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The dietary arginine requirement of channel catfish (*Ictalurus punctatus*) is influenced by endogenous synthesis of arginine from glutamic acid

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Abstract

Previous studies with young mammals have established that arginine synthesis from glutamate-derived citrulline can be a major endogenous source of arginine. Therefore, an experiment was conducted to re-assess the dietary arginine requirement of juvenile channel catfish and to determine the metabolic effects of including glutamate or glycine to maintain isonitrogenous levels among diets. Two sets of diets were formulated to contain 24 g crude protein/100 g dry weight from casein/gelatin and crystalline amino acids with arginine supplementation in 0.5 increments from 0.5 to 2.0 g/100 g diet. Amino acid nitrogen was maintained equal, within sets, by replacing arginine with aspartate and either glutamate or glycine. Each diet was fed to apparent satiation to triplicate groups of 12 fish initially averaging 11.4 g/fish for 8 weeks. Weight gain (WG), feed efficiency (FE), protein efficiency ratio (PER), protein retention (PR) and survival were significantly ($P < 0.05$) affected by arginine. At the suboptimal level of dietary arginine, glutamate appeared to contribute arginine through internally derived citrulline based on increased plasma citrulline and arginine concentrations. WG and plasma amino acid concentrations of fish fed diets with glycine suggested that it does not serve as a precursor for citrulline. Based on WG and FE, juvenile channel catfish were found to require arginine at 3.3% to 3.8% of dietary protein, when glutamate was included in the diet. The requirement estimate was 33% higher when glycine replaced glutamate in the diet and was similar to the previously determined arginine requirement of channel catfish at 4.3 g/100 g of dietary protein. These results strongly suggest that dietary

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glutamate is used for endogenous synthesis of arginine in channel catfish, especially when arginine is deficient in the diet.

Keywords: Arginine requirement; Amino acid metabolism; Channel catfish; Citrulline

1. Introduction

The amino acid arginine has important nutritional and physiological roles and is established as indispensable in the diet of many fish species (NRC, 1993). Experiments with young pigs, rats and dogs have indicated that renal arginine synthesis is a major endogenous source of arginine for these animals (Wu and Morris, 1998). The kidney readily synthesizes arginine from citrulline, which can arise from the intestinal metabolism of glutamate, the so-called intestinal–renal axis that converts glutamate to arginine. Endogenous synthesis via the intestinal–renal axis provides a reasonable explanation for arginine being dispensable for most mammalian species as arginine biosynthesis is sufficient to meet the needs of adult animals (Baker, 1994). However, young animals may require dietary arginine because the rate of endogenous synthesis is low compared with the metabolic requirement for this amino acid (Fuller, 1994). Studies with young pigs (Wu and Knabe, 1995; Wu et al., 1997) indicate that the endogenous synthesis of arginine can provide 50–69% of the total daily arginine requirement.

The quantitative arginine requirement of channel catfish has been determined to be 4.3% of dietary protein (Robinson et al., 1981). Diets in that study were formulated to contain 24% crude protein and were maintained isonitrogenous by adjusting the levels of aspartic acid and glutamate. Because glutamate has proven to be an important precursor for endogenous arginine synthesis in many species, this approach could affect the estimation of dietary arginine requirements. Therefore, the present study was conducted to re-evaluate the dietary requirement of channel catfish for arginine and to assess the metabolic effects of including glutamate or glycine in the experimental diets.

2. Materials and methods

2.1. Feeding trial

An 8-week feeding trial was conducted in which juvenile channel catfish, *Ictalurus punctatus*, were fed various levels of dietary arginine. Experimental diets were similar to those used by Robinson et al. (1981). The basal diet (Table 1) was formulated to contain 24% crude protein from casein, gelatin and crystalline L-amino acids to meet the amino acid requirements of channel catfish, except for arginine (NRC, 1993). The crude protein content of the experimental diets was confirmed by analysis according to established procedures (AOAC, 1990). Two different sets of diets were prepared by adding graded amounts of arginine · HCl to provide arginine at 0.5%, 1.0%, 1.5% or 2.0% of diet.

Table 1
Composition of the basal diet

Ingredients	Amount (%)
Casein[a]	10.0
Gelatin[a]	2.5
Amino acid premix[b]	6.7
Dextrin[a]	24.3
Celufil[a]	30.3
Corn oil[a]	4.0
Cod liver oil[a]	6.1
Vitamin premix[c]	3.0
Mineral premix[c]	4.0
$Ca(PO_4)_2$	1.0
Carboxymethylcellulose[a]	2.0
L-Aspartate	3.0
Glycine or L-glutamate	3.0
L-Arginine·HCl	–

[a]United States Biochemical, Cleveland, OH.
[b]Consisted of L-amino acids from Nutri-Quest, Chesterfield, MO and provided (as % of diet): histidine, 0.14; isoleucine, 0.19; leucine, 0.06; lysine, 0.64; methionine, 0.32; phenylalanine, 0.42; serine, 1.57; threonine, 0.13; tryptophan, 0.02; valine, 0.11; proline, 1.57; alanine, 1.57. Amino acids also were provided by 10% casein and 2.5% gelatin which in combination with the crystalline amino acids met channel catfish requirements (NRC, 1993).
[c]Same as Buentello et al. (1997).

Dietary arginine levels were determined by reversed phase HPLC; the basal diet contained 0.48% arginine and supplemental levels were confirmed by HPLC analysis. Amino acid nitrogen was maintained equal among diets, within each set, by adjusting the levels of arginine and either glutamate or glycine in combination with aspartate, resulting in a total of eight experimental diets. Although diets within each set were isonitrogenous, those containing glutamate had 6.9% less total nitrogen than those containing glycine, due to the lower nitrogen content of glutamate relative to glycine. Diets were supplemented with complete mineral and vitamin premixes and adjusted to pH 7.0 with 6 N NaOH. Procedures for diet preparation and storage were as previously described (Buentello et al., 1997).

All diets were fed twice a day to apparent satiation to triplicate groups of channel catfish each consisting of 12 fish initially weighing 137.5 ± 2.2 g/group. These juvenile channel catfish were obtained from ponds at the Aquacultural Research and Teaching Facility of the Texas A&M University System (Burleson, Texas) and acclimated to standardized conditions in aquaria for 2 weeks prior to the feeding trial, during which they were fed the basal diet containing glycine. The feeding trial was conducted in 24, 38-l aquaria operated as a flow-through system with well water flowing at 1 l/min. Water temperature was maintained at 27 ± 2°C. Supplemental aeration was provided to each aquarium to maintain dissolved oxygen levels near air saturation. Fluorescent illumination controlled by a timer provided a 12:12 h light:dark cycle. Water quality was monitored weekly for ammonia, nitrate, nitrite, hardness and pH and remained within

recommended levels for channel catfish (Lee, 1991) throughout the feeding trial. Procedures used in this study were approved by the Texas A&M University System Animal Care and Use Committee. Established formulas (Halver, 1989) were used to compute weight gain (WG), feed efficiency (FE) and protein efficiency ratio (PER) for fish at the end of the trial.

2.2. Sample collection and analyses

A sample of three fish at the start of the feeding trial and three fish per aquarium at termination were homogenized in a blender and stored frozen (-20°C) in polyethylene bags before determining proximate composition of carcass according to established procedures (AOAC, 1990). Based on carcass protein content, protein retention (PR) was computed as PR = 100 × ((fish final protein concentration × final biomass) − (fish initial protein concentration × initial biomass))/protein intake.

At the end of week 8, blood samples from three fish per aquarium were obtained from the caudal vasculature by heparinized needles, 14 h after the last feeding. Blood plasma was separated by centrifugation ($2000 \times g$, 10 min) and stored at -80°C. Analysis of plasma-free amino acids was performed using HPLC as described by Wu and Knabe (1995) but with a modified gradient program and plasma filtration to allow for better separation of threonine, citrulline and arginine. Briefly, plasma samples were deproteinized by addition of 1.5 mol/l $HClO_4$ (1 ml $HClO_4$/ml plasma) followed by neutralization with 2 mol/l K_2CO_3 (0.5 ml K_2CO_3/ml plasma). Deproteinized and neutralized samples were filtered through 0.22-μm polycarbonate syringe filters followed by precolumn derivatization with *o*-phthaldialdehyde (Sigma, St. Louis, MO). Identification and quantification of amino acids were accomplished using external standards (Sigma).

All statistical analyses were conducted using the Statistical Analysis System (SAS Institute, 1988). A 4×2 factorial analysis of variance was conducted to determine the effects of dietary arginine level and source of balancing nitrogen (glutamate or glycine) on WG, FE, PER, PR, plasma-free amino acids and survival of channel catfish. The minimum dietary arginine requirement of channel catfish was determined by subjecting the response variables to least-squares regression analysis using the broken-line method (Robbins, 1986). Contrast comparisons also were made between the diets containing 0.5% arginine and either glutamate or glycine. A P value of less than 0.05 was taken to indicate statistical significance.

3. Results

Survival was high for fish fed all diets (97% on average) except for those fed the diet containing 0.5% arginine with glycine as a nitrogen source (0.5 Arg–Gly) at only 73% (Table 2). Graded concentrations of dietary arginine significantly ($P < 0.05$) affected WG, FE, PER and PR of juvenile channel catfish, with fish fed both diets containing

Table 2
WG, FE, PER, PR and survival of channel catfish fed diets containing four arginine levels and either Gly or Glu to maintain them isonitrogenous[a]

Dietary arginine (% of diet)	Nitrogen source	WG (% initial weight)	FE (g gain/g feed offered)	PER (g gain/g protein fed)	PR (%)	Survival (%)
0.5	Gly	57	0.15[b,x]	1.19	15.5	73.3[b,x]
1.0	Gly	130	0.31	1.95	28.0	88.9
1.5	Gly	129	0.46	2.04	29.7	97.8
2.0	Gly	129	0.28	1.67	23.6	97.8
0.5	Glu	88	0.35[b,y]	1.44	20.2	97.8[b,y]
1.0	Glu	130	0.47	1.87	27.1	100
1.5	Glu	126	0.42	2.06	30.4	100
2.0	Glu	135	0.50	2.40	35.4	100
Factorial ANOVA, Pr > F[c]						
Arg level		0.001	0.0002	0.006	0.0044	0.17
Nitrogen source		0.39	0.0001	0.13	0.1444	0.03
Arg×Nitrogen		0.61	0.0028	0.25	0.1084	0.17
Pooled SEM		7.8	0.02	0.12	1.72	3.46

[a]Means of three replicate groups.
[b]Contrast comparisons between 0.5 glycine and 0.5 glutamate yielded significant ($P < 0.05$) differences for feed efficiency and survival as noted by the different superscripts while P values for WG, PER and PR were 0.09, 0.34 and 0.09, respectively.
[c]Probability associated with the F statistic.

0.5% arginine having the lowest responses (Table 2). WG of fish fed the diet with 0.5 Arg–Gly was about 66% of that achieved by fish fed the diet containing 0.5 Arg-Glu. Thus, glutamate in the diet limited the severity of arginine deficiency. FE, PER and PR of fish fed each diet containing 0.5% arginine followed a pattern similar to WG with fish fed 0.5 Arg–Glu having greater responses compared to those fed 0.5 Arg–Gly (Table 2). Broken-line analysis of WG and FE data from fish fed diets containing glycine as a nitrogen source provided arginine requirement estimates ($\pm$SE) of 1.0% ($\pm$0.07) and 1.2% ($\pm$0.05) of diet, respectively. These values correspond to 4.2% and 5% of dietary protein. However, using the same responses from fish fed diets containing glutamate, the broken-line analysis, fitted by the least-squares method, yielded arginine requirement estimates of 0.8 ($\pm$0.06) and 0.9 ($\pm$0.05), which correspond to 3.3% and 3.8% of dietary protein.

Plasma arginine concentration in fish was significantly ($P < 0.05$) increased 97% and 55% with an elevation of dietary arginine from 0.5% to 1.5% of diet in the presence of glycine and glutamate, respectively (Table 3). The highest concentration of plasma arginine was found in fish fed the 1.5 Arg–Glu diet. The use of glycine or glutamate as the nitrogen source also yielded significant ($P < 0.05$) differences in plasma concentrations of glutamate, glutamine, citrulline, arginine and ornithine (Table 3). Plasma glutamate levels did not vary significantly ($P < 0.05$) in fish fed diets containing glycine; however, a near significant ($P < 0.06$) step-wise reduction in plasma glutamate

Table 3
Plasma free arginine, citrulline, ornithine, glutamine and glutamate in juvenile channel catfish fed diets containing graded levels of arginine with either Gly or Glu to maintain them isonitrogenous[a]

Dietary arginine (% of diet)	Nitrogen source	Amino acid (μmol/l)				
		Glutamate	Glutamine	Citrulline	Arginine	Ornithine
0.5	Gly	25.5	91.0	49.9	45.1	30.7
1.0	Gly	29.2	105.9	56.5	71.6	26.1
1.5	Gly	28.4	102.9	50.9	88.7	28.4
2.0	Gly	20.7	102.8	49.6	82.3	24.7
0.5	Glu	43.7	150.6	88.2	65.2	55.0
1.0	Glu	33.1	145.0	72.9	86.5	47.6
1.5	Glu	28.2	134.1	69.4	101.2	39.7
2.0	Glu	29.6	135.4	65.5	95.7	37.1
Factorial ANOVA, Pr > F[b]						
Arg level		0.06	0.09	0.08	0.0001	0.08
Nitrogen source		0.0100	0.0001	0.0001	0.0012	0.0013
Arg×Nitrogen		0.12	0.37	0.24	0.90	0.70
Pooled SEM		2.16	5.06	3.50	3.15	3.63

[a]Values are means of three individual fish from each of three replicate groups.
[b]Probability associated with the *F* statistic.

was observed when glutamate was decreased to maintain the diets isonitrogenous as arginine was increased.

4. Discussion

4.1. Arginine requirement and glutamate sparing

WG achieved by channel catfish in this experiment was similar to that obtained by Robinson et al. (1981). Relatively low-protein diets comprised primarily of crystalline amino acids typically support less rapid growth of fish than diets composed of intact protein do (Wilson and Halver, 1986). Requirement estimates of 4.2% and 5.0% of dietary protein for glycine diets in the present study agree with the previously estimated arginine requirement of channel catfish at 4.3% of dietary protein (Robinson et al., 1981). However, glutamate appeared to spare up to 33% of the arginine requirement in channel catfish and had beneficial effects on fish fed the arginine-deficient diet compared to those fed the arginine-deficient diet with glycine. Presumably, fish fed the arginine-deficient diets had a greater need for arginine, and glutamate provided an adequate substrate for de novo synthesis of arginine.

Retarded growth, high mortality and fin erosion have been reported as signs of arginine deficiency in rainbow trout (Ketola, 1983) and channel catfish (Robinson et al., 1981). Similar conditions were observed in the present experiment only in fish fed the 0.5 Arg–Gly diet. The absence of these and other clinical deficiency signs in fish fed the 0.5 Arg–Glu diet may have been due to the use of glutamate for synthesis of ornithine

and citrulline before incorporation in arginine, as observed in one other fish species (Chiu et al., 1986). Although the glycine series diets contained about 6.9% more total nitrogen than the glutamate series, the glutamate-containing diets significantly ($P < 0.05$) out-performed the glycine-containing diets in terms of FE and survival as assessed by the contrast comparison (Table 2).

4.2. Plasma amino acids

Plasma amino acid concentrations of channel catfish in the present study were comparable to those noted in other experiments with juvenile channel catfish (Robinson et al., 1981; Wilson et al., 1985). Post-prandial changes in plasma-free amino acid concentrations have been used to investigate amino acid availability and utilization in fish species because these patterns reflect the net results of digestion, absorption and subsequent utilization (Wilson and Halver, 1986). However, the use of plasma-free amino acid responses to confirm dietary requirements assessed by growth trials have failed (Robinson et al., 1981; Walton et al., 1986; Cho et al., 1992) or succeeded (Kaushik, 1979; Lall et al., 1994; Berge et al., 1997). In the present study, blood collection took place 14 h after feeding and plasma arginine exhibited a dose-dependent response. This post-prandial time point was selected based on the time course of plasma amino acid concentrations of fish fed casein–caseinate mixes (Schuhmacher et al., 1995). Robinson et al. (1981) collected blood 15–20 h after feeding and found relatively constant levels of serum arginine. It also was suggested by Robinson et al. (1981) that serum levels of free arginine may have been dependent not only on dietary arginine concentration, but also on interrelations with other dietary amino acids. Indeed, circulating free arginine levels and overall arginine homeostasis in animals is influenced by endogenous synthesis and degradation, intracellular protein turnover and physiological state (Wu et al., 1997). Plasma-free arginine was responsive to dietary arginine in the present study by increasing with graded levels of arginine up to 1.5% of diet, then slightly declining at the highest arginine level. Griffin et al. (1994) noted a similar response while assessing the dietary arginine requirement of hybrid striped bass. Berge et al. (1997) also noted that arginine above 4.0% of dietary protein resulted in reduced (from peak values) circulating levels of arginine. This trend suggests the presence of a homeostatic mechanism by which fish regulate free plasma arginine. The liver would be a likely site for this regulatory action due to the presence of relatively high arginase activity (Anderson, 1995; Berge et al., 1997) exerting control on circulating levels of arginine. This hepatic regulatory effect has been proposed as a means by which extrahepatic tissues of fish can optimize the utilization of dietary amino acids for protein synthesis (Wilson et al., 1985).

4.3. Endogenous synthesis of arginine

Results from the present study suggest that diets containing glutamate contribute arginine through de novo synthesis, especially if dietary arginine is limited. This was reflected in a general improvement in WG, FE, PER and PR that occurred at suboptimal levels of arginine (0.5% of diet) if glutamate was included in the diet. It is now

generally accepted that endogenous synthesis of arginine in humans, pigs, dogs, sheep and rats involves the intestinal–renal axis in which citrulline released by the small intestine is converted to arginine in the kidney via argininosuccinate synthase and argininosuccinate lyase (Dhanakoti et al., 1990; Wu and Morris, 1998). Moreover, jejunal cells of newborn pigs synthesize arginine from glutamine to compensate for limited arginine present in the sow's milk (Blachier et al., 1993; Wu and Knabe, 1995).

The enteric section of the intestinal–renal axis for de novo arginine synthesis involves the conversion of dietary or circulating glutamine/glutamate along the following pathway: glutamine → glutamate → glutamyl-γ-phosphate → glutamyl-γ-semialdehyde → pyrroline-5-carboxylate (P5C) → ornithine → citrulline, catalyzed by phosphate-dependent glutaminase, Δ-P5C synthase, Δ-P5C synthase (bifunctional enzyme), spontaneous reaction, ornithine aminotransferase (OAT) and ornithine carbamoyltransferase (OCT), respectively (Wu et al., 1997). In mammals, these enzymes are found in liver as part of the urea cycle, but hepatic arginase activity is so high that arginine is split into ornithine and urea, preventing any net release of arginine from liver into circulation. Huggins et al. (1969) found appreciable levels of all enzymes of the urea cycle in several species of teleost fish. Wilson (1973) reported perceptible quantities of OCT and carbamoylphosphate synthase in channel catfish tissues. The fact that most fish species excrete ammonia as the end product of nitrogen metabolism seems to have an energetic basis, rather than the absence of required enzymes. Chiu et al. (1986) reported incorporation of L-[1-^{14}C] ornithine and L-[carbamoyl-^{14}C] citrulline into tissue arginine when rainbow trout were injected intraperitoneally. These authors, however, concluded that arginine biosynthesis occurred in the trout's liver as part of the urea cycle. The high arginase activity in the trout liver found by Chiu et al. (1986) and by Berge et al. (1997) makes the conclusion of Chiu et al. (1986) inconsistent with the current understanding of interorgan (intestine → kidney) de novo synthesis of arginine. It is well established that the kidney possesses a low arginase-to-arginine synthase ratio (Featherston et al., 1973) and arginine synthase (argininosuccinate synthase plus argininosuccinate lyase) is found predominantly in the renal cortex, making the kidney a major biosynthetic source of circulating arginine in rats and other mammals (Dhanakoti et al., 1990).

The full complement of enzymes of the urea cycle have been shown to exist in a number of ''typical'' teleost species (Huggins et al., 1969; Read, 1971; Wilson, 1973; Chiu et al., 1986; Mommsen and Walsh, 1989, 1991; Anderson, 1995). Taken together, these reports suggest the presence of all enzymes required for synthesizing arginine from glutamate in fish tissues (Wakabayashi et al., 1991). However, assessment of the enzymatic activities of the intestinal–renal axis in fish warrants further research. Interestingly, proline also may be a quantitatively important source of citrulline and arginine on the basis of recent findings that the α-amino group and the carbon skeletons of ornithine, citrulline and arginine can come from proline rather than from glutamine, glutamate or aspartate (Wu, 1997).

In the present study, plasma-free glutamate, glutamine, citrulline and ornithine in fish fed the Arg–Gly diets did not show significant changes as arginine was increased in diet (and glycine was decreased). This suggests that glycine does not have an important physiological role in the metabolism of these amino acids. In contrast, glutamine

concentration in plasma was significantly ($P < 0.0001$) higher in fish fed diets containing glutamate compared to glycine. This elevation (71.2% on average) of plasma glutamine could be explained by an up-regulated conversion of glutamate into glutamine by cytosolic glutamine synthase as observed in perivenous hepatocytes (Haussinger, 1990) or by reduced muscle-released glutamine utilization as a substrate for citrulline, ornithine and arginine synthesis (Wu and Knabe, 1995). Data on circulating plasma glutamate, glutamine, citrulline and ornithine indicate trends ($P < 0.06$, 0.09, 0.08 and 0.08, respectively) of an increased citrulline production in fish fed diets with glutamate, suggesting up-regulation of the reactions glutamate → P5C → OAT → ornithine → citrulline, in channel catfish fed diets with glutamate. The elevated circulating citrulline could be used for endogenous synthesis of arginine, possibly at the kidney, as in adult rats (Dhanakoti et al., 1990). The extent to which enteric citrulline is funneled into renal tissue (or elsewhere) or used for enterocyte conversion into arginine, for local use, remains to be elucidated. Because most of the structural features and physiological functions of the mammalian kidney are allocated to both the trunk kidney and head kidney in fish (Bond, 1979), the site of arginine biosynthesis needs to be further investigated in various fish species.

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