

Effects of dietary levels of protein on nitrogenous metabolism of *Rhamdia quelen* (Teleostei: Pimelodidae)

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Abstract

This manuscript reports changes in key enzymes and metabolites related to protein metabolism and nitrogen excretion in the liver of juveniles jundiá (*Rhamdia quelen*) fed on isocaloric diets containing 20%, 27%, 34% and 41% of crude protein. The hepatic activity of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), glutamate dehydrogenase (GDH), and arginase (ARG) increased with the content of protein in the diet, and the ratios among the aminotransferases and GDH allowed evaluating metabolic preference. The concentration of free amino acids, ammonia and urea also rose with the dietary protein content. Increase of plasma urea and ammonia was the resultant effect of over amino acids catabolism as consequence of dietary protein surplus. Since the increase of protein in the diets resulted in weight gain, the rise in the hepatic activity of protein-metabolising enzymes in the fish fed high protein diets denoted effective use of dietary amino acids for growth and as a substrate for gluconeogenesis. Analysis of changes on metabolite levels and key enzyme activities in amino acid metabolism is proposed as a tool for assessing the proper balance of diet macronutrients.

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1. Introduction

Fishes use amino acid carbon backbone not only for building proteins, but also for synthesis of sugars and fats (Wood, 1993). Metabolic pathways for amino acids, sugars and fats catabolism were firstly studied in mammals. However, the effects of dietary nutrients on the intermediary metabolism of teleost fishes remain scarcely known as compared to mammals.

In carnivorous fish glucose is usually synthesized from amino acids via gluconeogenesis (Cowey et al., 1977). In several species, glycogen mobilization is slow and glucose, for metabolic demands, is mainly obtained from amino acids. However, it is expected that omnivorous fishes can utilize carbohydrates more easily than carnivorous fish. The set of enzymes related to

both nutrient groups, sugar and protein, should be likely distinct concerning responses to dietary changes in both groups of fish. There are many reports concerning the direct relationship among enzymes of energetic metabolism, protein metabolism and the nutritional status (Baanante et al., 1991; Bonamusa et al., 1992; Moon and Foster, 1995; Metón et al., 1999).

In feeding fish, increasing contents of protein usually result in a rise in the growth rate. This is directly correlated to the increase in oxidative metabolism and protein synthesis likely as consequence of amino acids surplus and due to hormonal effects (Houlihan et al., 1993). The amino acids surplus from protein-rich diets cannot be directly stored in fishes; they are deaminated and converted into energetic compounds (Ballantyne, 2001; Stone et al., 2003).

The expression of key enzymes of intermediary metabolism is modulated by nutritional status in fish (Metón et al., 1999, 2003). In this regard, the levels of amino acid-metabolising enzymes and nitrogen excretion are reliable indicators of dietary protein

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availability. Increase in arginase activity is directly associated with protein in the diet of *Salmo salar* (Lall et al., 1994). The rise of protein catabolism due to high protein in diets has been also detected through the plasma concentration of free metabolic intermediates, such as amino acids (Ogata and Arai, 1985). In addition, low digestibility of the energetic foods increases nitrogenous excretion (Steffens, 1989).

Metabolism of carnivorous fish is adapted to high levels of dietary protein. By contrast, low ability of using sugars is reported (Covey and Walton, 1989; Caseras et al., 2000; Lundstedt et al., 2004). Chinook salmon *Oncorhynchus tshawytscha* was likely the first fish species studied on the protein demands (De Long et al., 1958). Afterwards, many other species, including omnivorous species, were studied and the protein requirement ranged around 22–40% for catfish *Ictalurus punctatus* (Garling and Wilson, 1976); 34% for the tilapia *Oreochromis aureus* (Winfree and Stickney, 1981); 30% for Nile tilapia *Oreochromis niloticus*; 40% for Mozambique tilapia *Oreochromis mosambicus* (Jauncey, 1982) and 32–40% for Neotropical freshwater fish piracanjuba *Brycon orbignyanus* (Do Carmo de Sá and Fracalossi, 2002) and *Pseudoplatystoma corruscans* (Lundstedt et al., 2004). Previously, we have observed that changes in the metabolic profile are a tool to assess the fish performance and ability to cope with different dietary conditions (Bidinotto and Moraes, 2000; Moraes and Bidinotto, 2004; Lundstedt et al., 2004). At present, most studies on fish protein requirements and metabolism are concerned with models for marine carnivorous coldwater fish. Therefore, knowledge on biochemical features in the warm freshwater fish will provide the basis for better understanding on the handling and rearing of these species.

The study of nutritional demands and the effect of dietary nutrients in the intermediary metabolism of the South American freshwater catfish jundiá (*Rhamdia quelen*) are of particular interest due to the aquaculture characteristics of the species: *R. quelen* accepts artificial feeding from hatching, shows large survival rates and fast growth (Piaia and Radünz-Neto, 1997; Melo et al., 2002). Little information concerning digestion and metabolism of species from the genus *Rhamdia* is available. *R. quelen* is omnivorous and able to use numerous kinds and sources of nutrients. The present work reports adaptive metabolic adjustments of key metabolites and related enzymes of protein metabolism of *R. quelen* fed increasing contents of crude protein.

2. Materials and methods

2.1. Experimental design

Juveniles of jundiá (*R. quelen*) ranging 32.6±3.0 g (S.D.) from artificial reproduction were fed with 6–8 mm dry pellets for 60 days at a feeding rate of 10% of biomass, twice a day. Periodical evaluation of growth was done each 15 days to adjust to that fish rate of feeding. We used four isocaloric diets (4,500 kcal gross energy/kg) with 20%, 27%, 34% and 41% of crude protein (CP) (Table 1). Fish ($N=160$) were equally divided into four fed groups and held in 2000 L tanks with thermo-regulated water at 26±2 °C, in a re-circulated closed system

Table 1

Ingredients and analyzed composition of the experimental diets (dry matter basis) for jundiá (*Rhamdia quelen*)

	Experimental diets			
	20% CP	27% CP	34% CP	41% CP
<i>Ingredients^a (%)</i>				
Fish meal ^b	14.2	24.2	19.2	35.2
Soy bran ^c	8.0	11.0	11.0	24.0
Yeast ^d	8.0	8.0	20.0	15.0
Corn ^e	38.0	30.0	26.0	8.0
Wheat bran ^f	14.0	14.0	14.0	10.0
Vegetable oil ^g	17.0	12.0	9.0	7.0
NaCl	0.5	0.5	0.5	0.5
Vitamin mixture ^h	0.2	0.2	0.2	0.2
Mineral mixture ⁱ	0.1	0.1	0.1	0.1
Vitamin C	0.05	0.05	0.05	0.05
<i>Chemical composition</i>				
Dry matter (%)	90.06	89.81	89.61	90.74
Crude protein (%)	19.37	26.33	33.2	40.14
Gross energy (kcal/kg)	4.419	4.610	4.504	4.438
Lipid (%)	18.57	14.76	11.98	9.70
Crude fiber (%)	3.24	3.24	3.14	3.92
Ash (%)	5.35	7.88	7.53	12.61
Carbohydrate (%) ^j	43.53	38.60	32.50	24.37

CP—crude protein; DM—dry matter; GE—gross energy; L—lipid; CF—crude fibre; CHO—carbohydrate as nitrogen free extract.

^a Ingredients used in the composition of experimental diets were purchased from animal food companies in Sao Paulo State, Brazil.

^b Fishmeal: 92.12% DM; 56.43% CP; 4100 kcal GE; 4.45% L; 0.34% CF; 21.4% Ash; 9.5% CHO.

^c Soy Bran: 86.54% DM; 46.02% CP; 3010 kcal GE; 0.96% L; 6.08% CF; 5.41% Ash; 28.07% CHO.

^d Yeast: 92.12% DM; 56.43% CP; 3522 kcal GE; 4.45% L; 0.34% CF; 21.4% Ash; 9.5% CHO.

^e Corn: 87.8% DM, 7.17% CP, 4004 kcal GE; 4.14% L; 1.5% CF; 1.15% Ash; 73.84% CHO.

^f Wheat Bran: 96.99% DM; 11.84% CP; 2700 kcal GE; 3.75% L; 8.4% CF; 4.82% Ash; 58.2 CHO.

^g Soy bean oil 9,800 kcal GE.

^h Composition of vitamin mixture/kg dietary: Vitamin A 10,000 UI, E 50 mg, D₃ 2000 UI, K-3 25 mg, B 12 50 mg, thiamine 15 mg, riboflavin 35 mg, pyridoxine 10 mg, biotin 250 mg, folic acid 5 mg, acid pantothenic 60 mg, niacin 60 mg, choline 120 g, antioxidant 50 g.

ⁱ Composition of mineral mixture/kg dietary: iron 40 mg, copper 12.5 mg, zinc 30 mg, cobalt 1 mg, iodine 1.4 mg and selenium 0.2 mg.

^j Carbohydrate (%)=Dry Matter–(Crude Protein+Fat+Crude Fibre+Ash).

provided with bio-filters. Water quality parameters were daily checked and kept within satisfactory range (pH 6.9±1.2; dissolved oxygen 5.6±1.3 mg/L; N–NH₃ 0.14±0.04 mg/L). Natural photoperiod was 14 h light. After 15 days, 10 fish per treatment were sampled, anaesthetized with benzocaine (50 mg/L) and killed by cervical punching. Liver were quickly excised, frozen in liquid nitrogen and kept at –80 °C. The remaining fish ($n=30$ per tank) continued in the tanks for 45 days more and were sampled for biometry.

2.2. Metabolites determination

Liver was homogenized (1:10 w/v) into 20% trichloroacetic acid (TCA acid extract) or in saline solution (neutral extracts). Metabolites were quantified in crude plasma and liver extracts.

Table 2
Growth parameters of jundiá (*Rhamdia quelen*)

	Dietary protein level (%)			
	20% CP	27% CP	34% CP	41% CP
Mean initial mass (g)	30.29±5.24	33.23±7.86	32.11±4.24	29.23±6.07
Mean initial length (cm)	15.82±1.07	16.08±1.06	15.97±0.87	15.61±1.31
Initial biomass (g)	1211	1329	1284	1169
Mean final mass (g)	60.04±17.04 ^d	76.12±13.89 ^c	87.64±11.71 ^b	101.17±17.58 ^a
Mean final length (cm)	18.52±2.14 ^b	19.67±1.51 ^{ab}	18.88±1.42 ^{ab}	20.91±1.78 ^a
Final biomass (g)	1801	2283	2629	3035
Weight gain (%)	98.2	129.1	172.9	246.1

CP: Crude Protein. Weight is expressed in grams, length in cm and weight gain in %. Different letters in the same row signify statistical differences ($P < 0.05$) (mean ± S.D.). Weight gain (%) = [(Mean Final Weight – Mean Initial Weight) / Mean Initial Weight] × 100.

Urea was determined at 525 nm in acid extract (Rahmatullah and Boyde, 1980). Glucose was determined at 340 nm by the glucose dehydrogenase method using a commercial kit (Roche). Free amino acids and ammonia were determined in neutral extracts at 570 nm (Copley, 1941) and 420 nm (Gentzkow and Masen, 1942), respectively.

2.3. Enzyme assays

Crude homogenates to assay enzyme activities were obtained by homogenization of powdered frozen liver (1/5, W/V) into 50 mM Tris–HCl pH 7.5, 4 mM EDTA (ethylenediamine tetraacetate), 50 mM NaF, 0.5 mM PMSF (Phenylmethylsulfonyl

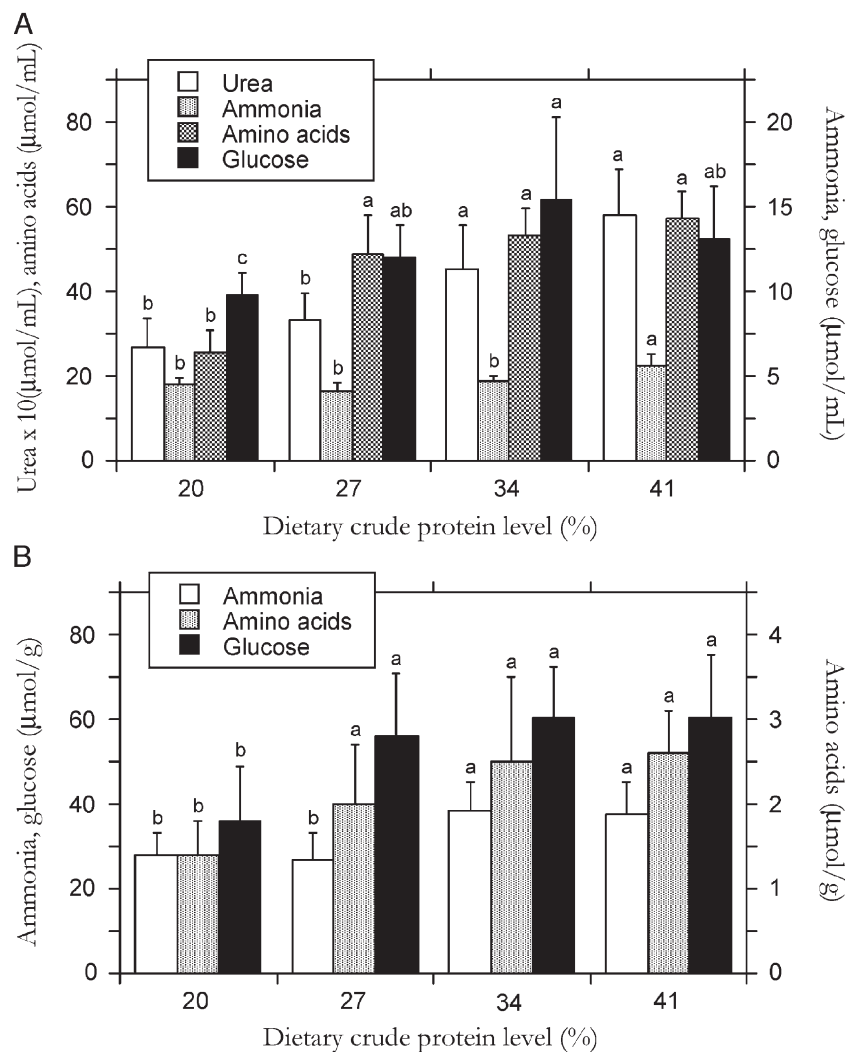


Fig. 1. Concentration of metabolites in plasma (A) and liver (B) of jundiá (*Rhamdia quelen*) fed different dietary crude protein levels (%). Distinct letters indicate statistical differences at $P < 0.05$ (mean ± S.D.; $n = 10$).

fluoride), 1 mM DTT (dithiothreitol) and 250 mM sucrose, in a tissue homogenator (PTA-7 Polytron position 3, 30 s). Homogenates were centrifuged at $20,000\times g$ for 30 min at 4 °C and the supernatants were used as source of crude enzyme. Enzymes activities are expressed as mU/mg of protein (1 mU = 1 nmol of enzyme product/min).

Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were assayed with the Roche-Kits for routine determinations in COBAS MIRA S auto-analyzer. Both reactions were carried out at 340 nm and 30 °C. Glutamate dehydrogenase (GDH) activity was determined through NADH extinction at 340 nm for 2 min of reaction (Hochachka et al., 1978). Assay was done at 25 °C and the reaction mixture contained: 0.05 M imidazole buffer (pH 7.0), 0.1 mM NADH, 1.0 mM ADP, 5 mM 2-ketoglutarate, 250 mM ammonium acetate and crude enzyme, previously adjusted to proper protein concentration to a final volume of 1.0 mL. Arginase (ARG) activity was determined at 25 °C for 30 min by a colorimetric method (Archibald, 1946). Assay mixture contained: 250 mM arginine, 50 mM Tris buffer pH 8.0 and 1 mM $MnCl_2$ and crude enzyme previously adjusted to the proper protein concentration to a final volume of 1.2 mL.

The enzyme reaction was stopped by addition of 0.1 mL 70% perchloric acid (PCA) and centrifuged at $12,000\times g$ for 3 min. Urea was colorimetrically determined in the supernatant at 525 nm (Rahmatullah and Boyde, 1980).

Total protein content in liver crude extracts was determined at 30 °C at 600 nm (Bradford, 1976), using bovine serum albumin as standard.

2.4. Statistics

Each tank was considered an experimental unity, and the experimental design was conceived for incomplete blocks considered each fish one replicate ($n=10$). All data were submitted to ANOVA, *F*-test and Tukey's test to compare the means. The accepted level of significance was $P<0.05$. All data were followed by \pm S.D.

3. Results

The mean weight and length, and the weight gain percent of *R. quelen* fed on increasing protein contents in the diet

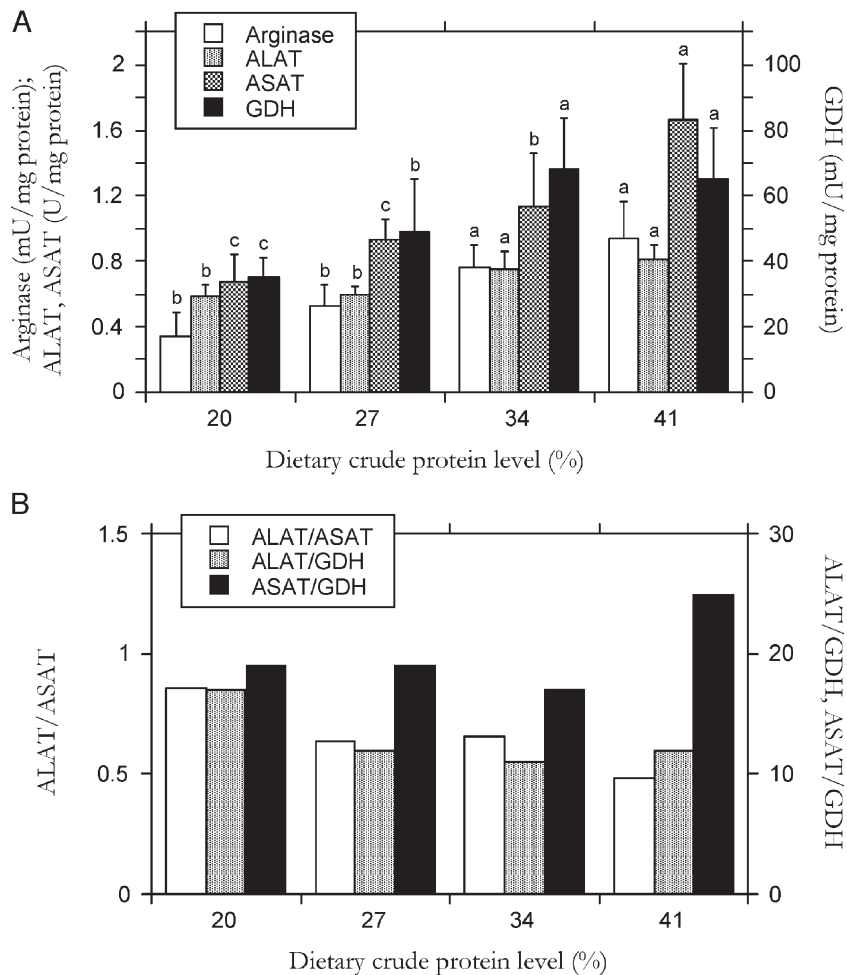


Fig. 2. Activities of liver key enzymes of amino acid metabolism (A) and the ratio between combined enzyme pairs (B) of jundiá (*Rhamdia quelen*). Enzyme activities are expressed in U/mg protein or mU/mg protein (1 U = 1000×1 mU = 1 nmol of enzyme product/min). Different letters in the same line signify statistical differences at $P<0.05$ (mean \pm S.D.; $n=10$).

significantly increased in the course of 60 days (Table 2). The percentage of weight gain in the fish fed with 27%, 34% and 41% of CP was about 1.5-, 2- and 3-fold, respectively, over the values observed in the group of fish fed with 20% of CP. The fish survival was 100% in all treatments.

Feeding *R. quelen* with different dietary protein levels also promoted significant changes in plasma and liver metabolites (Fig. 1), and enzyme activities involved in protein metabolism (Fig. 2). The concentration of plasma urea followed the increase of protein in the diets. The values rose from 266 to 581 $\mu\text{mol/mL}$. Such high increase was followed by hepatic arginase, which rose from 0.337 to 0.938 mU/mg of protein. A linear relationship was observed between the levels of dietary protein and activity of hepatic arginase, and levels of dietary protein and plasma urea concentration. The levels of plasma amino acids increased from 25 to 57 $\mu\text{mol/mL}$, and in the liver from 1.4 to 2.6 $\mu\text{mol/mg}$ protein. Both changes were directly related. The concentration of plasma ammonia increased from the average 4.4 $\mu\text{mol/mL}$ at the lower dietary protein to 5.5 $\mu\text{mol/mL}$ at 41% protein. Similarly, liver ammonia reached the higher concentrations when the fish were fed with 34 or 41% of CP, rising from about 27 $\mu\text{mol/mg}$ (20% and 27% of CP) to about 38 $\mu\text{mol/mg}$ of tissue protein.

Hepatic enzymes related to metabolism of amino acids were particularly affected. In the group of fish fed with 41% of CP, activity of ALAT increased nearly 30% from 587 to 813 mU/mg protein, ASAT more than doubled (from 687 to 1669 mU/mg protein), and GDH increased from 35 to 65 mU/mg protein, compared to fish fed with 20% of CP. Direct relationship can be observed between the levels of diet protein and the activities of those enzymes. Analysis of the ratios ALAT/ASAT, ALAT/GDH and ASAT/GDH indicate distinct efficiencies for ALAT, ASAT and GDH in response to the diet protein contents.

4. Discussion

In the present study, we used changes in the metabolic profile to predict the ability of *R. quelen* to cope with different dietary protein levels. The difference between the initial and final mean weight of *R. quelen* compared with the changes observed in the body length indicated a significant increase of muscular mass when dietary protein increased (Table 2). This fact can be attributed to the large offering of protein in the food and consequent conversion to body mass. However, a fraction of that protein may have been used as fuel. Relative deficiency of dietary protein can result in low food intake, impairing growth and development (Lovell, 1979), as a consequence of metabolic utilization of non-protein sources followed by amino acid deficiency (Winfrey and Stickney, 1981).

The muscle is a pivotal compartment directly linked to the amino acid turnover. This involves protein synthesis or breakdown of those molecules as energetic substrate. Any excess of amino acids can be converted into carbohydrates or, in smaller amounts, to fat (Driedzic and Hochachka, 1978). Metabolism of amino acids involves deamination and transamination reactions. The activities of transaminases and deaminases are useful to evaluate the feeding status in some fish (Alexis and Papapar-

askeva-Papoutsoglou, 1986; Moyano et al., 1991). The rise of hepatic ALAT, ASAT and GDH observed in *R. quelen* fed high protein diets may reflect the use of excess carbon backbones from amino acids to supply energetic demands. Likewise, high protein/carbohydrate ratios in the feeding of *Sparus aurata* bring about ALAT and ASAT to increase in the liver (Metón et al., 1999). Similar responses were observed in *Oncorhynchus mykiss* for ALAT and GDH (Sánchez-Muros et al., 1998) and in *Anguilla anguilla* for GDH (Suárez et al., 1995). The rise in the hepatic activity of protein-metabolizing enzymes when fish were fed high protein diets may denote use of excess dietary amino acids for growth as well as substrate for gluconeogenesis, particularly for GDH and ALAT activities.

Replacement of non-protein energetic nutrients with protein in the feeding of *R. quelen* showed adaptation of the metabolic pathways toward the consumption of proteins as energy source. Thus, substitution of dietary protein for carbohydrates demands special care because there is evidence that an excess of non-protein energy (carbohydrates and lipids) can impair growth (Winfrey and Stickney, 1981; Johnson et al., 2002). The use of ingested protein as energy source varies between 41% and 85% among fish (Van Waarde, 1983). Therefore, the ratio protein/carbohydrate must be adjusted to prevent the breakdown of amino acids just as energy substrate.

In the present study, the high contents of dietary protein, and the consequent low levels of carbohydrates, increased transaminations, deamination and nitrogenous metabolites in *R. quelen*. Apparently the increase of plasma glucose observed when the level of dietary carbohydrate decreased is inconsistent. Two possibilities are raised to explain such a fact, gluconeogenesis or lower digestibility of sugar from corn and wheat bran as higher contents are introduced. In spite of all enzyme activities were increased with the dietary protein, GDH, ASAT and ALAT were respectively more demanded. The enzymes GDH and ASAT are fully involved in amino acid metabolism; however, carbon residues directly from glycolysis can also supply ALAT. Therefore, by comparing ratios among transaminases and GDH in the liver of *R. quelen* it was possible to emphasize that the gluconeogenic processes were exacerbated. Consistently, higher levels of plasma and liver glucose were found in the fish fed with higher CP diets. Changes in nitrogen metabolism related enzymes associated with high increase of glucose observed in liver are very suggestive of gluconeogenesis due to CP increase in the diets. The increase in GDH, ASAT and ALAT denotes catabolism of amino acids and is associated with increased nitrogenous excretion. The augment of protein breakdown in fish, resulting in increase of plasma ammonia concentration, was previously observed in *Bidyanus bidyanus* and *Dicentrarchus labrax* (Yang et al., 2002; Peres and Oliva-Teles, 2001). Excess of ammonia is promptly excreted through the gills (Van Waarde et al., 1983). In some fish species, particularly under adverse environmental conditions when ammonia excretion is impaired, urea excretion is triggered (Wood et al., 1995; Saha and Ratha, 1998). High levels of environmental ammonia should contribute to plasma ammonia increased. However, the high quality of water in the trials prevented such undesirable effect. Therefore, the increase of ammonia and urea observed in *R. quelen* were certainly due to the increase of protein catabolism.

Urea cycle induction can be observed in fasting (Walsh et al., 1990), dehydration (Polez et al., 2003), confinement stress (Hopkins et al., 1995), water alkalinity (Polez et al., 2004), and external ammonia concentration (Saha and Ratha, 1994). Also, the increase of protein content in diets can enhance the activity of urea cycle enzymes in fish (Chiu et al., 1986). Arginase activity, urea excretion and plasma urea concentration in turbot (*Psetta maxima*) and rainbow trout (*O. mykiss*) are directly correlated to dietary arginine (Gouillou-Coustans et al., 2002; Fournier et al., 2003). Thus, enhancing of liver arginase activity and the plasma urea content in *R. quelen* fed high protein diets may result from increasing concentration of dietary arginine.

Plasma glucose and amino acids tend to increase in fish fed high protein levels as observed in *O. mykiss* (Lone et al., 1982) and European eel (Suárez et al., 1995). In the present study, the surplus of protein in the feeding of *R. quelen* can be inferred from the amino acids concentration in the tissues. This fact can be also associated to increased absorption of amino acids from protein digestion as stressed by Yamamoto et al. (1999). On the other hand, the increase of non-protein digestible energy enhances nitrogen retention (Engin and Carter, 2001). Although, the increase of amino acids in the plasma of jundiá followed by plasma glucose is likely driven to the synthesis of new proteins and/or sugar, the increase of ALAT, ASAT and GDH suggests protein catabolism at high protein levels in the diet. Blood and tissue levels of amino acids are dependent on factors such as digestion efficiency, fish size, bulk and composition of feeding, and temperature (Grove et al., 1981; Darcy, 1984). In this case, the rise of plasma amino acids versus dietary protein increase is likely due to enhancement of digestion (Lundstedt et al., 2002).

Increase of nitrogenous excretion is a consequence of using amino acids as energetic compounds (Hidalgo and Alliot, 1988; Kim et al., 1991). A rise in the ratio of protein/carbohydrate plus lipid increases nitrogen excretion in trout (Rychly, 1980). A direct relationship between protein intake and ammonia excretion has been found in fish (Li and Lowell, 1992; Chakraborty and Chakraborty, 1998), and ammonia excretion has been proposed as index for comparing the efficiency of dietary protein utilization among rainbow trout of different strains (Ming, 1985). Rychly (1980) reported that nitrogen excretion increased with high protein and low carbohydrate content in the diet. Therefore, the high catabolism of protein observed in *R. quelen* indicates the importance of carbohydrate addition in the feeding of this species for the purpose of sparing protein.

In conclusion, levels of ALAT, ASAT, GDH and arginase were significantly affected by diet composition in *R. quelen* and can be used as a metabolic tool for assessing the proper concentration of dietary protein in the feeding of this species. The ratios among transaminases and GDH enabled evaluating the metabolic preference in stated circumstances. The activity of arginase associated to urea and ammonia quantification is an easy form of establishing the surplus of nitrogen source in the feeding of *R. quelen*. In addition, the knowledge of the proper ratio protein/carbohydrate in the fish feeding is fundamental to establish the optimal nitrogen content in the food, to prevent nitrogen waste and to avoid environmental damages. The ratio

protein/energy in the *R. quelen* diets seems to be specific and further studies concerning types and sources of carbohydrates and lipids to replace protein as energy source are necessary.

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