Effect of different concentrations of protein on the digestive system of juvenile silver catfish

[*Efeito de diferentes concentrações de proteína sobre o sistema digestivo de juvenis de jundiá*]

J.F.B. *Melo*[1], L.M. *Lundstedt*[2], G. *Moraes*[3], L.A.K.A. *Inoue*[4]

[1]Universidade Federal do Vale do São Francisco – UNIVASF - Petrolina, PE
[2]EMBRAPA Pesca e Aquicultura – Palmas, TO
[3]Universidade Federal de São Carlos – UFSCAR - São Carlos, SP
[4]EMBRAPA Amazônia Ocidental – Manaus, AM

ABSTRACT

Aspects of digestion in juvenile silver catfish *Rhamdia quelen* fed with four levels of protein (20, 27, 34 and 41%) were studied. The studied traits were digestive enzymes (nonspecific proteases, trypsin, chymotrypsin, amylase and lipase) and weight gain. The experiment lasted 60 days; 160 juvenile catfish with initial body weight of 32 grams were transferred to the experimental system of 500L tanks with re-circulated and thermostatized water. The average weight of silver catfish increased with dietary protein and 41% of crude protein (CP) was accepted as the best one. Gastric protease activity increases with the CP level. The intestinal alkaline protease reached the peak at 27% CP and the highest activities were observed in the anterior section of the gut (AS). The trypsin peak was observed at 34% CP in the AS and medium section of the gut (MS). Amylase and lipase activities were inverse to the dietary CP and the highest values were observed in AS. In conclusion, the digestive enzymes of catfishes are responsive to the dietary protein level. The dietary CP required for highest weight gain of juvenile silver catfish is 41%.

Keywords: digestive enzymes, nutrition, fish, *Rhamdia quelen*

RESUMO

Foram estudados aspectos digestivos de juvenis de jundiá Rhamdia quelen *alimentados com quatro níveis de proteína (20, 27, 34 e 41 %). As características estudadas foram enzimas digestivas (proteases inespecíficas, tripsina, quimiotripsina, amilase e lipase) e ganho de peso. O período experimental foi de 60 dias; 160 juvenis de jundiá com peso médio inicial de 32 gramas foram transferidos para um sistema experimental de tanques de 500L, com recirculação de água termostatizada. O peso médio dos jundiás aumentou com o teor de proteínas da dieta até 41% de proteína bruta (PB), valor esse considerado o melhor. A atividade de protease gástrica aumentou com o nível de PB. A protease alcalina intestinal atingiu o pico em 27% de PB e as maiores atividades foram observadas na secção anterior do intestino (SA). O pico de tripsina foi observado com 34% de PB na SA e na secção média do intestino (SM). As atividades de amilase e lipase foram inversas ao teor de PB, e os valores mais elevados foram observados na SA. Em conclusão, as enzimas digestivas de jundiá são responsivas ao nível de proteína da dieta. O teor mais alto de PB da dieta necessário ao maior ganho de peso em juvenis de jundiá é 41%.*

Palavras-chave: enzimas digestivas, nutrição, peixe, Rhamdia quelen

INTRODUCTION

Among the macronutrients, proteins play a pivotal role in many biological functions. Fish, in particular, present meaningful requirements of dietary protein. This fact deserves special attention when the diets are formulated in intensive fish rearing systems. Nutritional needs must look at many aspects, such as: fish species, metabolic demands, growth phase and potential to adapt to environmental changes.

Several studies have evaluated the enzyme behavior of the gastrointestinal tract of fish correlated to the use of nutrients aiming for the best understanding of the processes involving nutrition, growth, health and performance on the fish husbandry (Kuz'mina, 1981; Dabrowski *et al.*, 1992; Lundstedt *et al.*, 2004; Xiong *et al.*, 2010). In spite of reports on digestive enzymes of fish affirming they are adaptable to dietary protein contents (Fish, 1960; Kawai and Ikeda, 1972; Mukhopadhyay, 1977; Hofer and Schiemer, 1981), this fact remained as a matter of discussion for a while. Glass *et al.*, (1989) assumed that the knowledge on the exact amount and specificity of digestive enzymes should be a tool to precisely predict the digestive process in fish. This assumption has been observed and accepted in studies with warm water tropical fishes (Lundstedt *et al.*, 2004; De Almeida *et al.*, 2006; Correa *et al.*, 2007). Thereby, to improve fish digestions and nutrient intake the adaptive range of responses must be established (Segner *et al.*, 1989).

The South American catfish "jundia" (*Rhamdia quelen*) is a Neotropical Siluriform-Pimelodidae reported from Mexico to Argentina (Silfvergrip, 1996). This species displays several farming qualities and belongs to a group of eleven species, presently included in the Rhamdia genus. The *R. quelen* fish is an omnivorous species which accepts a number of different nutrients, including artificial feeding, since hatching. It presents good survival rates, and fast growth and development (Piaia *et al.*, 1997; Melo *et al.*, 2002). Nowadays, there is little information concerning digestion in species from the Rhamdia genus. In the present study, adaptations of the digestive profile of *R. quelen* fed distinct contents of crude protein (CP) were investigated.

MATERIAL AND METHODS

Juvenile jundiá with initial average weight of 44.98g±13.32 and 16.92cm±1.44 length were held in four 2.000L tanks with filtered, aerated and temperature-controlled water in a closed system. Forty fish per tank were fed isocaloric diets (4500kcal gross energy/kg) with 20, 27, 34 and 41% of crude protein (CP). The

nutrient composition was previously determined (Table 1) and the diet ingredients were analyzed according to the Official... (1990). The fish were fed twice a day at the rate of 10% of the biomass and sampled on the 60th day. Ten fish were sampled per treatment for enzyme assays and growth evaluation through the expression:

$$G = \overline{x}_{Fw} - \overline{x}_{Iw}$$ where (G= growth; $\overline{x}_{Fw}$= average final weight; $\overline{x}_{Iw}$= average initial weight).

The water quality was preserved through continuous recirculation, mechanic filtration through bio-beds and constant aeration. The water conditions were monitored and maintained in the course of the experiments as: temperature 25°C±1; pH 7.2; dissolved oxygen 5.4mg/L; alkalinity 42mg/L and ammonia 0.12mg/L. Biological variability was minimized using fish from the same strain. No death was observed in the whole experimental period.

Fish were sampled, anaesthetized with 100mg/L of benzocaine (Inoue *et al.*, 2003), gauged for size and weight, and blood samples were withdrawn from the caudal vein. Then the fish were killed through cervical pinch and samples of white muscle, kidney and liver were collected. Plasma aliquots were separated after blood centrifugation at 4°C for 10min at 5000×G. The samples were immediately frozen in liquid nitrogen and kept at −20°C. The digestive tract was excised, divided into stomach, anterior, middle and posterior intestine, and stored at −20°C.

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Table 1. Ingredients and analyzed composition of the experimental diets

	Experimental diets			
	20% CP	27% CP	34% CP	41% CP
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[1]	0.2	0.2	0.2	0.2
Mineral mixture[2]	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 (%)	43.53	38.60	32.5	24.37

CP: crude protein;
[1]Composition of vitamin mixture/kg diet: Vitamin A 10.000UI. E 50mg. D_3 2000UI. K-3 25mg. B 12 50mg. Thiamine 15mg. Riboflavin 35mg. Pyridoxine 10 mg. Biotin 250mg. Folic acid 5mg. Acid Pantothenic 60mg. Niacin 60mg. Choline 120g. Antioxidante 50g.
[2]Composition of mineral mixture/kg diet: Iron 40mg. Copper 12.5mg. Zinc 30mg. Cobalt 1mg. Iodo 1.4mg e Selenium 0.2mg.

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Samples from each gastrointestinal section were homogenized in an ice bath at a ratio of 1:10 (tissue:homogenization buffer) with a Teflon pestle of a motor-driven tissue-cell disrupter. The homogenization buffer solution was 0.02M Tris/0.01M phosphate pH 7.0 in v/v glycerol. The homogenates were centrifuged at 11000×G for 3min and the supernatants (crude homogenate) were used as the enzyme resource. The protein content of the homogenates was

determined colorimetrically at 660nm (Lowry *et al*., 1951) and albumin solution was used as standard.

Enzymes were previously assayed in the different sections of the gut to optimize the pH and temperature reaction, the incubation time, enzyme aliquot, and substrate saturation. Enzyme reaction mixture for Non-specific Acid Protease was 1% casein in 0.1M glycine/HCl (pH 2.0) with the proper aliquot of crude homogenate. After incubation for 30 min at 30ºC the reaction was stopped by 15% TCA (trichloroacetic acid) solution. The reaction mixture was centrifuged at 14400×G for 3min and the optical density of the supernatant was determined at 280nm (Walter, 1984). Tyrosine was used as standard and one unit was defined as the amount of enzyme to hydrolyze 1 μmol of substrate per minute. Specific activity is expressed in units per mg of protein (U/mg protein). Enzyme reaction mixture for Non-specific Alkaline Proteases in the intestine sections was: 1% azocasein in 0.1M Tris-HCl

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(pH 8.5) with proper aliquot of crude homogenate. After incubation for 30 minutes at 30ºC the reaction was stopped by 15% TCA solution. The samples were centrifuged at 14400×G for 3min; 1mL was transferred to 1mL of 1.3N NaOH and kept for 15min at room temperature. The optical density was read at 440nm (Sarath *et al*., 1989). One unit of enzyme activity was defined as the amount of enzyme to hydrolyze 1μmol of substrate/min. Specific activity is expressed in units/mg of protein (U/mg protein).

Both trypsinolytic and chymotrypsinolytic activities were determined according to Hummel (1959) at 25ºC. The reaction mixture for *Trypsin* was 1.04mM TAME (p-toluenesulfonyl-L-arginine ethyl ester) and 0.01M $CaCl_2$ in 0.2M Tris pH 8.1. The reaction product was followed at 247nm. One unit of enzyme was established as the amount to hydrolyze 1μmol of TAME/min. (corresponding to 1μmol of arginine/min) and the specific activity was expressed in unit/mg of protein (U/mg protein). The reaction mixture for *Chymotrypsin* was 0.001M BTEE (N-benzoill-L-tyrosine ethyl ester), $CaCl_2$ 0.10M in 0.10M TRIS- HCl pH 7.8. The reaction product was followed at 256nm. One unit of enzyme was defined as the amount to hydrolyze 1μmol of BTEE/min (corresponding to 1μmol of tyrosine/min) and the specific activity was expressed in unit/mg of protein (U/mg of protein).

Amylase reaction mixture was 5% starch solution in 0.2M citrate-phosphate buffer pH 7.0 with 0.5% NaCl and suitable aliquot of tissue homogenate (Bernfeld, 1995). The mixture was incubated for 15min. at 37°C. Reaction was stopped by addition of 5% $ZnSO_4$/0.3N $Ba(OH)_2$ (v:v) solution and the precipitate removed by centrifugation at 3000 × G for 2min. Free glucose concentration was quantified (Park and Johnson 1949) and 1 unit was defined as the amount of enzyme to produce 1μmol of reducing sugar/min. The specific activity was expressed in unit/milligram of protein (U/mg protein).

Lipase activity was assayed according to Albro *et al*. (1985). Suitable aliquots of homogenate were incubated with 0.4mM p-nitrophenyl myristate in 24mM ammonium bicarbonate pH 7.8, containing 0.5% Triton X-100 as emulsifier.

Control reactions were run without enzyme and/or substrate. Reaction was stopped after 30min by addition of 25mM NaOH and the absorbance was read at 405nm. One unit was defined as the amount of enzyme to hydrolyze 1μmol of substrate/min and the specific activity was expressed unit/milligram of protein (U/mg protein).

The experimental design was established as incomplete blocks formed by four treatments and ten repetitions per treatment. The enzymes average activities were analyzed as the variance and compared through Tukey's test. The growth rate and fish performance analyses were evaluated through descriptive statistics for $P < 0.05$.

RESULTS AND DISCUSSION

The weight of silver catfish fed increasing levels of protein increased significantly ($P < 0.05$) over 60 days. The protein content required for optimum weight gain was found to be 41% CP. The average weight increased from 63.48 to 73.12, 73.28, 77.72 and 97.72 grams with the increasing levels of dietary protein. Increase of dietary CP usually results in growth rate and weight increase within specific ranges (Melo *et al*., 2006; McGoogan and Gatlin III, 1999). Although considerable progress has been made in determining nutritional requirements for maximum growth of fish, it is important to consider that dietary manipulations may also influence other responses that are of aquacultural importance such as digestive enzyme activities.

In the stomach, the unspecific acid proteolytic activity followed the levels of dietary protein (Table 2). In the anterior, middle and posterior section of the gut (AS, MS and PM) the alkaline protease activity increased from 20 to 27% CP, and the highest activities were observed in the AS. The stomach presented alkaline proteolytic activity and the highest values were observed for 27% CP. In the AS and MS of the gut trypsin activity increased from 20 to 34% CP. In the gut, chymotrypsin activity was proportional to the increase of dietary protein. Amylase and lipase activities were proportionally converse to the content of dietary CP, and the highest values were observed in the AS.

Table 2. Digestive enzyme activities in the gastrointestinal sections of *Rhamdia quelen* with different dietary compositions. The values are means SD for n=10; significant differences ($P < 0.05$) are presented by distinct letters

	CP %	Acid proteolytic U/mg protein	Alkalina proteolytic U/mg protein	Trypsin U/mg protein	Chymotripsin U/mg protein	Amylase U/mg protein	Lipase U/mg protein
Stomach	20	9.64d±3.67		0.33a±0.06	0.34±0.01	0.08a±0.03	0.32a±0.06
	27	19.78c±4.61	-	0.31a±0.05	0.39±0.01	0.05b±0.02	0.31a±0.05
	34	27.21b±4.61		0.35a±0.05	0.82±0.01	0.02c±0.01	0.35a±0.05
	41	43.46a±9.89		0.36a±0.08	1.03±0.08	0.02c±0.02	0.33a±0.08
Anterior intestine	20		15.02b±2.91	2.12a±0.47	3.51b±0.85	0.19a±0.03	20.59a±3.61
	27		25.60a±2.91	3.21b±0.50	6.14a±0.96	0.14b±0.03	13.61b±2.98
	34	-	22.96a±2.77	3.31b±0.49	5.78a±1.01	0.08c±0.02	8.54c±1.35
	41		18.24b±2.96	2.16a±0.62	3.92b±0.93	0.07c±0.01	8.33c±1.76
Middle intestine	20		5.38b±2.12	0.56b±0.14	5.75b±1.08	0.05a±0.02	6.21a±1.21
	27		13.35a±4.44	0.99b±0.17	11.1a±1.96	0.04a±0.01	4.49b±0.92
	34	-	12.85a±3.53	1.51a±0.54	12.4a±1.69	0.02b±0.008	1.89c±0.56
	41		9.79b±3.18	1.17ab±0.38	10.98a±2.02	0.02b±0.007	1.73c±0.96
Posterior intestine	20		4.11b±1.89	1.28b±0.29	0.65c±0.20	0.06a±0.005	10.84a±0.82
	27		8.91a±2.16	1.63a±0.39	2.79b±0.68	0.04ab±0.007	5.46b±0.88
	34	-	9.69a±1.64	1.29ab±0.36	5.15a±0.99	0.03b±0.005	3.48c±1.10
	41		7.82a±2.81	1.31ab±0.28	4.82a±1.01	0.03b±0.004	1.67d±1.13

The activity of the most digestive enzymes of the gastrointestinal tract of fish may change with the alimentary habit (Hofer and Shiemer, 1981; Kuz'mina and Smirnova, 1992). Pepsin is the main protease in the stomach of fishes; it has been reported in several species such as *Anguilla japonica* (Morishita *et al*., 1964), *Tilapia mossambica* (Fish, 1960; Nagasse, 1964), *Oncorhynchus mykiss* (Kitamikado and Tachino, 1960), *Ictalurus sp* (Nordlie, 1966; Smith, 1967) *Dicentrachus labrax* (Cuvier-Péres, 2001) and many other species. The optimum pH activity of pepsin is usually between 2.0 and 3.3 (Fänge and Grove, 1969; Lovell, 1988; Kuz'mina, 1991, Xiong *et al*. 2011), however its proteolytic activity has been reported in a larger pH range, varying from 1.5 to 8.5 (Kuz'mina, 1991). The responsiveness of pepsin to environmental factors such as light, husbandry and nutrient variation has been previously reported in fish (Kapoor *et al*., 1975; Cuvier-Péres, 2001). Pepsin from jundiá was responsive to dietary CP. This kind of response observed in jundia suggests that this enzyme ensures a plentiful utilization of the ingested protein. The source of protein, either animal or vegetal, may influence the yield of digestive processes in fish. Not only may the level of dietary CP affect the enzyme response of the digestive tract, but the kind or source of protein can. However, some species are not affected. The pepsin activity of tambaqui *Colossoma macropomum*, is not influenced by vegetal or animal protein (Kohla *et al*., 1992). In this particular study, jundiá was also unaffected by the source of protein since the effects observed were for fish fed wheat or soy, as well as animal protein.

The alkaline proteases observed in the digestive tract of jundiá showed greater enzymatic activities in the anterior section of the intestine. Similar results are reported in other species such as *Gptosternum maculatum* (Xiong *et al*., 2011). In rainbow trout the alkaline proteases are also responsive to dietary protein (Kawai and Ikeda, 1972). That response is straightly related to the protein content. The unspecific alkaline proteolytic activity observed along all the intestine of jundiá is probably due chiefly to the presence of trypsin and chymotrypsin, since the substrate azocasein is a target of both enzymes. These enzymes were responsive to the dietary CP level; however, the responsiveness seems to be coarser, or limited, compared to pepsin. These enzymes are not often reported in the stomach of fish (Cuvier-Péres, 2001), however, they are present in the stomach of *Pseudoplatystoma corruscans* (Lundstedt *et al*., 2004) as well as in jundiá (Melo *et al*., 2006).

Responsiveness of amylase to the content of dietary polysaccharides is only reported in fish (Kawai and Ikeda, 1972; Moraes and Bidinotto, 1995). In jundiá, amylase was induced by

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increasing levels of corn. Studies on matase activity of *Sparus aurata* and *Carassius auratus* have shown that this carbohydrase is affected by the source of dietary carbohydrate (Silva *et al*., 2010). The influence of diet processing (extrusion or pelletization) of wheat and corn on the nutritional characteristics of the feeding is also reported. Structural changes on the dietary starch can affect the enzyme activity of amylase as reported for pacu *Pyaractus mesopotamicus* (Honorato *et al*., 2009). The amylase activity of jundiá was similar in all sections of the gastrointestinal tract. Likewise, lipase was induced by the content of dietary lipids. Lipase was expressed in all gastrointestinal tract of jundiá and the degree of responsiveness was similar among all sections. The highest lipase activity was observed in the anterior section of the intestine, decreasing toward the posterior section. This indicates the intestine as the main site for lipid digestion. These results were similar to those reported on other fish species *Scophthalmus maximus* (Fu *et al*., 2005) and *Boleophthalmus pectinirostris* (Wu *et al*., 2007).

The induction of lipase through the content of dietary fat in the fish was observed in Tilapia *mossambica* (Nagase, 1964), *Ctenopharyngodon idella* (Das and Tripathi, 1991) and *P.corruscans* (Lundstedt *et al*., 2004). Therefore, the increase of carbohydrates and fat in the diet of jundiá raised the amylase and lipase activities. This fact can be properly used to adjust the content of these nutrients in the formulation of diets. Considering the sum of digestive enzymes and the response to the level of nutrients we assume that the best amount of dietary protein is near 27%.

CONCLUSIONS

Changes in the level of dietary protein, carbohydrate and fat in the feeding of jundiá led to digestive adjustments. Gastric proteolysis was proportional to the dietary protein. The anterior intestine holds the chief amount of alkaline proteases, which are fully expressed at 27-34% crude protein. Amylase and lipase were responsive to the carbohydrate and fat contents in the feeding. The present data encourages measurements and adjustments of the protein/energy ratio to achieve the best yield in the nutrition of *R. quelen*.

ACKNOWLEDGMENTS

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