The Journal of Experimental Biology 208, 2885-2894
Published by The Company of Biologists 2005
doi:10.1242/jeb.01689

A concept of dietary dipeptides: a step to resolve the problem of amino acid availability in the early life of vertebrates

Konrad Dabrowski[1,*], Bendik F. Terjesen[1–3], Yongfang Zhang[1,4], James M. Phang[5] and Kyeong-Jun Lee[1,6]

[1]*School of Natural Resources, The Ohio State University, Columbus, OH 43210, USA*, [2]*Institute of Aquaculture Research* and [3]*Aquaculture Protein Centre – CoE, Protein and Amino Acid Section, Sunndalsøra, N-6600, Norway*, [4]*The Ohio State University Interdisciplinary Program in Nutrition, Columbus, OH 43210, USA*, [5]*Metabolism and Cancer Susceptibility Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick, MD 21702, USA* and [6]*Faculty of Applied Marine Science, Cheju National University, Jeju 690-756, South Korea*

*Author for correspondence (e-mail: dabrowski.1@osu.edu)

Accepted 17 May 2005

Summary

The premise that a dietary dipeptide approach will improve the understanding of amino acid utilization in the fastest-growing vertebrate, the teleost fish, was tested by examining the muscle free amino acid (FAA) pool and enzyme activities, in concert with growth response, when dietary amino acids were provided in free, dipeptide or protein molecular forms. We present the first evidence in fish that, in response to a synthetic dipeptide diet, muscle FAA varies as a result of both growth rate and amino acid availability of specific peptides. We demonstrate significantly diminished muscle indispensable FAA (3–10-fold) in rainbow trout alevins fed a dipeptide-based diet compared with a protein-based diet. The dipeptide-based diet did not contain proline, resulting in 10–27-fold less muscle free proline and hydroxyproline in alevins. The response of alevins fed FAA-based or peptide-based diets can be indicative of collagen turnover (Hyp/Pro ratio) and showed significant differences between dietary treatments. Pyrroline-5-carboxylate (P5C) reductase activity was detected, suggesting that P5C may ameliorate proline deficiency, but synthesis from glutamate could not maintain free proline levels in muscle. This finding will provide an impetus to test whether proline is conditionally indispensable in young fish, as in mammals and birds. This study shows that amino acids given entirely as dipeptides can sustain fish growth, result in muscle FAA and enzyme responses in line with dietary levels and identify growth-limiting amino acids. The understanding of these factors necessitates a diet formulation that will improve the accuracy of determining amino acid requirements in the early life stages of vertebrates.

Key words: dipeptide, protein, amino acid, proline, teleost, rainbow trout, *Oncorhynchus mykiss*.

Introduction

One of the most disputed concepts of digestive physiology at present is the role of dipeptides in accretion of indispensable amino acids (IDAA) in animal and human nutrition in states of health and disease (Fürst and Kuhn, 2000). As mentioned in the above review, “the dipeptides are innovative, true, new substrates” destined “to prepare complete, well balanced” diets that will become a solution to problems in the field of clinical nutrition. We propose to use teleost larval fish as a miniature vertebrate model *in vivo* because of their unique features demonstrated *in vitro* (see Verri et al., 2003) in order to understand peptide amino acid transport and regulations of absorption mechanisms.

Requirements of protein for maximum growth in teleost larval and juvenile stages are nearly twice as high as in older fish (Dabrowski, 1986). The reason for this may be due to underestimated IDAA requirements because of suboptimal growth rates in juvenile fish when purified diets were used (Dabrowski, 1986; NRC, 1993). There is essentially no data for larval, pre-metamorphosed fish amino acid requirements because of limitations in formulating acceptable diets.

Determination of tissue free amino acid (FAA) levels during requirement studies or comparison with dietary amino acid composition has met with variable success in fish (Schumacher et al., 1997; Yamamoto et al., 2000). However, most of these measurements have been made for plasma, while in fish the white musculature is quantitatively the most important site for protein accretion (Carter and Houlihan, 2001). In humans, muscle FAAs have been found to vary according to dietary levels and pathological and catabolic conditions (Fürst and Stehle, 2004). Ontogenetic, dietary and post-prandial effects on

muscle FAAs are also important in rainbow trout (Carter et al., 1995).

We documented previously that a synthetic dipeptide-based diet supported growth of rainbow trout alevins (first-feeding) whereas a free amino acid mixture diet did not (Dabrowski et al., 2003). Hydrolysates as sources of peptides are insufficient, since such peptides are impaired by their characteristics, i.e. the range of molecular sizes and difficulties in controlling their amino acid composition in requirement studies. There is evidence that tetra- and larger peptides, in the absence of pancreatic enzymes and deficiencies of brush border peptidase activity, become incapable of covering nitrogen requirements (Grimble, 1994; Daniel, 2004). Losses of IDAA during hydrolysate preparation are frequently responsible for nutritional inadequacies resulting in growth depression (Langar et al., 1993). This may have been the case when the proportion of hydrolysate was higher than 20% protein replacement in larval fish diets (Cahu et al., 1999).

Advantages of using specific di- or tripeptides as substrates for particular peptide transporters have been determined based on the affinity of PEPT1 transporters (Doring et al., 1998). These transporters were found to be expressed in larval teleosts prior to the first exogenous feeding (Verri et al., 2003). We hypothesize that synthetic dipeptide diets can be instrumental in defining amino acid requirements for pre-metamorphosed fish, which is largely unknown. As a first step, we set out to determine whether physiological indices such as muscle FAA and enzyme activities would respond to such diets, in comparison with a dietary mixture of synthetic amino acids or other molecular forms. The specific amino acid composition of the dipeptide diets was based on the capacity of cytosolic peptidases in fish intestinal epithelial cells (Aranishi et al., 1998) and on known IDAA requirements for rainbow trout juveniles (NRC, 1993). A series of feeding trials was carried out with first-feeding alevins and larger, juvenile rainbow trout (Dabrowski et al., 2003). We hypothesized that the concentrations of FAA in fish muscle (constituting 65–80% of body mass; Weatherley and Gill, 1987) would be an integrated measure of availability of dietary amino acids for protein synthesis and, at the same time, an indication of protein synthesis and degradation rates. Although not anticipated, we discovered that free proline concentration in muscle showed a strong dietary dependency and was possibly a conditional indispensable amino acid, similar to in young mammals (Kirchgessner et al., 1995). We followed this line of inquiry and, for the first time, analyzed pyrroline-5-carboxylate reductase (P5CR) activity in fish, the final step in proline biosynthesis.

Materials and methods

Feeding trials

The design of the experiments and diet formulations where tissue samples for FAA and enzyme analysis were collected were described earlier (Dabrowski et al., 2003). Briefly, dietary amino acids were provided in free, peptide or protein molecular forms (Table 1). Diets were isonitrogenous and isolipidic and contained either (1) casein-gelatin as the protein source [‘casein’ diets, with (+) or without (–) inclusion of maca meal; Lee et al., 2004], (2) a mixture of synthetic dipeptides (‘dipeptide’ diet) or (3) a mixture of synthetic amino acids (‘Free AA’ diet). The levels of amino acids in the experimental diets (g 100 g^{-1}) were calculated from added amounts (Dabrowski et al., 2003), Sigma (St Louis, MO, USA) and ICN

Table 1. *Compositions (%) of the four experimental diets (modified after Dabrowski et al., 2003)*

Ingredients/diets	Casein(–)	Casein(+)	Free AA	Dipeptide
Casein	40.00	40.00	–	–
Gelatin	8.00	8.00	–	–
Free AA mix[1]	–	–	44.60	–
Dipeptide mix[2]	–	–	–	44.60
Wheat meal	15.00	–	–	–
Maca meal	–	15.00	15.00	15.00
Dextrin	6.25	6.25	6.25	6.25
CPSP 90[3]	5.00	5.00	5.00	5.00
Cod liver oil	14.00	14.00	14.00	14.00
Vitamin mix[4]	4.00	4.00	4.00	4.00
Mineral mix[5]	3.00	3.00	3.00	3.00
Vitamin C[6]	0.05	0.05	0.05	0.05
CMC[7]	2.00	2.00	2.00	2.00
L-Arg	0.50	0.50	0.50	0.50
L-Met	0.40	0.40	0.40	0.40
L-Lys	0.80	0.80	0.80	0.80
Choline chloride	1.00	1.00	1.00	1.00
Cellulose	0	0	3.40	3.40

Casein(–): casein-gelatin as the protein source. Casein(+) is the same diet as casein(–) except for the inclusion of maca meal. Dipeptide: a mixture of synthetic dipeptides. Free AA: a mixture of synthetic amino acids.

[1]Free AA mix composition (g 44.6 g^{-1}; all L-form AA unless otherwise indicated): Arg, 1.5; His, 0.7; Ile, 0.9; Leu, 2.9; Lys, 1.8; Met, 1.0; Phe, 1.8; Thr, 0.8; Trp, 0.2; Val, 0.2; Pro, 11.1; Ser, 11.1; DL-Ala, 11.1.

[2]Dipeptide mix composition (g 44.6 g^{-1}): Arg–Val, 2.87; His–Leu, 1.21; Gly–Ile, 1.41; Lys–Gly, 2.96; Gly–Met, 1.38; Phe–Leu, 3.03; Thr–Leu, 1.56; Gly–Trp, 0.26; Val–Leu, 0.45; Ala–Gly, 10; Ala–Gln, 15; Gly–Tyr, 4.95.

[3]Soluble fish protein hydrolysate; Sopropeche S. A., Boulogne Sur Mer, France.

[4]Roche Performance Premix composition (g kg^{-1} of vitamin mixture): vitamin A acetate, 7.56; cholecalciferol, 0.0055; α-tocopheryl acetate, 66.1; vitamin B_{12}, 0.0013; riboflavin, 13.2; niacin, 61.7; *d*-pantothenic acid, 22.1; menadione, 1.32; folic acid, 1.76; pyridoxine, 4.42; thiamin, 7.95; *d*-biotin, 0.31 (Hoffman-La Roche, Inc., Nutley, NJ).

[5]Five mg Se in the form of sodium selenite per kg Bernhart Tomarelli salt mixture (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA).

[6]Mg-L-ascorbyl-2-phosphate; Showa Denko K. K., Tokyo, Japan.

[7]Carboxymethylcellulose; ICN Biomedicals, Inc., Costa Mesa, CA, USA.

Biomedicals, Inc. (Costa Mesa, CA, USA) product sheets of dietary ingredients, and literature (Kim et al., 1992; Berge and Storebakken, 1996; Yamamoto et al., 2000; Halver, 2002). All diets covered the requirements for IDAA in rainbow trout (NRC, 1993).

Experiment 1

Rainbow trout *Oncorhynchus mykiss* Walbaum of a local strain (London, OH, USA) were hatched and, at the first-feeding stage (114±16 mg wet mass individual^{-1}), distributed randomly into triplicate tanks for each dietary treatment. After 5 weeks of feeding, fish were collected from each tank and transferred into an ice water bath and then individually frozen on dry ice and stored at –80°C for FAA analyses.

Experiment 2

Juvenile rainbow trout of 0.78±0.08 g initial mass individual^{-1} and approximately 5 weeks old were used. Three diets were tested: Free AA, dipeptide and casein(–). At the completion of the 2-week experiment, sampling tissues for FAA and P5CR activity analyses commenced in the morning, 24 h after the last feeding, and were conducted randomly across tanks and treatments. Fish were killed and samples stored in the same manner as in Experiment 1.

Experiment 3

This experiment aimed to determine changes in P5CR activity with time duration of the assay, i.e. to optimize kinetics. Furthermore, it aimed to analyze tissue distribution of P5CR activity between week 2 and 6 after the first feeding. Thus, it would include the time-periods of rainbow trout ontogeny studied in Experiments 1 and 2. Rainbow trout alevins were reared as described earlier and fed a casein-based diet (30% casein, 6% gelatin, 6% casein-hydrolysate; K. Dabrowski and M. Penn, personal communication) until sampling at weeks 2 and 6. Fish were collected from each tank at sampling and individually frozen in liquid nitrogen and stored at –80°C. To establish the assay conditions and presence of P5CR activity in fish, we used fresh tissues and individuals of larger size to separate organs of interest: liver and intestine. Tissue samples of 12-month-old rainbow trout were therefore assayed. These fish were fed a standard commercial diet (Zeigler-Bros., Gardners, PA, USA). After 48 h fasting, four fish (53±12 g individual^{-1}, 17.6±1.5 cm length) were killed by a sharp blow to the head, and the intestine (including pyloric caeca) and the liver dissected out, rinsed in 0.9% NaCl and assayed immediately.

FAA analysis

Individual fish from Experiments 1 and 2 were rapidly dissected while still frozen on ice-cooled boards. The head and tail (beyond the abdominal cavity) were removed, and the dorsal body region, anterior and posterior to the dorsal fin, was dissected out (28±8% of body mass; N=76). Care was taken to avoid remains of kidney tissue by scraping any blood containing tissue from the upper part of the body cavity. The tissue samples were weighed and re-frozen at –80°C. Thus, these muscle tissues also contained skin, cartilage and bone, but were largely composed of white muscle, and are, for simplicity, termed 'muscle'. Within two days, muscle samples were extracted in 0.1 mol l^{-1} HCl containing 160 μmol l^{-1} norleucine, using a tissue:extraction medium ratio of 1:4 (juveniles) or 1:10 (alevins) (w/v), according to Cohen et al. (1989). The norleucine recovery was 104±28% (N=52). Tissue extracts were spun at 12 000 ***g*** (4°C, 15 min), and supernatants filtered (Millipore, Billerica, MA, USA; 10 kDa cut-off at 2000 ***g***, 4°C, 90 min). Samples of blanks and external standards (Sigma acid/neutral and basic amino acids), supplemented with glutamine, were prepared at the same time as sample extraction by adjusting appropriately with distilled and deionized H_2O, and 0.1 mol l^{-1} HCl containing 160 μmol l^{-1} norleucine. Tissue extracts, standards and blanks were then stored at –80°C, for later analysis using the Waters (Milford, MA, USA) PicoTag method with pre-column derivatization and reverse-phase high-performance liquid chromatography (RP-HPLC; Cohen et al., 1989).

Enzyme activity

P5CR (EC 1.5.1.2) activity was measured using modifications of mammalian protocols (Herzfeld et al., 1977; Dekaney et al., 2003). DL-P5C was produced from its 2,4-dinitrophenylhydrazone (Sigma), purified by cation-exchange chromatography, analyzed with *o*-aminobenzaldehyde and stored at 4°C in 0.5 mol l^{-1} HCl (Mezl and Knox, 1977). Liver and intestine from juvenile trout (assays in fresh tissues), or liver and intestine from alevins or juveniles from Experiment 3 (N=3 tanks), for P5CR activity analysis were obtained using dissection techniques described above. The whole intestine, including pyloric caeca and pancreas, was used. Liver constituted 1–2%, and intestine 8–9%, of total body mass, and no changes in hepatic or intestinal indices were found when comparing Experiment 3 alevins fed for 2 weeks (0.27±0.03 g body mass) with those fed for 6 weeks (1.08±0.09 g body mass).

To assess total P5CR activity in juvenile trout fed the different experimental diets (Experiment 2), assays were run on the whole fish body (Terjesen et al., 2001). Tissues to be assayed for P5CR activity were homogenized on ice (Potter-Elvehjem at 40 rev min^{-1}, 60 s, two strokes) in 250 mmol l^{-1} sucrose, 50 mmol l^{-1} phosphate buffer (pH 7.2), 1 mmol l^{-1} EDTA, 2.6 mmol l^{-1} dithiothreitol, and 2.5 μg ml^{-1} each of aprotinin, pepstatin A, chymostatin and phenyl methyl sulphonyl fluoride. Whole-body samples were gently disrupted prior to the Potter-Elvehjem by an Omni GLH homogenizer (4000 rev min^{-1}, 15 s). All extracts were spun at 600 ***g*** (10 min, 4°C), the supernatant further centrifuged at 15 000 ***g*** (10 min, 4°C), and the final supernatant used for measurements. This approach (Dekaney et al., 2003) was followed assuming that P5CR has a cytosolic location in fishes, as in mammals. Proline oxidase, which could utilize the P5CR reaction product, has a mitochondrial location in mammals (Wu and Morris, 1998). Immediately before assay, the P5C substrate was neutralized

on ice, using 1 mol l^{-1} Hepes (pH 7.0) and 1 mol l^{-1} NaOH. Duplicate reactions were run in 100 µl total volume (10 µl extract), containing 4.5 mmol l^{-1} DL-P5C, 2 mmol l^{-1} NADH and 45 mmol l^{-1} phosphate buffer (pH 6.8). After 0–15 min at 26°C (Terjesen et al., 2001), reactions were terminated by 25 µl of 1.5 mol l^{-1} $HClO_4$ and neutralized with 12.5 µl of 2 mol l^{-1} K_2CO_3. After centrifugation (600 ***g***), proline was determined by PicoTag RP-HPLC (Cohen et al., 1989), and protein determined by the Bradford method (Bradford, 1976). The amount of proline formed was linear with time and protein concentrations, and control assays without P5C or enzyme extract did not result in detectable activity (chromatograms not shown).

Data analysis

Data are presented as means ± S.D. Tank means were used as the statistical unit, and data were analyzed in SPSS version 12.0.1. (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) for all tests except for Experiment 3 regarding P5CR tissue distribution and age since first-feeding (two-way ANOVA). If significant (P<0.05), Duncan's multiple range tests were employed.

Results

Except for feed particle size, the specific diets given to both life stages were identical and were rapidly ingested (Dabrowski et al., 2003). Rainbow trout showed life-stage-specific muscle FAA responses to dietary amino acids (Figs 1, 2). Rainbow trout alevins (Experiment 1) fed Free AA or dipeptide diets had significantly lower total FAA muscle concentrations than alevins fed the casein(+/–) diets (Table 2). The sum of dispensable amino acid (DAA) was twice as high in juveniles (Experiment 2) compared with alevins fed the Free AA diet, whereas the IDAA concentrations were not significantly different between the life stages (Tables 2, 3). Concerning specific FAA and differences between the life stages, the high lysine concentrations in alevins fed the Free AA diet (Table 2) may have reflected diminished protein synthesis since it corresponded to a low growth rate (Fig. 1) in comparison with juveniles (Fig. 2; Table 3). The high proline level in the Free AA diet did not result in elevated free proline in muscle of alevins (Fig. 1C). This was in contrast to muscle of juvenile trout fed the Free AA diet (Fig. 2C). Similarly, the dipeptide diet, rich in glycine, resulted in only a modest level of free glycine in rainbow trout alevins (Fig. 1A), whereas the same diet amounted to 20 mmol kg^{-1} wet mass glycine in muscle of juveniles, the highest level of a single FAA (Fig. 2A).

Several FAAs in muscle, such as histidine, threonine, valine, arginine and alanine, corresponded to dietary amino acid profiles in Experiments 1 and 2. The casein(+/–) diets had a higher IDAA level than the Free AA and dipeptide diets, and this was mirrored in both alevin and juvenile muscle FAA concentrations (Figs 1B, 2B; Tables 2, 3), and resulted in the highest growth rates (Figs 1, 2, top diagram). However, these differences in muscle free IDAA, e.g. histidine (3–8-fold), were in many cases several folds larger than the dietary level differences (Figs 1B, 2B; Tables 2, 3). Among free DAA, alanine, which was high in the Free AA diet, reached 18 mmol kg^{-1} wet mass in muscle of juveniles (Fig. 2A), and alanine concentrations were significantly higher in fish of both life stages fed the Free AA diet compared with trout fed other diets. Free aspartic or glutamic acids in muscle did not indicate any impact of dietary levels (Figs 1A, 2A).

The muscle concentrations of several free IDAA were affected by dietary molecular form. In particular, isoleucine and methionine were present in muscle at higher levels when given a Free AA diet, in both alevin (Experiment 1) and juvenile trout (Experiment 2), compared with the dipeptide diet fed fish, despite similar dietary levels (Figs 1B, 2B; Tables 2, 3). This observation may imply that an excess of amino acids was not utilized for protein synthesis in the Free AA diet fed alevins (Experiment 1), since negligible weight gains (Fig. 1) and elevated ammonia excretion were observed (Dabrowski et al., 2003). To the contrary, there was no significant difference in ammonia excretion rate in juveniles fed Free AA and casein(–) diets, and improved, although low, weight gain was observed in juveniles fed a Free AA diet (Fig. 2; Dabrowski et al., 2003).

The assays of fresh rainbow trout juvenile tissues showed that P5CR activity was present in liver and intestine (data not shown). P5CR activity was present in the intestine and liver of rainbow trout alevins fed for 2 weeks (Fig. 3A). Liver had significantly higher P5CR activity than intestine (P<0.01), but no significant effects of age, or age × tissue, were noted (two-way ANOVA). The effects of different dietary sources of amino acids on P5CR activity were investigated in Experiment 2 (Fig. 3B). The juvenile trout fed the dipeptide diet showed numerically higher P5CR activity than juveniles fed Free AA- and casein-based diets, although differences were not significant (P=0.25; one-way ANOVA). When the total P5CR activity in the whole body was estimated in the casein-diet-fed trout (0.7 U g^{-1} wet mass) and compared with the activity of intestine (1.0 U g^{-1} wet mass) and liver (3.1 U g^{-1} wet mass), it was calculated that these two tissues (11% of body mass) accounted for ~20% of whole-body P5CR activity. In conclusion, rainbow trout alevins and juveniles expressed

Fig. 1. Muscle free amino acid (FAA) concentrations in rainbow trout alevins subjected to Experiment 1 treatments. (A) Examples of dispensable amino acids (DAA) in relation to dietary levels. (B) Examples of indispensable amino acids (IDAA) suggesting limitations in dietary peptide availability. (C) Amino acids involved in the proline–ornithine–arginine pathway. Data given as means ± S.D., two samples of 2 fish each were assayed per tank, three tanks per treatment (N=3 per group), except Free AA (N=2). The top graph (specific growth rate) is modified from Dabrowski et al. (2003). The lower parts of each figure (bars) show muscle concentrations of individual FAA (y_1 axis title to the left). The upper parts of each figure (broken lines) indicate the level of each amino acid in the diets (g 100 g^{-1}; y_2 axis title to the right). Significant differences between treatments (P<0.05) are indicated by differing letters. Figures with no letters next to the bars indicate non-significant one-way ANOVAs.

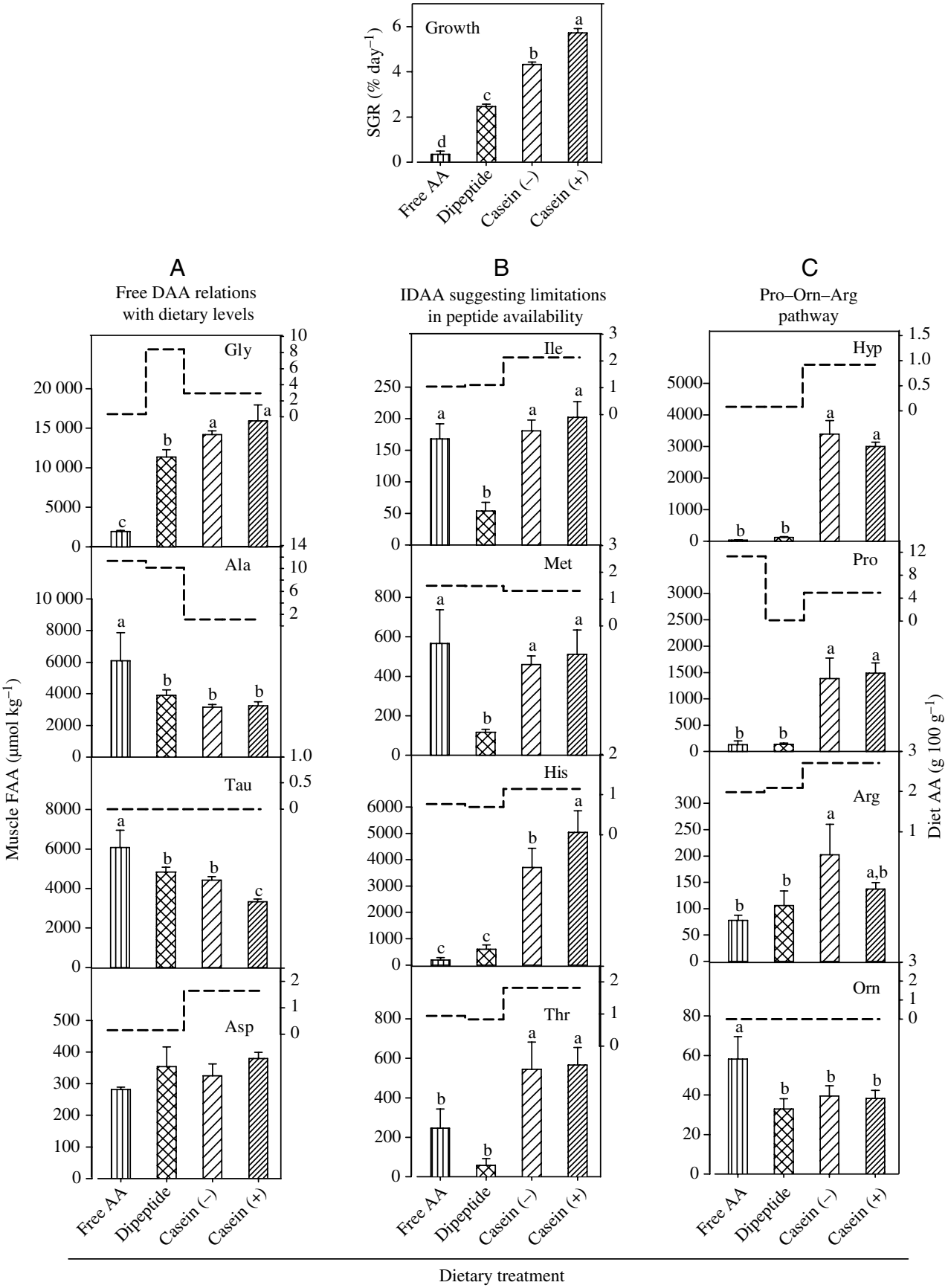

Fig. 1. See previous page for legend.

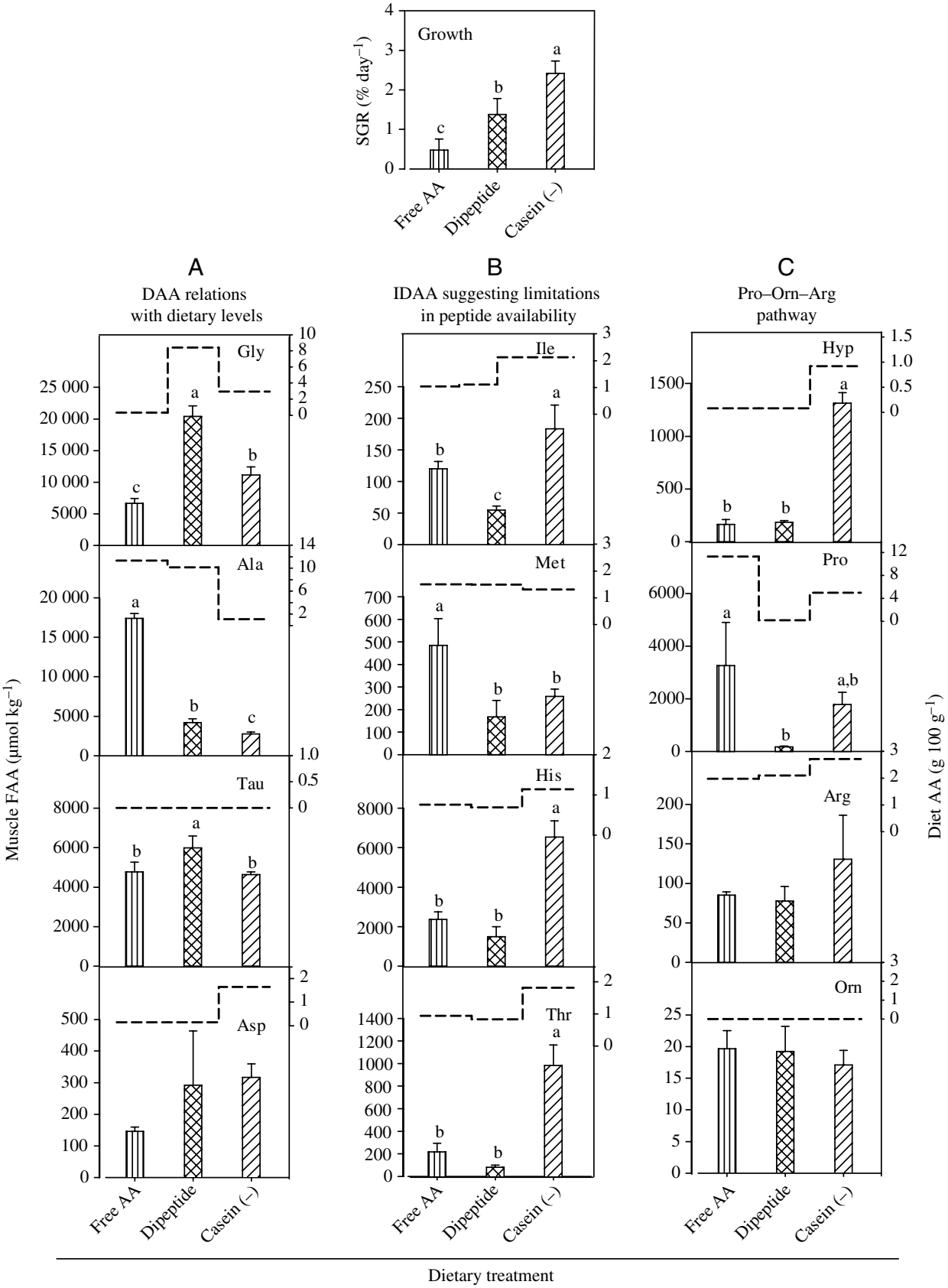

Fig. 2. Muscle free amino acid concentrations in rainbow trout juveniles subjected to Experiment 2 treatments. Data given as means ± S.D., two samples of 2 fish each were assayed per tank, three tanks per treatment, N=3 for all groups. Significant differences between treatments (P<0.05) are indicated by differing letters. See Fig. 1 for further details.

Table 2. *FAA concentrations and selected ratios in muscle of rainbow trout alevins (Experiment 1) not shown in Fig. 1*

	Dietary treatment			
Amino acid	Free AA	Dipeptide	Casein(–)	Casein(+)
Gln*	467±100^{b}	835±110^{a}	858±76^{a}	902±92^{a}
Glu*	1423±11^{b}	1425±176^{b}	1865±77^{a}	2167±217^{a}
Ser*	239±53^{c}	992±115^{b}	1865±87^{a}	1652±411^{a}
Tyr*	98±38^{c}	295±21^{b}	423±56^{a,b}	516±56^{a}
Val*	307±61^{b}	131±27^{c}	473±39^{a}	522±76^{a}
Leu*	316±78^{b}	164±28^{c}	455±54^{a}	499±82^{a}
Phe*	63±6^{b}	78±8^{b}	138±23^{a}	124±22^{a}
Trp*	50±11^{c}	61±9^{c}	167±4^{a}	137±20^{b}
Lys*	1416±247^{b}	1449±142^{b}	4079±1406^{a}	818±115^{b}
Sum DAA†	17.1±3.2^{c}	24.4±1.4^{b}	32.3±0.2^{a}	32.9±2.6^{a}
Sum IDAA†	3.4±0.8^{b}	2.8±0.2^{b}	10.4±2.1^{a}	8.6±0.8^{a}
Sum FAA†	20.5±4.0^{c}	27.2±1.2^{b}	42.7±1.9^{a}	41.4±3.4^{a}
Orn/Pro‡	0.58±0.41^{a}	0.29±0.12^{a,b}	0.03±0.00^{b}	0.04±0.01^{b}
Hyp/Pro‡	0.34±0.17^{b}	1.11±0.55^{b}	2.71±0.77^{a}	2.62±0.29^{a}

*Data are given as tank means ± S.D. in μmol kg^{-1} wet muscle mass (N=3), except for the Free AA diet (N=2).

Sums of DAA (dispensable amino acids), IDAA (indispensable amino acids) and total FAA (free amino acids) (mmol kg^{-1} wet mass) represent the sum of FAA given here and in Fig. 1.

‡Orn/Pro and Hyp/Pro data were calculated from values given in Fig. 1.

Superscript letters represent significant effects of dietary treatment; data within an experiment not sharing a superscript letter are significantly different at P<0.05.

P5CR activity in liver and intestine, but fish could not maintain muscle free proline when fed proline-deficient dipeptide diets (free proline muscle levels were 10-fold lower) in comparison with fish fed proline-containing casein(–) (juveniles) or both casein(+) and (–) diets (alevins) (Figs 1C, 2C).

Rainbow trout alevins fed Free AA- or dipeptide-based diets (Experiment 1) showed different responses in muscle levels of ornithine and proline and interactions with growth. Ornithine, which may be synthesized from P5C, was higher in alevins fed Free AA diet than in alevins fed other diets (Fig. 1C; Table 2), concurrent with a negligible growth rate (Fig. 1) and very low proline muscle concentrations (Fig. 1C), despite being given a diet high in proline. By contrast, dipeptide-fed alevins showed significantly higher growth rates than the FAA-fed groups (Fig. 1; Dabrowski et al., 2003), and the dipeptide-fed fish had comparable muscle ornithine levels to that of the casein(+/–)-fed fish (Fig. 1C). Despite being given a dipeptide diet, devoid of peptide proline, these fish produced significantly higher [Orn]/[Pro] ratios (Table 2). In regard to hydroxyproline, in juveniles (Experiment 2) the free muscle levels were comparable in the Free AA- and dipeptide-fed groups, but 7-fold lower than in fish fed the casein(–)-based diet (Fig. 2C). In alevins (Experiment 1), the dipeptide-fed fish (126±20 μmol kg^{-1} wet mass) had a numerically higher free hydroxyproline concentration in muscle than did the Free AA-fed fish (37±2 μmol kg^{-1} wet mass); however, this was 27-fold lower (3394±430 μmol kg^{-1} wet mass) than in the fast-growing (4.5% day^{-1}) rainbow trout alevins fed the casein(–) diet (Fig. 1C).

Discussion

The concept that small peptides are absorbed in the vertebrate intestine has recently been the subject of many in-depth reviews (Grimble, 1994; Daniel, 2004). However, *in vivo* evidence of the nutritional and metabolic significance of complete (all IDAA) peptide diets remains elusive. The finding that a vertebrate can grow on a diet exclusively composed of synthetic dipeptides of known composition was recently reported for the first time in teleost fish by our laboratory (Dabrowski et al., 2003). There is evidence that a single peptide can be more efficiently absorbed than the mixture of identical amino acids both in fish (Reshkin and Ahearn, 1991; Boge et al., 2002) and mammals (Matthews, 1991). The

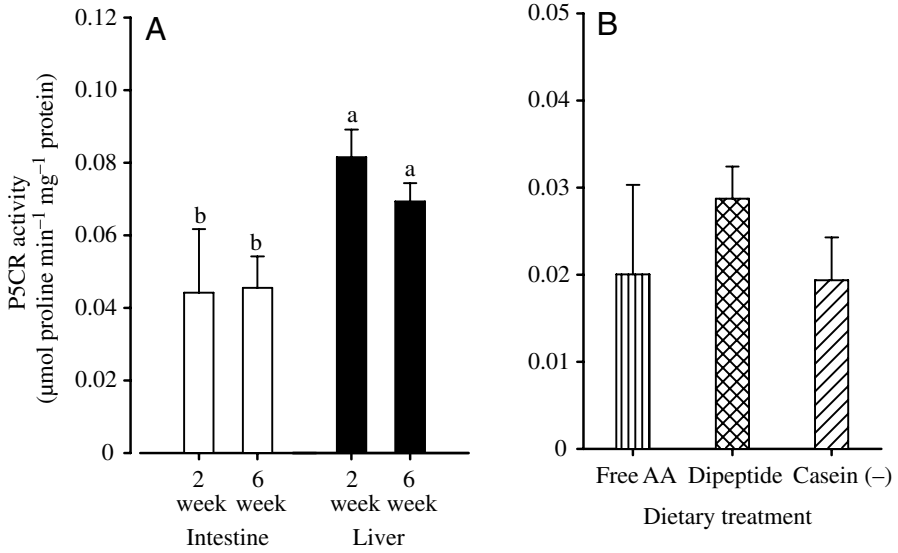

Fig. 3. Pyrroline-5-carboxylate reductase (P5CR) activity in rainbow trout alevins and juveniles. (A) Liver and intestinal P5CR activity in rainbow trout alevins at 2 weeks and 6 weeks post first-feeding (Experiment 3). (B) P5CR activity in whole body of juvenile rainbow trout fed amino acids in different molecular forms (Experiment 2). Data given as means ± S.D., N=3 per group. Data with superscripts indicate significant ANOVA; means not sharing a similar letter are significantly different (P<0.05).

Table 3. *FAA concentrations and selected ratios in muscle of rainbow trout juveniles (Experiment 2) not shown in Fig. 2*

	Dietary treatment		
Amino acid	Free AA	Dipeptide	Casein(–)
Gln*	949±43[b]	1211±150[a]	1363±86[a]
Glu*	1216±101	1746±493	1905±24
Ser*	875±262	1530±529	1414±44
Tyr*	96±22[b]	254±135[a,b]	350±63[a]
Val*	211±31[b]	173±29[b]	510±94[a]
Leu*	156±20[b]	204±91[b]	441±96[a]
Phe*	31±2	69±28	57±8
Trp*	33±6[b]	48±11[a,b]	56±4[a]
Lys*	426±53[b]	1328±579[a]	1945±138[a]
Sum DAA[†]	35.6±1.0[a]	36.0±2.3[a]	27.0±1.8[b]
Sum IDAA[†]	4.1±0.3[b]	3.7±0.7[b]	11.1±1.0[a]
Sum FAA[†]	39.7±1.1	39.7±3.0	38.1±2.6
Orn/Pro[‡]	0.01±0.01[b]	0.12±0.02[a]	0.01±0.01[b]
Hyp/Pro[‡]	0.09±0.09[b]	1.14±0.15[a]	0.86±0.22[a]

*Data are given as tank means ± S.D. in μmol kg^{-1} wet muscle mass (N=3).

Sums of DAA (dispensable amino acids), IDAA (indispensable amino acids) and total FAA (free amino acids) (mmol kg^{-1} wet mass) represent the sum of FAA given here and in Fig. 2.

[‡]Orn/Pro and Hyp/Pro data were calculated from values given in Fig. 2.

Superscript letters represent significant effects of dietary treatment; data within an experiment not sharing a superscript letter are significantly different at P<0.05.

present report provides the first evidence using synthetic dipeptide diets on the regulatory mechanisms affecting amino acid absorption and utilization resulting in distinct patterns of muscle FAA.

In fed animals, weight gains usually arise as a result of increased protein synthesis and decreased protein degradation in comparison with fasted animals (Waterlow, 1999). Several factors are pivotal in determining the effectiveness of these processes, such as concentrations and correct proportions, i.e. balanced amino acid composition to achieve protein accretion, i.e. growth. We submit that the differences in muscle FAA shown here (1) are due to differences in amino acid absorption rates from free, dipeptide or protein dietary sources, (2) result in uneven accumulation rates and post-prandial peak times for muscular FAA and (3) consequently result in different metabolic handling of the amino acids and availability for protein synthesis. Superimposed on these are differences in growth rate, protein synthesis rate and developmental stages, which demand varying amounts of amino acids at the quantitatively most important site for protein accretion, white muscle. This set of factors, together with enzyme expression data, provides more insight into amino acid nutrition than nitrogen balance or growth rate alone. As an example, methionine was provided in comparable amounts in all three diets, but methionine given in dipeptide form was present in muscle of alevins in significantly lower amounts than in fish with essentially no growth (Free AA group) and higher growth rates [casein(+/–) groups], suggesting limitations of the Gly–Met dipeptide availability (Fig. 1B).

A major finding in the present study is the strong dependence of free proline in muscle tissue on dietary level of proline in dipeptide- and casein-based diets (Figs 1C, 2C), since it can be assumed that any excess of proline would be catabolized for energy *via* P5C and ornithine or glutamate. In rainbow trout juveniles fed a dipeptide-based diet devoid of proline, endogenous synthesis did not maintain proline levels comparable with fish fed diets with proline [casein(+/–)]. The free proline levels in muscle of the dipeptide-fed group are at the lower end of the range reported in fish muscle (Torrissen et al., 1994; Yamamoto et al., 2000; Ogata, 2002). In large rainbow trout fed a low-protein, 5% casein-containing diet, only 'trace' amounts of free proline were found in muscle, whereas in fish on a 50% casein diet, the proline concentration was 3400 μmol kg^{-1} (Yokoyama and Nakazoe, 1991). In mammals, although proline can be synthesized from ornithine or glutamate *via* P5C, young mammals, rats and pigs still require a dietary source of proline for maximum growth and protein retention (Ball et al., 1986). Furthermore, increased dietary proline levels in young rats and pigs correlate with plasma proline concentrations (Samuels et al., 1989; Kirchgessner et al., 1995), and no significant response was found in P5CR activity when comparing pigs fed control and proline-deficient diets. The findings in young mammals correspond to the present study on rainbow trout. In fish, a 10-fold drop in the free proline in muscle was associated with a 48% increase (ANOVA not significant) in P5CR activity in the dipeptide-fed rainbow trout, a diet devoid of proline (Fig. 3B). In other fish (Pacific salmon), juveniles fed AA-based diets were reported for the first time by Halver et al. (1957) to grow at the rate of 0.45% day^{-1}. That is similar to rainbow trout juveniles in the present study (0.48% day^{-1}; Fig. 2). The growth data for fish fed diets with no proline in the Halver et al. study, however, ended much earlier than with other treatments where AA were omitted from the diets. Therefore, results cannot be compared directly. Similarly, growth of young tilapia on an AA-based diet devoid of proline was shown to be inferior (Aoe et al., 1970). However, these authors discontinued the study. Since larger fish were used in most amino acid requirement studies, a possible proline conditional indispensability for optimal growth of larval fish was not demonstrated. The classical study (Halver and Shanks, 1960) indicates that the final mass of sockeye salmon fed a proline-deficient diet was less than that of fish fed a control diet, and the experiment was shortened to only 5 weeks. In addition, in this previous study (Halver and Shanks, 1960) and earlier experiments with Chinook salmon (Halver et al., 1957), a proline-deficient diet was supplemented with more than a generous amount of arginine (3.6–5%). Interconversion of arginine to ornithine and then ornithine to proline in neonatal mammals can reach 25 and 57%, respectively (Bertolo et al.,

2003). Regarding fish, early life stages are characterized by high protein synthesis rates (170–300%), and protein deposition rates of 50% body protein per day have been observed in larval catfish (Terjesen et al., 1997) and other species (Fauconneau et al., 1986). Furthermore, during the saltatory development of larval fish, muscle fibres and skeleton undergo a considerable change (Blaxter, 1988), demanding collagen synthesis. Since proline and hydroxyproline constitute more than 20% of the amino acid residues in collagen in mammals (Smith and Phang, 1978), and in fish hydroxyproline alone is estimated at 7% (Sato et al., 1989), a requirement for dietary proline supplementation should be investigated in exogenous feeding during the early life stages. We submit that dipeptide-based diets can be instrumental in determining dietary requirements for IDAA, including conditional indispensability for proline, in larval and juvenile fish.

High concentrations of hydroxyproline in muscle of fast-growing animals is an indicator of a high rate of collagen turnover (Adams and Frank, 1980). Since hydroxyproline released by collagen breakdown and subsequent hydrolysis of Hyp-containing peptides is destined for catabolism and excretion, present results in rainbow trout are the best indication thus far of the muscle limiting degradation of collagen when there is no dietary supply of proline (Figs 1C, 2C). In juvenile salmon, muscle hydroxyproline was the only FAA that correlated with growth rates (Sunde et al., 2001). Although we indicate the presence of hydroxyproline in the casein(+/–)-based diets, we must assume that transport of dietary hydroxyproline from intestine to muscle is limited (Adams and Frank, 1980), and thus high concentrations in muscle of a casein/gelatin-based diet-fed fish would reflect high protein turnover rates. This would suggest that low proline and hydroxyproline concentrations are indicative of either fasting (the Free AA diet fed alevins) or dietary limitation (the dipeptide diet). We submit that this paradox is related to the only cursory understanding of the proline–ornithine–arginine pathway in fish, conditional indispensability and interconversion of these amino acids.

In conclusion, present observations and data collected with the use of dipeptide–protein (1:1 ratio)-based diets (B.F.T. and K.D., unpublished data) suggest that dietary peptide transport and hydrolysis in rainbow trout early life stages can be improved by the inclusion of protein supplement and more efficiently hydrolysable dipeptides. Consequently, monitoring disproportions of FAA in muscle and expression of glutamate–proline–ornithine pathway enzymes, in concert with growth indices, is the right approach to address basic mechanisms of amino acid utilization in larval fishes. The modest success of nutrient administration in the form of dipeptides points to the potential for evaluation of amino acid requirements in early life stages of fishes that has not been possible thus far.

We are grateful to G. Wu, Texas A&M University for mammalian P5CR assay protocols. We acknowledge The Ohio State University Post-doctoral Fellowship Program, and The Norwegian Research Council (project no. 120/159934) for funding.

References

Adams, E. and Frank, L. (1980). Metabolism of proline and the hydroxyprolines. *Annu. Rev. Biochem.* **49**, 1005-1061.

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

Aranishi, F., Watanabe, T., Osatomi, K., Cao, M., Hara, K. and Ishihara, T. (1998). Purification and characterization of thermostable dipeptidase from carp intestine. *J. Mar. Biotechnol.* **6**, 116-123.

Ball, R., Atkinson, J. and Bayley, H. (1986). Proline as an essential amino acid for the young pig. *Br. J. Nutr.* **55**, 659-668.

Berge, G. M. and Storebakken, T. (1996). Fish protein hydrolyzate in starter diets for Atlantic salmon (*Salmo salar*) fry. *Aquaculture* **145**, 205-212.

Bertolo, R. F. P., Brunton, J. A., Pencharz, P. B. and Ball, R. O. (2003). Arginine, ornithine, and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. *Am. J. Physiol. Endocrinol. Metab.* **284**, E915-E922.

Blaxter, J. H. S. (1988). Pattern and variety in development. In *Fish Physiology*, vol. XIA (ed. W. S. Hoar and D. J. Randall), pp. 1-58. London, New York: Academic Press.

Boge, G., Roche, H. and Balocco, C. (2002). Amino acid transport by intestinal brush border vesicles of a marine fish, *Boops salpa*. *Comp. Biochem. Physiol. B* **131**, 19-26.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of the protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Cahu, C., Zambonino-Infante, J. L., Quazuguel, P. and Le Gall, M. M. (1999). Protein hydrolyzate vs. fish meal in compound diets for 10-day old sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* **171**, 109-119.

Carter, C. G. and Houlihan, D. F. (2001). Protein synthesis. In *Fish Physiology*. Vol. 20 (ed. P. A. Wright and P. M. Anderson), pp. 31-75. San Diego, CA: Academic Press.

Carter, C., He, Z.-Y., Houlihan, D., McCarthy, I. and Davidson, I. (1995). Effect of feeding on the tissue free amino acid concentrations in rainbow trout (*Oncorhynchus mykiss* Walbaum). *Fish Physiol. Biochem.* **14**, 153-164.

Cohen, S., Meys, M. and Tarvin, T. (1989). *The Pico-Tag® Method: A Manual of Advanced Techniques for Amino Acid Analysis*. 124pp. Milford, USA: Millipore Corporation.

Dabrowski, K. (1986). Ontogenetical aspects of nutritional requirements in fish. *Comp. Biochem Physiol. A* **85**, 639-655.

Dabrowski, K., Lee, K.-J. and Rinchard, J. (2003). The smallest vertebrate, teleost fish, can utilize synthetic dipeptide-based diets. *J. Nutr.* **133**, 4225-4229.

Daniel, H. (2004). Molecular and integrative physiology of intestinal peptide transport. *Annu. Rev. Physiol.* **66**, 361-384.

Dekaney, C. M., Wu, G. and Jaeger, L. A. (2003). Gene expression and activity of enzymes in the arginine biosynthetic pathway in porcine fetal small intestine. *Pediatr. Res.* **53**, 274-280.

Doring, F., Walter, J., Will, J., Focking, M., Boll, M., Amasheh, S., Clauss, W. and Daniel, H. (1998). Delta-aminolevulinic acid transport by intestinal and renal peptide transporters and its physiological and clinical implications. *J. Clin. Invest.* **101**, 2761-2767.

Fauconneau, B., Aguirre, P. and Bergot, P. (1986). Protein synthesis in early life of coregonids: Influence of temperature and feeding. *Arch. Hydrobiol. Beih. Ergebn. Limnol.* **22**, 171-188.

Fürst, P. and Kuhn, K. S. (2000). Amino-acid substrates in new bottles: Implications for clinical nutrition in the 21st century. *Nutrition* **16**, 603-606.

Fürst, P. and Stehle, P. (2004). What are the essential elements needed for the determination of amino acid requirements in humans? *J. Nutr.* **134**, 1558S-1565S.

Grimble, G. (1994). The significance of peptides in clinical nutrition. *Annu. Rev. Nutr.* **14**, 419-447.

Halver, J. (2002). *Fish Nutrition*. San Diego, CA: Academic Press.

Halver, J. E. and Shanks, W. (1960). Nutrition of salmonoid fishes. VIII. Indispensable amino acids for sockeye salmon. *J. Nutr.* **72**, 340-346.

Halver, J. E., Delong, D. and Mertz, E. (1957). Nutrition of salmonoid fishes.

V. Classification of essential amino acids for Chinook salmon. *J. Nutr.* **63**, 95-105.

Herzfeld, A., Mezl, V. and Knox, W. (1977). Enzymes metabolizing 1-pyrroline-5-carboxylate in rat tissues. *Biochem. J.* **166**, 95-103.

Kim, K. I., Grimshaw, T. W., Kayes, T. B. and Amundson, C. H. (1992). 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**, 89-106.

Kirchgessner, v. M., Fickler, J. and Roth, F. (1995). Effect of dietary proline supply on N-balance of piglets. 3. Communication on the importance of non-essential amino acids for protein retention (in German). *J. Anim. Physiol. A Anim. Nutr.* **73**, 57-65.

Langar, H., Guillaume, J., Metailler, R. and Fauconneau, B. (1993). Augmentation of protein synthesis and degradation by poor dietary amino acid balance in European sea bass (*Dicentrarchus labrax*). *J. Nutr.* **123**, 1754-1761.

Lee, K.-J., Dabrowski, K., Rinchard, J., Gomez, C., Guz, L. and Vilchez, C. (2004). Supplementation of maca (*Lepidium meyenii*) tuber meal in diets improves growth rate and survival of rainbowtrout *Oncorhynchus mykiss* (Walbaum) alevins and juveniles. *Aquac. Res.* **35**, 215-223.

Matthews, D. (1991). *Protein absorption*. New York: Wiley-Liss.

Mezl, V. A. and Knox, W. E. (1977). Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. *Anal. Biochem.* **74**, 430-440.

NRC (1993). Nutrient requirements of fishes. In *National Research Council, Board of Agriculture* (ed. C. Cowey, Y. C. Cho, K. Dabrowski, S. Hughes, S. Lall, R. Lovell, T. Murai and R. Wilson). Washington, DC: National Academy Press.

Ogata, H. (2002). Muscle buffering capacity of yellowtail fed diets supplemented with crystalline histidine. *J. Fish Biol.* **61**, 1504-1512.

Reshkin, S. and Ahearn, G. (1991). Intestinal glycyl-L-phenylalanine and L-phenylalanine trasnport in a euryhaline teleost. *Am. J. Physiol.* **260**, R563-R569.

Samuels, S., Aarts, H. and Ball, R. O. (1989). Effect of dietary proline on proline metabolism in the neonatal pig. *J. Nutr.* **119**, 1900-1906.

Sato, K., Yoshinaka, R. and Sato, M. (1989). Hydroxyproline content in the acid-soluble collagen from muscle of several fishes. *Nippon Suisan Gakkaishi* **55**, 1467.

Schumacher, A., Wax, C. and Gropp, J. (1997). Plasma amino acids in rainbow trout (*Oncorhynchus mykiss*) fed intact protein or a crystalline amino acid diet. *Aquaculture* **151**, 15-28.

Smith, R. and Phang, J. (1978). Proline metabolism in cartilage: the importance of proline biosynthesis. *Metab. Clin. Exp.* **27**, 685-694.

Sunde, J., Taranger, G. and Rungruangsak-Torissen, K. (2001). Digestive protease activities and free amino acids in white muscle as indicators for feed conversion efficiency and growth rate in Atlantic salmon (*Salmo salar* L.). *Fish Physiol. Biochem.* **25**, 335-345.

Terjesen, B. F., Verreth, J. and Fyhn, H. J. (1997). Urea and ammonia excretion by embryos and larvae of the African Catfish *Clarias gariepinus* (Burchell 1822). *Fish Physiol. Biochem.* **16**, 311-321.

Terjesen, B. F., Chadwick, T. D., Verreth, J. A. J., Rønnestad, I. and Wright, P. A. (2001). Pathways for urea production during early life of an air-breathing teleost, the African catfish *Clarias gariepinus* Burchell. *J. Exp. Biol.* **204**, 2155-2165.

Torrissen, K., Lied, E. and Espe, M. (1994). Differences in digestion and absorption of dietary protein in Atlantic salmon (*Salmo salar*) with genetically different trypsin isozymes. *J. Fish Biol.* **45**, 1087-1104.

Verri, T., Kottra, G., Romano, A., Tiso, N., Peric, M., Maffia, M., Boll, M., Argenton, F., Daniel, H. and Storelli, C. (2003). Molecular and functional characterisation of the zebrafish (*Danio rerio*) PEPT1-type peptide transporter. *FEBS Lett.* **549**, 115-122.

Waterlow, J. (1999). The nature and significance of nutritional adaptation. *Eur. J. Clin. Nutr.* **53**, S2-S5.

Weatherley, A. H. and Gill, H. S. (1987). *The Biology of Fish Growth*. London: Academic Press.

Wu, G. and Morris, S. M. (1998). Arginine metabolism: nitric oxide and beyond. *Biochem. J.* **336**, 1-17.

Yamamoto, T., Unuma, T. and Akiyama, T. (2000). The influence of dietary protein and fat levels on tissue free amino acid levels of fingerling rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **182**, 353-372.

Yokoyama, M. and Nakazoe, J.-I. (1991). Effects of dietary protein levels on free amino acid and glutathione contents in the tissues of rainbwo trout. *Comp. Biochem. Physiol.* **99**, 203-206.