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Glycolysis and gluconeogenesis in the liver of catfish fed with different concentrations of proteins, lipids and carbohydrates

[*Glicólise e gliconeogênese no fígado de jundiá alimentado com diferentes concentrações de proteínas, lipídeos e carboidratos*]

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ABSTRACT

The activities of enzymes from a number of metabolic pathways have been used as a tool to evaluate the best use of nutrients on fish performance. In the present study the catfish *Rhamdia quelen* was fed with diets containing crude protein-lipid-carbohydrate (%) as follows: treatment (T) T1: 19-19-44; T2: 26-15-39; T3: 33-12-33; and T4: 40-10-24. The fish were held in tanks of re-circulated, filtered water with controlled temperature and aeration in 2000L experimental units. The feeding experiment lasted 30 days. The following enzymes of the carbohydrate metabolism were determined: Glucokinase (GK), Phosphofructokinase 1 (PFK-1), Pyruvate kinase (PK), Fructose-1,6-biphosphatase 1 (FBP-1). The activities of 6 phosphogluconate dehydrogenase (6PGDH) and glucose 6 phosphate dehydrogenase (G6PDH) were also assayed. The influence of nutrient levels on the enzyme activities is reported. The increase of dietary protein plus reduction of carbohydrates and lipids attenuates the glycolytic activity and induces hepatic gluconeogenesis as a strategy to provide metabolic energy from amino acids. The fish performance was affected by the concentrations of protein, lipid and carbohydrates in the diet. The greatest weight gain was obtained in fish fed diet T4 containing 40.14% of crude protein, 9.70% of lipids, and 24.37% of carbohydrate, respectively.

Keywords: metabolism, enzymes, nutrition, adaptation

RESUMO

As atividades de enzimas das vias metabólicas têm sido utilizadas como uma ferramenta para avaliar a melhor utilização dos nutrientes e o desempenho dos peixes. No presente estudo, o jundiá foi alimentado com rações contendo diferentes concentrações de proteína bruta, lipídeos e carboidratos (%), da seguinte forma: tratamento (T) T1: 19-19-44; T2: 26-15-39; T3: 33-12-33; e T4: 40-10-24. Os peixes foram mantidos em tanques de recirculação, com água filtrada, temperatura controlada e aeração em unidades experimentais de 2.000L. O período experimental foi de 30 dias. Foram aferidas as atividades das enzimas glicoquinase (GK), fosfofrutoquinase 1 (PFK-1), piruvato quinase (PK) e frutose-1,6-difosfatase (FBP-1). Também foram aferidas as atividades da 6-fosfogluconato desidrogenase (6PGDH) e glicose-6-fosfato desidrogenase (G6PDH) da via das pentoses. É relatado que níveis de nutrientes influenciam as atividades enzimáticas das vias metabólicas. No presente estudo, o aumento da proteína da dieta e a redução de hidratos de carbono e lipídeos reduziram a atividade glicolítica e induziram a gliconeogênese hepática como uma estratégia para fornecer energia pelos aminoácidos. O desempenho dos peixes foi afetado pelas concentrações de proteínas, lipídeos e carboidratos na dieta. O maior ganho de peso foi obtido em peixes alimentados com dieta T4 contendo 40,14% de proteína bruta, 9,70% de lipídeos, e 24,37% de carboidratos, respectivamente.

Palavras-chave: metabolismo, enzimas, nutrição, adaptação

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INTRODUCTION

Among the macronutrients, proteins play a pivotal role in many biological functions. In particular, fish present meaningful dietary protein requirements. Lipids are assumed as the main source of metabolical energy; however, tropical fresh water species are usually able to use great deals of carbohydrate while most fish burn proteins as ordinary fuel. These facts deserve special attention when a diet is designed for an intensive fish rearing system. Nutritional requirements must look upon many aspects such as: fish species, metabolic demands, growth phase and potential to adapt to environmental changes. In essence, adaptation to external changes is related to two factors, gene assembly and the array of expressed enzymes; and both are directly related to metabolism.

To date, the number of studies on metabolism of fish related to nutritional status is scarce. However, many studies have focused on the comprehension of the metabolic phenomena toward the improvement of nutrient utilization and the fish performance in aquaculture systems. Among carnivorous animals, fishes require higher levels of dietary protein, ordering amino acids toward protein synthesis and glucose formation for energetic requirements (Sánchez-Muros *et al*., 1998). Increases of plasma amino acids after high protein feeding or aestivation make up the large source of energy for carnivorous fish. Studies on regulatory aspects of gluconeogenesis in *Oncorhyncus mykiss* fed high protein levels report the rise of plasma amino acids (Cowey *et al*., 1977). Partial replacement of protein by dietary carbohydrates is well tolerated in *Sparus aurata* (Metón *et al*., 2003). The omnivorous *Rhamdia hilari* also adapts its metabolism to high levels of dietary carbohydrates, using dietary lipid and protein as the main source of energy in the muscle during starvation. Moreover, lipid and protein synthesis in muscle and liver are remarkably higher after refeeding or insulin administration (Machado *et al*., 1988).

This is a consequence of the use of carbohydrate for energetic processes sparing amino acids for muscle protein synthesis. Therefore, the possibility of feeding fish with different contents of carbohydrates to improve the use of nutrients is a promising item, particularly in omnivorous species.

The South American catfish "jundiá" *Rhamdia quelen* displays several farming qualities. Eleven species are presently included in the *Rhamdia* genus, a Neotropical Siluriform-Pimelodidae reported from Mexico to Argentina (Silfvergrip, 1996.). The *R. quelen* accepts artificial feeding since hatching, presenting good survival rates and fast growth and development. Nowadays, there is little information concerning metabolism of species from the genus *Rhamdia*. Recently, we have reported that the levels of dietary protein affect the protein metabolism and nitrogen excretion in juveniles of that species (Melo *et al*., 2006), and hypothesized that the metabolic machinery is adapted to changes in diet composition. We have been proposing the study of metabolic changes combined with digestive profiles as a tool to infer the fish performance and evaluate its effort to cope with different nutritional circumstances (Lundstedt, *et al*., 2004). In the present study, adaptive adjustments of metabolic enzymes in *R. quelen* fed distinct contents of nutrients were investigated. The changes observed in the metabolic frames of *R. quelen* were used in attempt to match the levels of dietary macronutrients with the best growth performance.

MATERIALS AND METHODS

The present study was approved by the bioethics committee of the University of Barcelona-ES, under number 349002-ES/2003. One-hundred-and-sixty juvenile jundiá *R. quelen* from the same strain (44.98±13.32g and 16.92±1.44cm) were equally distributed in four 2000L tanks in a close, re-circulated water system and acclimated for one week and fed commercial pellets. Afterwards, feeding was discontinued and the fish were fed to satiety for 30 days twice a day with the experimental diets. The water quality was monitored and kept at: temperature 25±1°C, pH 7.2, dissolved oxygen 5.4mgL^{-1}, alkalinity 42mgL^{-1} and ammonia 0.12mgL-^{1}. No fish mortality was observed during the experiment.

The experimental feeding consisted of four diets containing: 19, 26, 33, and 40% of crude protein (CP); 19, 15, 12, and 10 % of lipids, and 44, 39, 33, and 24% of carbohydrate, respectively (Table 1). We conducted a control treatment,

where the animals were fasted during the experimental period. Dietary average gross energy was 4490Kcal Kg^{-1}. Nutrient composition was previously determined and the diet ingredients were analyzed according to the Association of Official Analytical Chemists. After the trial period, the feeding was discontinued for 24h and 10 fish per treatment were randomly netted, anaesthetized with benzocaine (100mgL^{-1} water), and killed by cervical pinch. The fish were dissected and liver were collected and immediately frozen into liquid nitrogen. All samples were preserved at -80°C until analyses.

Table 1. Composition of the experimental diets

	Experimental treatments[1]			
	T1 (19-19-44)	T2 (26-15-39)	T3 (33-12-33)	T4 (40-10-24)
Formulation (%)				
Fish meal	14.2	24.2	19.2	35.2
Soy bran	8.0	11.0	11.0	24.0
Yeast	8.0	8.0	20.0	15.0
Corn	38.0	30.0	26.0	8.0
Wheat	14.0	14.0	14.0	10.0
Vegetal Oil	17.0	12.0	9.0	7.0
Salt	0.5	0.5	0.5	0.5
Vitamin mixture[2]	0.2	0.2	0.2	0.2
Mineral mixture[3]	0.1	0.1	0.1	0.1
Vitamin C	0.05	0.05	0.05	0.05
Chemical Analysis				
Crude protein (%)	19.37	26.33	33.2	40.14
Gross Energy (kcal/kg)	4.419	4.610	4.504	4.438
Fat (%)	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
Dry matter (%)	90.06	89.81	89.61	90.74
Carbohydrate (%)[4]	43.53	38.60	32.5	24.37

[1]Experimental treatments (dietary protein-lipid-carbohydrate content, respectively);
[2]Composition of vitamin mixture/Kg diet: Vitamin A 10.000 UI, E 50mg, D_3 2000 UI, K-3 25mg, B-12 50mg, Thiamin 15mg, Riboflavin 35mg, Pyridoxine 10mg, Biotin 250mg, Folic acid 5mg, Pantothenic Acid 60mg, Niacin 60mg, Choline 120g. Antioxidant 50g.
[3]Composition of the mineral mixture/Kg of diet: Iron 40mg, Copper 12,5mg, Zinc 30mg, Cobalt 1mg, Iodine 1,4mg and Selenium 0,2mg.
[4]Carbohydrate (%) = Dry matter – (Crude protein + fat +ash + crude fiber)

Liver samples were powdered under liquid nitrogen, homogenized in 50mM Tris-HCl pH 7.5, 4mM EDTA, 50mM NaF, 0.5mM phenylmethylsulfonyl fluoride, 1mM 1,4-dithiothreitol and 250mM sucrose with a PTA-7 Polytron (Kinematica GmbH, Littau-Luzern, Switzerland) position 3 for 30 sec, and centrifuged at 20000x g for 30 min at 4°C. Supernatants were used as crude enzyme source.

Glucokinase (GK), phosphofructokinase 1 (PFK-1), pyruvate kinase (PK), fructose-1,6-bisphosphatase 1 (FBP-1), 6 phosphogluconate dehydrogenase (6-PGDH) and glucose 6 phosphate dehydrogenase (G6PDH) were assayed in a COBAS MIRAS spectrophotometric analyzer (Roche, Basel, Switzerland). Enzyme assays were carried out at 30°C and followed at 340nm.

GK reaction mixture was: 0.2mL of 100mM Tris-HCl pH 7.75, 7.5mM $MgCl_2$, 100mM KCl, 2.5mM DTT, 1mM NADP, 1mU.mL^{-1} yeast glucose-6-phosphate dehydrogenase and 5U/L of crude enzyme source previously filtered in Sephadex G-25. Enzyme reaction was started by 6mM ATP and 100mM or 0.5mM glucose. Enzyme activity was calculated from the rate of

NADPH formation at 340nm for 100mM glucose minus the rate for 0.5mM glucose (hexokinase activity), after correcting the interferences from glucose dehydrogenase (Tranulis *et al*. 1996).

PFK-1 reaction mixture was: 0.2mL of 100mM Tris-HCl pH 8.25, 5mM $MgCl_2$, 50mM KCl, 0.15mM ammonium sulfate, 4mM 2-mercaptoethanol, 10mM fructose 6-phosphate, 30mM glucose 6-phosphate, 0.675UI mL^{-1}aldolase, 5UI mL^{-1} triose phosphate isomerase, 2 UI mL^{-1} glycerol 3-phosphate dehydrogenase, (NADH 0,15mM**)** and 4µL of crude enzyme source. Enzyme reaction was started by the addition of 1mM ATP and calculated from the rate of NAD formation at 340nm (Castaño *et al*., 1979).

PK reaction mixture was: 0.25mL of 70mM glycyl-glycine pH 7.4, 10mM $MgCl_2$, 100mM KCl, 0.15mM NADH, 2.8mM phosphoenolpyruvate, 21UI mL^{-1} lactate dehydrogenase and 2.5µL of crude enzyme source. Enzyme reaction was started by the addition of 2.5mM ADP and calculated from the rate of NAD formation at 340nm (Staal *et al*., 1975).

FBP-1 reaction mixture was: 0.2mL of 85mM imidazole-HCl pH 7.7, 5mM $MgCl_2$, 0.5mM NADP, 12mM 2-mercaptoethanol, 0.05mM fructose 1,6-bisphosphate, 2.5UI mL^{-1} glucose-6-phosphate isomerase, 0.48 UI mL^{-1} glucose-6-phosphate dehydrogenase and 4µL of crude enzyme source. Enzyme reaction was started by the enzyme addition and calculated from the rate of NADPH formation at 340nm (Bartrons *et al*., 1983).

6-PGDH reaction mixture was: 83mM imidazole-HCl buffer at pH 7.7, 3mM $MgCl_2$, 0.5mM NADP and 2mM 6-P gluconate. Enzyme reaction was triggered by the addition of the crude enzyme extract and followed by the NADPH formation at 340nm (Bergmeyer, 1984).

G6PDH reaction mixture was: 78mM imidazole-HCl buffer at pH 7.7, 5mM $MgCl_2$, 1mM NADP and 1mM glucose 6-P. Enzyme reaction was triggered by the addition of the crude enzyme extract and followed by the NADPH formation at 340nm (Metón, 1996).

One enzyme unit (UI) was defined as the enzyme amount needed to catalyze one mole of substrate per min. The enzyme specific activity was calculated based on the protein concentration of every sample of crude enzyme extract, which was performed according to the method of using bovine serum albumin as standard.

The experimental design was established as randomized incomplete blocks-RIB formed by four blocks and ten fish per block, where each fish was assigned as an experimental unit. The data were submitted to ANOVA and differences among treatment means were checked by the Turkey post-test Tukey for $P<0.05$.

RESULTS

Different amounts of dietary protein, lipids and carbohydrates brought about responses on the activity of glycolytic and gluconeogenic enzymes (Fig. 1). The activity of the glycolytic enzymes GK, PK and PFK-1 lessened in fish fed with diets with decreasing carbohydrate contents (Figure 2). The activity of the gluconeogenic enzyme FBP-1 displayed an opposite profile. Such activity increased in fish fed with rising amounts of protein (Figure 2).

The fish performance was affected by dietary protein, carbohydrate and lipid (Table 2). The greatest weight gain was obtained in fish fed T4 diet with 40.14 % crude protein, 9.70% of lipids and 24.37% carbohydrate. The fish kept starved over the trial showed reduction in weight gain.

Activities of 6-PGDH and G6PDH were not affected by dietary contents of protein and carbohydrates (Table 2). The levels of these nutrients gave rise to reduction in the activity of these enzymes in the fish that remained fasted.

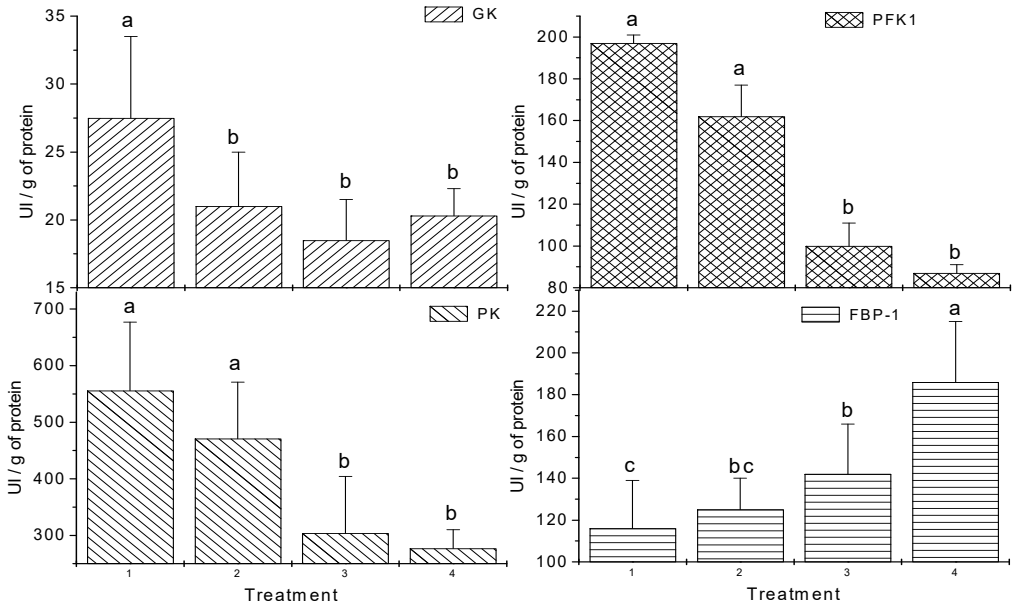
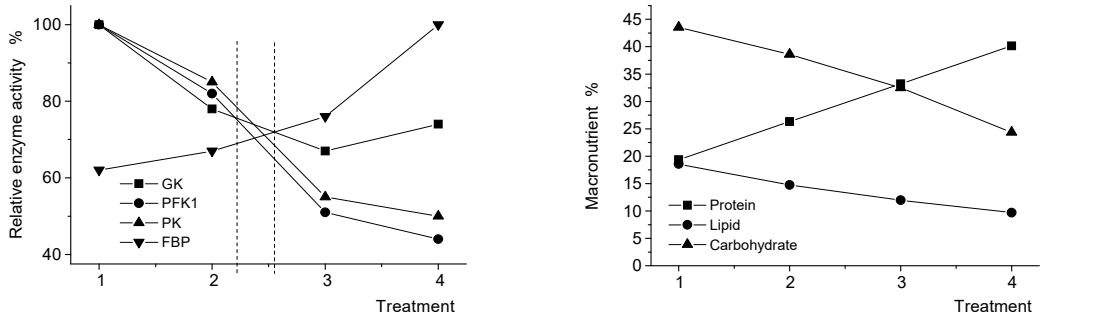


Figure 1. Glucokinase (GK), phosphofructokinase 1 (PFK-1), pyruvate kinase (PK) and fructose-1,6-bisphosphatase 1 (FBP-1) activities in the liver of *R. quelen* fed with diferent contents of macronutrients. The enzyme activities are expressed in UI/g of protein as mean ± SD (n=10); significant differences (P<0.05) are presented as distinct letters. T1 (19-19-44), T2 (26-15-39), T3 (33-12-33), T4 (40-10-24) (crude protein-lipid-carbohydrate, %).



(a) (b)

Figure 2. (a) Relative activities (%) of the glycolytic and gluneocogenic enzymes: glucokinase (GK), phosphofructokinase 1 (PFK-1), pyruvate kinase (PK) and fructose-1,6-bisphosphatase 1 (FBP-1). (b) Content (%) of dietary macronutrients (protein, lipid and carbohydrate) in the four treatments are allowed by interpolating their values.

Table 2. Enzymatic activity of G6PDH, 6-PGDH (mU/mg protein) in liver of *Rhamdia quelen* and weight gain fed different protein-lipid-carbohydrate contents and fasting

Treatments[1]	G6PDH	6PGDH	Weight gain(g)
Fasted	140.97b±11.85	31.57b±7.61	32.53d±5.32
T1 (19-19-44)	191.59a±33.64	48.55a±11.35	63.40c±4.56
T2 (26-15-39)	265.89a±66.89	52.42a±6.99	73.28b±5.12
T3 (33-12-33)	268.97a±79.95	47.16a±17.93	77.72b±6.48
T4 (40-10-24)	233.48a±68.93	51.65a±14.40	97.72a±5.09

[1]Protein-lipid-carbohydrate levels (%)

DISCUSSION

It is widely known that fishes are remarkable protein consumers. This macronutrient, pivotal for the most vital functions, takes place as provider of radicals and or building blocks of several biomolecules. However, the surplus of protein works as an amino acid source to supply energetic demands. Thus, well-suited diet composition is needed to prevent losses of amino acids as caloric substrate. High dietary protein levels are also associated to inconvenient environmental impacts due to potential nitrogenous waste resulting in water eutrophication. Protein requirement is also dependent upon the levels of other non-protein energy sources (Ruohonen and Kettunen, 2004). Besides, the content variation of macronutrients is essential to maintain the energetic equivalence based on their bio-availability.

The activities of glycolytic enzymes may fluctuate in response to the concentration and/or type of dietary carbohydrates. In *Labeo rohita*, the ratio between dietary non-gelatinized and gelatinized starch do not affect the glycolytic activity (Vikas *et al.*, 2010). However, higher activities of glycolytic enzymes are observed in gilthead sea bream fed diets with gelatinized corn starch (Fernandez *et al*., 2007). Controversial results were posteriorly observed on the hepatic HK and PK activities (Enes *et al*., 2008. Notwithstanding, when the type of carbohydrate – glucose and maltose – is changed, an increase of GK and PK activities is observed in that species (Enes *et al*., 2010).

In this work, decrease of dietary carbohydrates lessened the activity of glycolytic enzymes. The highest levels of those enzymes were found in the fish fed with lower CP or higher-carbohydrate diets. Consistently, the hepatic activity of the gluconeogenic enzyme FBP-1 was correlated with the dietary CP content (PC = 0.94; $P<0.05$). Increased protein in the diets enhanced this enzymatic activity in the liver.

The pattern of glycolytic and gluconeogenic enzymes on grounds of dietary nutrients suggests catabolism of amino acids (Fig. 2). This pattern was followed by a shifting of the amino-acids-carbon-backbones toward glucose synthesis. This framework is in agreement with the previous assumption of gluconeogenesis following the CP increase. Similar results are observed in *S. aurata* and *O. mykiss* fed with high levels of protein (Caseras *et al*. 2002; Metón *et al*. 2003). The gluconeogenic pathway is widely reported in liver and also in other tissues (Tam *et al*., 1998). Gluconeogenesis is an important strategy to regulate the blood glucose concentration, even in fish. The balance of plasma glucose may reflect the gluconeogenic activity. Variations of the plasma glucose concentrations in fish, as also observed from results with glucose tolerance tests, emphasizes the role of adaptation to the diets and are interpreted in the context of secondary carbohydrate metabolism (Hemre *et al*., 2002).

The FBP-1 is a regulatory enzyme playing a key role in this process to gain glucose from non-sugar molecules. The gluconeogenic strategy proposed in the present dietary conditions is substantiated in the changes observed in such enzyme activity, also reported in other fishes (Panserat *et al*., 2000). Depending on the type and the amount of carbohydrate and protein in the fish diet, the gluconeogenesis can be induced. The use of no-gelatinized starch in the diet to *L. rohita* induces gluconeogenesis from amino acids metabolism (Kumar *et al.,* 2010). In addition, the gluconeogenesis may occur due to the inability to utilize carbohydrates from the feeding (Enes *et al*., 2009).

Regarding the enzymatic activities of G6PDH and 6GPDH in the liver of catfish, the only response was obtained in fasted fish. The concentrations of protein, carbohydrates and lipids did not affect these enzymes. However, it must be considered that such enzymes may not be responsive to nutrients from food or fasting (Metón *et al*., 2003).

The couple of intersections in the family of the curves for the enzymes assayed in this work (Fig. 2a) are inserted into a range between treatments 2 and 3. The values for such range correspond to about 29 % of CP, 37 % of carbohydrate and 14 % of lipid (Fig. 2b). This set of macronutrient values does not match the performance measurements. One must consider that inferences from the performance data do not take into account several conditions such as waste of amino acids, excessive excretion of nitrogen, ability of using different nutrients, metabolic plasticity to diets, etc. On the other hand, it suggests the possibility of fine adjustments to

achieve the best formulation on dietary macronutrient and fish performance subsequently. The optimal concentration of dietary protein is different among fishes and depends on the food habit (omnivorous or carnivorous). For example, the best weight gains are in pintado *Pseudoplatystoma corruscans* (Lundstedt *et al*., 2004), *Mugil platanus* (Carvalho *et al*., 2010), *Sparus macrocephalus* (Zhang *et al*., 2010). Concerning carbohydrates, the recommended amount to reach the best performance is also distinct among fishes. The optimal levels of carbohydrate for *Puntius gonionotus* (Habieb *et al*., 1994), *Clarias batrachus* (Mollah and Allam, 1990) and *Mystus montanus* (Raj *et al*., 2008) are 30, 15-20, 9.5 %, respectively.

CONCLUSION

In conclusion, *R. quelen* is a freshwater fish species able to adapt the carbohydrate and amino acid metabolic pathways according to the dietary content of protein and carbohydrate. Such ability allows us to risk that 40% of CP, 24% of carbohydrate and 10% of lipid are good amounts of such nutrients to reach the best weight gain. Instead of using only the growth parameters to establish the best performance, the present work encourages the use of biochemical evaluations to adjust the content of macronutrient components in the diet and to prevent undesirable, physiological effects and achieve inaccurate results.

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