasted for 24 h. Thirty fish were transferred from each of three triplicate aquaria into 1 L containers and 10 sampled prior to feeding (before a meal). Remaining fish were fed with the FAA mixture diet at 10–15 min intervals with 3 meals and then sampled by blotting on paper, weighing and freezing on dry ice. Sampling was conducted randomly across tanks and treatments.

In the second experiment common carp juveniles fed on live *Artemia* nauplii for 4 weeks were used (before experiment individual weight was 23.7 ± 6.4 mg). After 24 h fasting fish were offered either 1) live *Artemia salina*, nauplii, 2) a mixture of synthetic dipeptides ("Peptide" diet) or 3) a mixture of synthetic amino acids ("FAA"diet) with 10–15 min interval within one hour. Both diets were isonitrogenous and isolipidic (Dabrowski et al., 2003) and covered the requirements for indispensable amino acids (IDAA) in common carp (NRC, 1993). Whole fish were sampled and wet weight measured within 0.1 mg accuracy, frozen on dry ice rapidly and freeze-dried. Values for fasting fish free amino acid composition were those of the same age fish (4 weeks) but of the smaller body size (AA-2W; see Fig. 1).

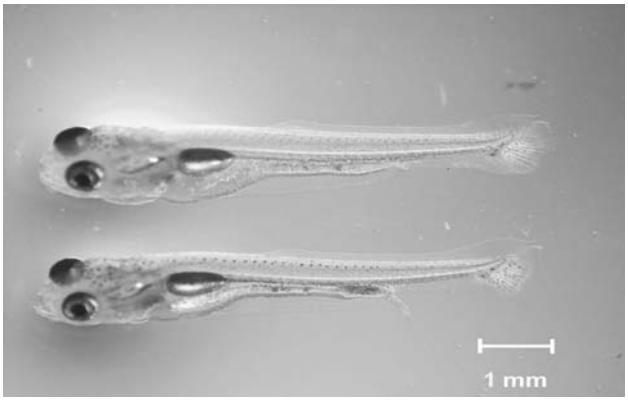

Fig. 1. Common carp larvae after 4 weeks on FAA-diet. Note presence of larval fin fold (the sign of arrested metamorphosis in fish) and intestinal content in the posterior intestine in comparison to non-feeding larvae

Dry samples from both experiments were kept at $-20\,^{\circ}$C until they were weighed again prior to homogenization and dry weight measured.

FAA analysis

Dry samples were extracted with 0.1 mol L^{-1} HCl in 1:75 (w/v) containing 160 µmol L^{-1} norleucine (recovery of $104 \pm 28\%$) internal standard, according to Cohen et al. Samples were subsequently spun at $12{,}000 \times g$ ($4\,^{\circ}$C, 15 min), and supernatants filtered (Millipore, 10 kDa cutoff at $2{,}000 \times g$, $4\,^{\circ}$C, 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 sample preparation. Samples, blanks and external standards were stored at $-80\,^{\circ}$C until analysis.

The same concentration of glutamine in 0.1 M HCl as external standards were prepared on the analysis day and added into the basic amino acids standard. Amino acids were pre-column derivatized with phenylisothiocyanate (PITC) (Cohen et al., 1989). Sample precipitates were removed by 10 min centrifugation at $10{,}000 \times g$ (Terjesen et al., 2004). Free amino acids were qualified by a Waters Pico Tag RP-HPLC 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 $46\,^{\circ}$C. Eluent 1 and eluent 2 purchased from Waters were used throughout the whole analysis. Each amino acid was identified using spiking with known amino acids and retention time of external standards. FAA concentrations (expressed as µmol/100 ml body fluid) were calculated using internal and external standards (Cohen et al., 1989).

Data analysis

Data are presented as means ± SD. Tank means were used as the statistical unit. Data of Experiment 1 were analyzed using *t*-test, and data of Experiment 2 were analyzed using one-way ANOVAs, LSD multiple comparison if significant using SPSS ver. 13.0. (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significantly different.

Results

Experiment 1

Carp larvae fed Free AA diet had a negligible growth rate, the final weight after 4 weeks amounted to 2.9 ± 0.4 mg

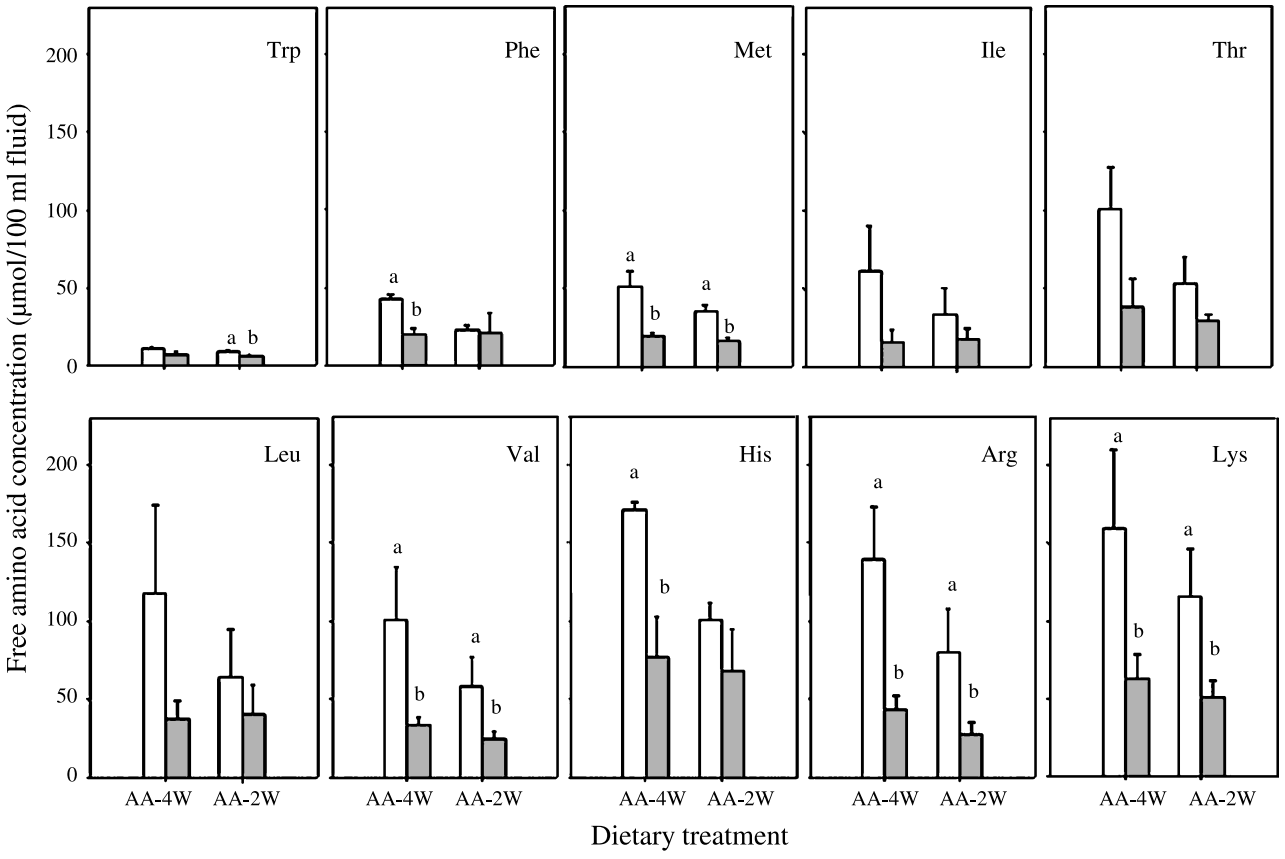

Fig. 2. Body indispensable amino acid (IDAA) concentrations in common carp; (□-before a meal; ▩-after feeding). Common carp were offered an FAA-based diet after a feeding trial of 28 days duration. (groups AA-4W and AA-2W prior FAA-base diet fed fish individual body weight were 2.9 ± 0.2 mg and 8.9 ± 2.1 mg respectively. IDAA concentrations are means $\pm$ SD, $n = 3$ except group AA-4W (Before) $n = 2$ (because one tank data was missing). Different letters indicate there are significant differences between before a meal and after feeding ($P < 0.05$)

and fish did not metamorphose maintaining their larval finfold (Fig. 1). Carp juveniles similarly had only negligible growth rate when offered a FAA diet at the age of 2 weeks. However, the evidence is provided based on the digesta content in the posterior intestine (Fig. 1) that fish actually ate diets. Microscopical observations also revealed that 15 min interval of ''feeding frenzy'' results in similar food intake in live *Artemia* and experimental diets fed fish.

Most of the fish whole body indispensable free amino acid concentrations after being fed a FAA-based diet decreased in both the 4-week FAA diet treatment group (AA-4W) and the 2-week FAA diet treatment group (AA-2W). At the age of 4 weeks, the mean fish wet weight of group AA-2W was 8.9 ± 2.1 mg. For the group 4W, the concentration of 6 indispensable amino acids out of 10 IDAAs (Phe, Met, Val, His, Arg and Lys) significantly decreased compared with that before a meal, and others (Trp, Ile, Thr and Leu) showed the same trend (Fig. 2). The body concentrations of 9 DAAs (except H-Pro, Tau and Asn) out of 12 detectable DAAs decreased after a meal; among them, Gln, Ser, Ala and Pro decreased significantly. There was, however, no significant difference for the total body free amino acids between fish before and after feeding, although numerically the levels decreased substantially (Table 1). For AA-2W group, concentrations of Trp, Met, Val, Arg and Lys after feeding were significantly lower than that before a meal; the concentration of Phe, Ile, Thr, Leu, and His showed the same trend. The concentrations of 9 DAAs (except for Asp, Orn and Asn) out of 12 detectable DAAs decreased after feeding; Ala concentration decreased significantly (Table 1). Tau showed the most significant change in concentration between AA-2W and AA-4W groups (Table 1) and this trend seemed to be related to fish size. If low concentration of Tau is indicative of deficiency, it may suggest that Tau is a conditionally indispensable amino acid in larval freshwater fish as earlier shown in marine fish larvae (Takeuchi, 2001). In summary, the indispensable amino acid concentrations were greatly negatively affected by

Table 1. Dispensable amino acid (DAA) concentration (μmol/100 ml fluid) of carp larva in Experiment 1

FAA	Dietary treatment			
	AA-2W (before)	AA-2W (after)	AA-4W (before)	AA-4W (after)
Gln	139.1 ± 16.3	106.6 ± 33.7	221.6 ± 7.6[a]	138.4 ± 24.4[b]
Asp	42.3 ± 12.7	52.8 ± 16.1	99.4 ± 24.8	93.5 ± 34.7
Glu	161.5 ± 30.9	148.3 ± 30.6	257.6 ± 86.7	172.1 ± 51.8
H-pro	14.7 ± 8.0	5.7 ± 1.0	7.3 ± 0.7	8.8 ± 3.6
Ser	106.5 ± 31.5	52.8 ± 15.3	163.2 ± 46.8[a]	72.4 ± 11.7[b]
Gly	154.0 ± 41.7	102.4 ± 36.2	279.5 ± 127.5	146.5 ± 61.5
Tau	876.3 ± 129.2	819.4 ± 61.4	190.6 ± 19.6	211.3 ± 68.8
Ala	163.1 ± 28.1[a]	97.8 ± 22.3[b]	249.7 ± 14.5[a]	113.1 ± 20.0[b]
Pro	58.8 ± 18.2	37.1 ± 10.4	100.1 ± 28.7[a]	43.9 ± 2.9[b]
Tyr	32.2 ± 6.3	25.1 ± 6.1	59.4 ± 23.4	35.9 ± 5.2
Orn	7.3 ± 2.0	7.5 ± 0.6	11.0 ± 0.1	9.4 ± 6.1
Asn	14.2 ± 2.6	23.5 ± 11.3	15.4 ± 1.1	18.8 ± 3.6
DAA	1770.4 ± 306.7	1479.6 ± 221.1	1655.3 ± 323.7	1020.6 ± 175.1
Total FAA	2347.62 ± 463.82	1784.76 ± 271.14	2611.51 ± 572.17	1377.34 ± 260.11

Values are expressed as means ± SD, $n = 3$ except group AA-4W(before) $n = 2$ (because one tank data was missing); Different letters mean there are significant differences among different dietary treatments

the FAA-based diet, and the total amino acid concentration was balanced to a certain extent by a less significant decrease in dispensable amino acids.

Experiment 2

Following the ingestion of FAA-based or dipeptide-based diets, the concentration of 8 indispensable amino acids,

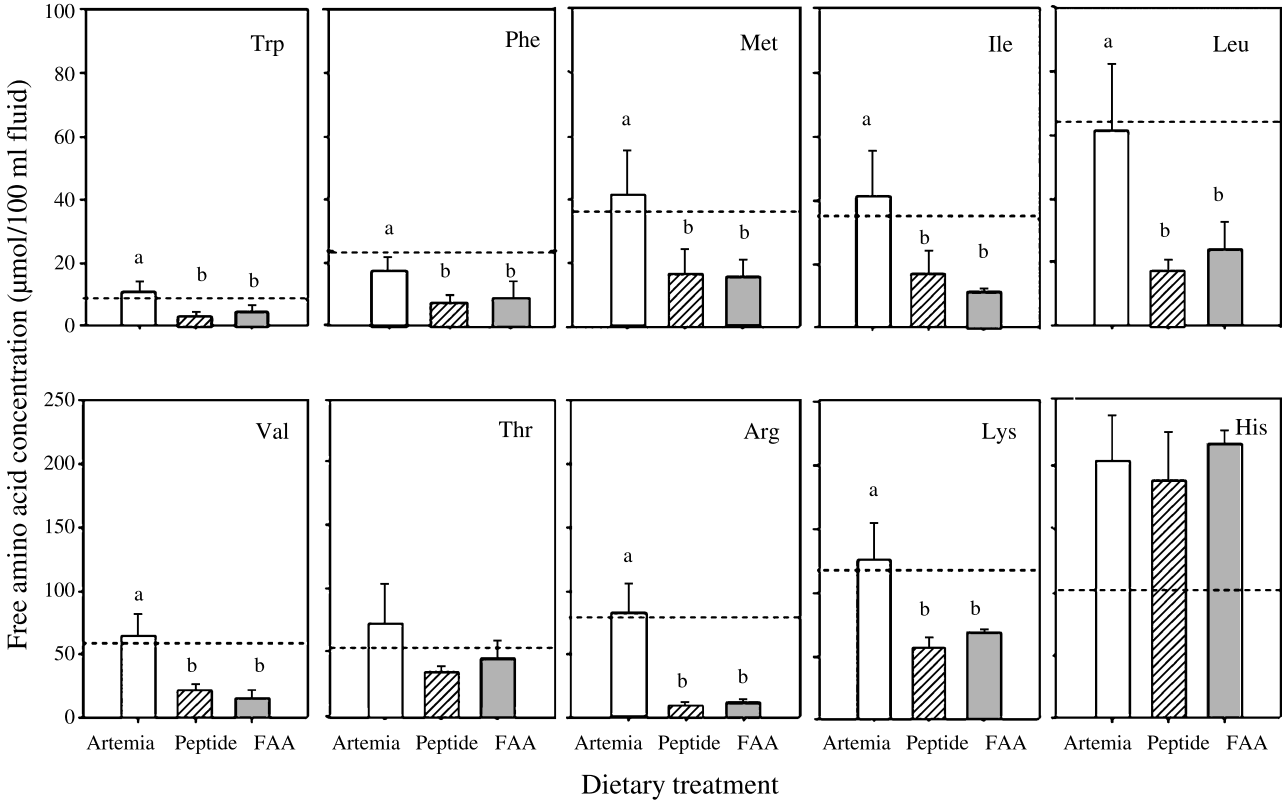

Fig. 3. Body indispensable amino acid (IDAA) concentrations in common carp juveniles. Values are expressed as means ± SD; $n = 3$; Different letters indicate there are significant differences between treatments ($P < 0.05$). Dash line indicates IDAA concentration of AA-2W diet treatment (24-h fasting fish)

Table 2. Dispensable amino acid (DAA) concentrations (μmol/100 ml fluid) of carp larva in Experiment 2

FAA	Dietary treatment		
	Artemia	Peptide	FAA
Gln	282.8 ± 84.5	293.5 ± 66.5	325.1 ± 28.2
Asp	51.1 ± 13.5[a]	33.5 ± 6.5[a]	161.2 ± 86.6[b]
Glu	270.4 ± 83.3	178.4 ± 22.6	254.6 ± 21.6
H-pro	14.5 ± 5.0	14.3 ± 4.3	17.3 ± 2.9
Ser	143.9 ± 36.7[a]	57.7 ± 6.7[b]	65.6 ± 4.5[b]
Gly	251.1 ± 63.2	196.9 ± 12.3	213.9 ± 17.9
Tau	982.0 ± 249.8	1159.7 ± 196.1	1278.4 ± 44.4
Ala	361.5 ± 98.7[a]	195.5 ± 33.0[b]	299.9 ± 26.1[ab]
Pro	216.9 ± 69.8[a]	55.2 ± 21.2[b]	71.3 ± 9.7[b]
Tyr	42.9 ± 13.2[a]	10.1 ± 3.2[b]	17.6 ± 2.3[b]
Orn	22.4 ± 4.6[a]	3.7 ± 0.9[b]	4.5 ± 0.8[b]
Asn	51.1 ± 21.6	23.3 ± 7.2	88.1 ± 7.1
DAA	2690.7 ± 709.5	2221.8 ± 361.3	2797.4 ± 120.1
Total FAA	3404.1 ± 865.3	2592.5 ± 408.1	3222.9 ± 116.4

Values from fasting fish were assumed to be not significantly different between 2 and 4 week old fish (see Table 1 for details). Values are expressed as means ± SD, $n = 3$; Different letters mean there are significant differences among different dietary treatments

Trp, Phe, Met, Ile, Leu, Val, Arg, Lys, were significantly lower than that of Artemia group fish. The concentration of Thr also showed a decreasing trend after ingestion of FAA- and dipeptide-based diets compared with fish fed with *Artemia* diet. Except His, IDAA levels of FAA and peptide group were much lower than that of 24 h fasting fish (AA-2W group before a meal). There were no significant differences for all 10 IDAA concentrations between the FAA-based diet group and peptide-based diet group. For the *Artemia* fed group, the concentrations of Trp, Met, Ile, leu Val, Thr, Arg, Lys and His were higher or very close to that of 24 h fasting level (Fig. 3). The concentrations of 4 DAAs in the *Artemia* fed group, Ser, Pro, Tyr and Orn, were significantly higher than that of fish ingesting peptide- or FAA-based diets. However, the concentration of Tau showed a trend to increase in all dietary treatments in comparison to fasted fish (Table 2). In short, the concentrations of IDAAs rather than DAA were greatly affected by the FAA-based or peptide-based diets. There was no significant difference in the total FAA among the three groups.

Discussion

The present study showed that most of the IDAA concentrations in the carp larval/juvenile body, independent of size (ranging from 2.9 to 23.7 mg) and nutritional history (purified or live food diets), significantly decreased after the ingestion of FAA- or peptide-based diets. This is a counterintuitive result in many respects, as intracellular depletion of free amino acids is regarded as a sign of deficiency (Furst and Stehle, 2004). Many studies on the utilization of FAA-based diets that had been carried out with common carp indicated inferior utilization of amino acids in comparison to equivalent protein-based diets in respect to fish growth (Murai, 1982; Murai et al., 1983; Kaushik and Dabrowski, 1983). This dilemma was in part resolved when it was indicated that in stomachless fish such as common carp (Murai et al., 1984) but also in stomach possessing fish, such as sturgeon (Ng et al., 1996), the majority of dietary free amino acids are excreted directly through the gills and/or with the urine. The FAA-based diet resulted in no body weight gain in first feeding rainbow trout alevins, whereas juvenile trout showed growth when the same diet was offered (Dabrowski et al., 2003). This FAA-based diet was, however, not expected to lead to a severe hypoaminoacidemia as demonstrated in the present work in common carp. It is also important to note that in common carp larvae, a peptide-based diet failed to support fish growth and signs of the loss of body amino acid pool was similar as in a FAA-based diet fed fish (Fig. 3). This is contrary to earlier results with rainbow trout alevins offered a dipeptide-based diet as the first food (Dabrowski et al., 2003). The dipeptide-based diet led to significant body weight gains and an increase in the IDAA pool in the rainbow trout body (Dabrowski et al., 2005).

Because of their potential use in larval/juvenile fish diets, free dietary amino acids need in depth studies in the early life stages of fish and explanations of FAA possible absorption and utilization mechanisms. Experiments with juvenile rainbow trout (*Oncorhynchus mykiss*) and Atlantic cod (*Gadus morhua*) (Cowey and Walton, 1998; Berge et al., 1994) showed faster FAA absorption rates compared with protein bound AA. It is indicative of the kinetics of the dietary free amino acid absorption that, for instance in warm water fish such as tilapia, the peak of most IDAA occurs in blood plasma in the first 1–2 hours after ingestion (Yamada et al., 1982). However, ordinarily in studies in which the amino acid absorption is analyzed the relation to the data on growth rate are not considered; therefore, the data are truncated and conclusions are difficult to draw (Murai and Ogata, 1990). Ronnestad et al. (2000) studied the use of intubation techniques where the authors showed that an FAA suspension (lacking other dietary ingredients) containing L-[^{35}S] methionine was absorbed with a 3.5-times higher transfer rate from the gut into the body tissue compared with the protein diet

containing L-[methylated-^{14}C] in Senegal sole (*Solea senegalensis*). This may be, however, related to the fact that methylated proteins tend to be undigested (in principle prepared for use in immunoassays) and were proven to be unavailable even to bacteria (Keil and Kirchman, 1992). Other studies showed, however, that FAA-based diets, with only 4.5% protein supplementation could sustain tilapia juvenile growth over 20–50 fold body weight increases (Santiago and Lovell, 1988). It has been argued that rapid amino acid absorption rates may lead to amino acid imbalances, increasing amino acids catabolism instead of protein synthesis, which result in lower or zero growth rates. Murai et al. (1984) reported that 36% of total nitrogen excretion through the gills and kidney was composed of FAA when carp juveniles were fed with a 38.4% free amino acids diet. The excretion dropped to 12.8% in carp fed with the comparable casein diet. This result indicated that the FAA flood increased amino acid excretion rather than the amino acid utilization (protein synthesis), which may contribute to our current results and ultimately lead fish to deplete body proteins resulting in lower growth.

In respect to mammals, the Na^+-dependent amino acid transport system and the Na^+-independent system are recognized as major systems that are responsible for amino acid uptake and release from the gut. In general, several amino acids share the same transporters, which can cause the transport of a particular amino acid to be competitively inhibited by the presence of other amino acids (Stipanuk, 2000). The free amino acids and peptides are transported with different systems. The mammalian peptide transporter, oligopeptide transporter (Pept-1), is abundantly expressed in larval zebrafish (*Danio rerio*) (Verri et al., 2003). Utilization of a mixture of FAA, peptides and protein seems to be promising to improve artificial diets. The optimum proportion of peptide and free amino acids in respect to a major protein source for different fish species and different life stages needs to be addressed in the future (Carvalho et al., 1997). Similarly, it has been demonstrated in common carp that postprandial changes in free amino acids following FAA-based diet ingestion are tissue specific and are regulated by insulin (Murai and Ogata, 1990). In carp of 81 g, amino acid peaks that occurred in blood plasma within 1 h, took over 2.5 h to appear in muscle. The presence of large amounts of dietary FAA in a relatively short time (1/4 h) may significantly increase the requirement for the FAA transporters and lead to competitive advantages of some amino acids (DAA) for transporters. This may have led to the decrease of IDAA concentration after FAA diet ingestion.

Numerous studies have shown that intact small peptides are taken up by the intestinal mucosal cells and absorbed more rapidly than free amino acids in rats, hamsters, pigs, humans and fish (Burston et al., 1972; Mathews et al., 1974; Li et al., 1999; Adibi and Soleimanpour, 1974; Boge et al., 1981). One of the advantages of peptide inclusion in common carp diets was an earlier finding by Carvalho et al. (1997) that protein hydrolyzates can be effectively utilized in this species for growth. However, in vivo evidence of the nutritional and metabolic significance of the complete (all IDAA) peptide-based diets in larval fish remains illusive. Espe et al. (1999) showed that with an increase in proportion of small peptides, muscle protein synthesis decreased 1.5–2.0 fold and the growth rate significantly decreased. More recent studies conducted by Aragao et al. (2004) showed that dipeptides could improve dietary amino acid balance. This corroborates findings in our laboratory with pacu (*Piaractus masopotamicus*) juveniles (Tesser et al., 2005). Dabrowski et al. (2003) reported that synthetic peptide diets sustain rainbow trout alevin and juvenile high growth rates. Unexpectedly, in the current experiment with common carp larvae (8.2 mg), a dipeptide-based diet did not improve low weight gains of carp in comparison to the first feeding larval fish. Changing the live diet (*Artemia*) to artificial diets (peptide-based diet or FAA-based diets) may be an additional stressor for fish. This may have resulted in the observation of lower body IDAA concentrations compared to the 24 h fasting *Artemia* group.

Taurine is evidently the major amino acid responsible for the buffering capacity in carp larvae tissues (Tables 1 and 2). Taurine can be assigned the conditionally indispensable amino acid function in larval fish nutrition (Takeuchi, 2001). Chen et al. (2005) reported in marine larval flounder that live food enriched with taurine resulted in 4–8 fold increase in taurine concentration in fish body. The taurine level in carp body (Table 1) was 4 fold higher in larger fish suggesting that synthesis of taurine from cysthationine may be impaired also in freshwater larvae as indicated in marine fish (Takeuchi et al., 2001). Evidence also comes from high intracellular/extracellular taurine ratios (8–25 fold) indicated in common carp juveniles (Murai and Ogata, 1990). A supplement of taurine, currently incorporated to all neonate diets (Furst and Stehle, 2004) is worth examining in freshwater fish larvae and may substantially change the body tissue free amino acid pool and subsequent dietary amino acid utilization.

In conclusion, our observations strongly suggest that dietary peptide transport and/or hydrolysis in common carp early life stages cannot be viewed as similar to rain-

bow trout alevins possessing functional stomachs at the time of first feeding. Neither FAA-based diets nor dipeptide-based diet can meet carp larval dietary AA requirements for optimal growth. We hypothesize that synthetic FAA- and peptide-based diet accelerated postprandial intact amino acid excretion and decreased protein synthesis rate. The evidence seems to emerge that provision of free amino acid flood in the form of FAA- or peptide-based diets disregulated natural degradation (proteasome)-renewal balance in tissues (Vabulas and Hartl, 2005) and resulted in rapid amino acid depletion followed by impairment of protein synthesis.

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