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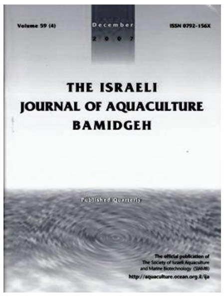
Published under auspices of
The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB)
&
University of Hawai'i at Mānoa
&
AquacultureHub
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_68.2016.1268, 9 pages



Effect of Dietary Alanine and Glycine Supplementation on Growth Performance, Body Composition and Apparent Nutrient Digestibility of Juvenile Grass Carp (*Ctenopharyngodon idella*)

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Keywords: *Ctenopharyngodon idella*; alanine; glycine; growth performance; apparent digestibility.

Abstract

A 42-day growth trial was undertaken to estimate the effects of supplemented alanine and glycine on growth performance, feed utilization, and apparent digestibility of nutrients in juvenile grass carp (*Ctenopharyngodon idella*).
Three diets were compared: 1) fishmeal (FM), 2) soybean meal (SBM) and 3) alanine and glycine supplemented (NEAA) where alanine and glycine were added to the soybean diet to achieve the desired alanine and glycine content in FM diet.
Results showed that NEAA diet decreased the feed conversion ratio (FCR) by 15.3%, improved average final weight by 13.4%, and increased 14.0% of protein retention compared with the SBM diet ($P<0.05$). In grass carp fed the SBM diet muscle protein and lipid content were significantly higher than those fed the FM and NEAA diets ($P<0.05$). Serum AST, ALT, ALP activities of fish fed the FM diet were significantly lower than the SBM diet ($P<0.05$), and similar to the NEAA diet ($P>0.05$). Serum urea content from the NEAA diet was significantly lower than FM and SBM diets ($P<0.05$). In conclusion, while adding alanine and glycine to a plant based diet can improve the growth performance, liver function, and protein utilization of juvenile grass carp, it does not improve performance to the levels of an FM diet.

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Introduction

In 1950, global production of cultured grass carp was 10,527 tons. By 2013 it had increased over 500 times reaching 5,226,202 tons (FAO, 2013). Grass carp is one of the most important species cultured in inland bodies of water in China.

Due to the use of pelleted and extruded feeds the species grows quickly in China. Fishmeal (FM) is widely thought to be the most ideal protein source for aquatic animals. However, since global supplies of FM are limited, soybean meal (SBM) with its high protein content and advantageous amino acid profile, has become the most commonly used substitute for FM. However, compared to the FM based feeds, SBM based feeds often lead to decreased growth and poor feed conversion rate.

In recent years, research has shown that the addition of essential amino acids such as lysine and methionine to plant based feeds can improve growth, feed efficiency and protein digestibility in aquatic animals (Viola et al., 1992; Nwanna et al., 2012). These feeds are still inferior to FM based diets, and growth performance is inferior even when essential amino acids are added. Non-essential amino acids also are important regulators of key metabolic pathways. Alanine can stimulate the feeding response of certain fish (Shamushak et al., 2007) and also carries nitrogen for inter-organ amino acid metabolism (Mommsen et al., 1980). Several studies have shown that alanine increases plasma levels largely independent of dietary protein levels (Petzke et al., 2000; Holecek and Kovarik, 2011). Glycine participates in gluconeogenesis, sulfur amino acid metabolism, one-carbon unit metabolism, and fat digestion (Fang et al., 2002); it also stimulates feed intake in many fish (Shamushaki et al., 2007). The purpose of this study was to examine the effect of alanine and glycine on growth performance and body composition of juvenile grass carp.

Materials and Methods

Experimental diets. Formulations of experimental diets are shown in Table 1. Two practical experimental diets (SBM and FM diets) were formulated with 310 g/kg (dry matter) crude protein without supplementation of crystalline amino acid. In another diet (NEAA diet), alanine and glycine were added to the SBM diet. According to the raw material database from our laboratory, available alanine and glycine in the FM diet and NEAA diet were comparable. All diets were isoenergetic.

Table1 Formulation and approximate composition of practical diets for grass carp.

Ingredient(g/kg)	*SBM diet*	*FM diet*	*NEAA diet*
Double low rapeseed meal[a]	350	350	350
Cotton meal[a]	200	200	200
Wheat flour[a]	201.9	201.9	200.4
Rice bran meal[a]	150	150	150
Soybean meal[a]	50	0	50
Fish meal[a]	-	50	-
Phospholipid[a]	5	5	5
Monocalcium phosphate[a]	20	20	20
Mineral mix[b]	5	5	5
Vitamin mix[c]	5	5	5
Choline chlorine(50%)[a]	2	2	2
Vc ascorbic acid	1	1	1
Corn oil[a]	10	10	10
Y_2O_3[d]	0.1	0.1	0.1
99%Glycine[e]	-	-	0.8
99%Alanine[e]	-	-	0.7
Total	1000	1000	1000
Approximate composition(g/kg diet)			
Crude protein	287.8	290.9	290.0
Crude lipid	25.7	27.7	25.2
Ash	81.1	83.2	79.3
Moisture	83.6	82.0	97.2
Available alanine [f]	7.8	8.5	8.4
Available glycine [f]	8.1	8.8	8.8
Gross energy kJ/kg	1115.5	1113.3	1112.4

a Zhuhai Shihai Feed Corporation Ltd, Zhuhai, China.
b Mineral mix(mg/kg diet): $MgSO_4·7H_2O$, 315; $ZnSO_4·7H_2O$, 285; $CaHPO_4·2H_2O$, 250; $FeSO_4·7H_2O$, 200; $MnSO_4·H_2O$, 25; $CoSO_4·7H_2O$, 25; $CaIO_3$, 25; $CuSO_4·5H_2O$, 15; Na_2SeO_3, 10. (Guangzhou Chengyi Aquatic Technology Ltd, Guangzhou, China).
c Vitamin mix (mg/kg diet): thiamin, 3; riboflavin, 8; vitmin A, 1 500 IU; vitamin E, 40; vitamin D3, 2000 IU; menadione, 6; pyridoxine, 4; cyanocobalamin, 2; biotin, 2; calcium pantothenate, 25; folic acid, 2; niacin, 12; inositol, 50. (Guangzhou Chengyi Aquatic Technology Ltd, Guangzhou, China).
d Y_2O_3 (Yttrium oxide), analytical pure. (Weibo Chemical Ltd, Guangzhou, China).
e Glycine contained glycine >99% and Alanine contained alanine >99% (CJ Co., Ltd., Liaocheng, China)
f available amino acid=raw material content%×dry matter%×amino acid content%×digestibility%×100

All dry ingredients were finely ground, weighed, and mixed manually for 5 min, then transferred to a Hobart mixer (A-200T Mixer Bench Model unit, Resell Food Equipment Ltd, Ottawa, ON, Canada) where they were mixed for another 15 min. Oil and phospholipid were then slowly added as mixing continued for another 10 minutes. Distilled water (about 300 g/kg of diet) was then added to the mixture to form a dough-like consistency. The wet dough was placed in a dual-screw extruder (Institute of Chemical Engineering, South China University of Technology, Guangzhou, China) and pellets were extruded through a 1.25-mm die. The diets were dried with forced air at 16°C for 48 h, and the moisture reduced to less than 100 g/kg. The dry pellets were placed in plastic bags and stored at -20°C in a deep freezer until used.

Experimental fish and experimental conditions. Prior to the commencement of the experiment, the fish were acclimated to the experimental conditions for 2 weeks and fed to satiation with a commercial diet containing 310 g/kg protein and 40 g/kg lipid. The fish were fasted for 24h before being randomly distributed to each of the 9 (three treatments X 3 replications each) fiberglass tanks (98 L × 48 W × 42 H cm, water volume of 200 L) at an equal stocking rate of 25 fish per tank connected to a recirculation system. Initial body weight averaged 8.01±0.03 g. During the trial, the fish were fed twice daily, 7 days per week at a rate of 30-40 g/kg of body weight for 42 days. Water exchange in each tank was maintained at 10 L/min. Water was oxygenated, passed through artificial sponge (3 cm thickness), coral sand (25 cm thickness), and active-carbon filter (25 cm thickness) to remove chlorine. During the trial period, the diurnal cycle of 12 h light/12 h dark was maintained. Water quality parameters were monitored weekly: temperature, 26.7 ± 1.5 °C; dissolved oxygen, 7.3 ± 0.38 mg/L; TAN, 0.091 ± 0.005 mg/L; pH, 7.7 ± 0.2, respectively. Feces were collected daily during the final 2 weeks, dried at 105°C, and stored at -80°C for determination of digestibility with Y_2O_3 (Weibo Chemical Ltd, Guangzhou, China) as indicator.

Sampling and analytical methods. At the beginning of the feeding trial, 12 fish were randomly sampled from the initial fish batch and sacrificed for analysis of whole body composition. At the end of the 42-day experiment, the fish were fasted for 24 h before sample collection. All fish from each tank were counted and weighed to determine WG, specific growth rate, and feed efficiency rate. Nine fish from each tank were randomly collected for proximate analysis, three for analysis of whole body composition, and six were anaesthetized with tricaine methane sulphonate (MS222, sigma, America) (50 mg/L) for individual body length and weight, and for blood collection. This was extracted from the hearts with heparinized syringes. Blood samples were centrifuged (4000 g, 10 min) at 4°C (centrifuge MR23i, Jouan, France). The plasma was separated and stored at -80°C until analyzed. The exsanguinated fish were dissected to extract viscera, liver, mesenteric fat, and hypothalamus, as well as white muscle.

Diets and fish samples (including white muscle and liver) were analyzed in triplicate for proximate composition. Crude protein, crude lipid, moisture, crude ash, and gross energy (GE) were determined following standard methods (AOAC, 2005). Crude protein (N × 6.25) was determined by the Kjeldahl method after acid digestion using an Auto Kjeldahl System (1030-Auto-analyzer, Tecator, Sweden). Crude lipid was determined by ether extraction using a Soxtec System HT (Soxtec System HT6, Tecator, Sweden). Moisture was determined by oven-drying at 105°C for 24 h. Crude ash was determined by incineration in a muffle furnace at 550°C for 24 h. GE was determined using an adiabatic bomb calorimeter. Amino acids were analyzed after acid hydrolysis using high-pressure liquid chromatography (HPLC; Hewlett Packard 1090, Palo Alto, CA, USA). The concentrations of dietary and fecal Yttrium oxide (Y_2O_3) were determined with an inductively coupled plasma atomic emission spectrophotometer (ICP; model: IRIS Advantage, HR; Thermo Jarrel Ash Corporation, Boston, MA, USA) after perchloric acid digestion (Bolin et al., 1952). Activities of plasma aspartate aminotransferase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) along with the concentrations of plasma triglyceride (TG), cholesterol (CHO), total protein (TP) and urea were assayed within 3 days by enzymatic procedure using automatic biochemical analyzer (Hitachi

7170; DAICHI, Tokyo, Japan) in a clinical laboratory of the First Affiliated Hospital, Sun Yat-sen University.

Statistical analysis. All data are presented as means ± SEM and subjected to one-way analysis of variance to determine whether significant differences were observed between treatments. If a significant difference was identified, differences among means were compared by Duncan's multiple range test (Duncan, 1955) at $P<0.05$.

Results

Growth performance, feed utilization and morphometric index. During the feeding trial, only one fish died in the NEAA diet group. This appeared to be unrelated to diet. The grass carp grew from an average weight of 8.4 g to 23.6 g during the 42 days (Table 2), with significantly higher WG in the FM diet group ($P<0.05$). The NEAA diet improved average final weight by 13.4% compared to the SBM diet. The specific growth ratio (SGR) in the SBM diet group was significantly lower than FM and NEAA diet groups ($P<0.05$). Food intake was similar among all of the experimental diets, however the SBM diet group tended to eat less. FCR of the SBM diet group was higher FCR then the other groups. FCR decreased by 15.3% in the NEAA diet group compared to the SBM diet group.

Table 2 Growth performance, feed utilization and morphometric index of grass carp fed with experiment diets for 42 days[a].

	SBM diet	*FM diet*	*NEAA diet*
Growth performance			
Initial weight(g)	8.4±0.02	8.4±0.02	8.4±0.03
Final weight(g)	20.9±0.61[a]	26.1±0.05[c]	23.7±0.31[b]
WG (%)[b]	147.8±7.68[a]	210.6±1.36[c]	180.4±5.12[b]
SGR(day^{-1})[c]	2.16±0.07[a]	2.70±0.01[c]	2.45±0.04[b]
FCR[d]	1.57±0.03[a]	1.20±0.00[b]	1.33±0.14[ab]
Food intake(g/fish)[e]	19.52±1.34	21.14±0.06	20.29±1.74
Morphometry[f]			
VSI[g]	7.89±0.25	8.66±0.19	8.33±0.04
HIS[h]	2.17±0.09	2.43±0.19	2.15±0.10
IPF[i]	2.34±0.08	2.03±0.26	2.19±0.20
CF[j]	2.16±0.04	2.19±0.12	2.17±0.02

a Means ± SEM of three replicates and values within the same column with different letters are significantly different($P<0.05$).
b WG = 100 × (FBW − initial body weight)/initial body weight.
c specific growth ratio (SGR)= 100 × ln (final weight/initial weight)/days of the experiment.
d Feed conversion ratio (FCR) = Feed consumed/(FBW − IBW).
e Feed intake (FI) = dry feed consumed /number of fish
f Means ± SEM of 24 replicates.
g Viscerasomatic index (VSI) = 100 × viscerasomatic weight (g)/body weight (g).
h Hepatopancreasomatic index (HSI) = 100 × liver weight (g)/body weight (g).
i Intraperitoneal fat ratio (IPF) = 100 × intraperitoneal fat weight (g)/body weight (g).
j Condition factor (CF) = 100 × body weight (g)/body length (cm)^3.

Condition factor (CF), hepatopancreasomatic index (HSI), intraperitoneal fat ratio (IPF) and viscerasomatic index (VSI) of juvenile grass carp fed the experiment diets are presented in Table 2. The morphometric index was not significantly affected by the treatments ($P>0.05$). VSI tended to be lower in SBM group than the other two groups. Highest IPF was in the SBM diet group, while the lowest IPF was found in FM diet group.

Whole body and muscle composition. As shown in Table 3, grass carp fed the SBM diet had highest muscle protein and lipid content, which were significantly higher than FM and NEAA diet groups ($P<0.05$). Muscle moisture content of FM diet group was significantly higher than SBM and NEAA diet groups ($P<0.05$). There were no significant differences ($P>0.05$) in whole body composition and muscle ash content among the groups.

Table 3 Whole body and white muscle composition of grass carp after 42 days feeding trial[a].

	SBM diet	*FM diet*	*NEAA diet*
Whole body(g/kg)			
moisture	743.9±9.41	750.8±9.80	751.1±2.00
crude protein	138.8±0.50	140.9±1.85	139.4±0.55
crude lipid	76.9±2.11	74.0±5.22	74.0±1.13
ash	3.20±0.51	3.03±0.08	2.84±0.10
Muscle (g/kg)			
moisture	794.7±0.96[a]	802.9±0.84[c]	797.8±0.44[b]
crude protein	176.1±0.34[c]	169.0±0.54[a]	172.3±1.61[b]
crude lipid	17.90±0.26[b]	13.04±0.83[a]	13.60±1.86[a]
ash	1.18±0.01	1.14±0.04	1.13±0.05

a Means ± SEM of three replicates and values within the same row with different letters are significantly different($P<0.05$) .

ADC and retention of nutrients. Apparent digestibility of dry matter, protein, and retention of protein lipids (see Table 4) were not significantly affected by the treatments ($P>0.05$). ADC of nutrients in the FM and NEAA diet groups tended to be better than SBM diet group. Protein retention was highest, and lipid retention was lowest, in the FM diet group. Lipid retention in the NEAA diet group decreased by 15.0% compared to the SBM diet group but protein retention increased by 14.1%.

Table 4 Apparent digestibility and nutrients retentions of juvenile grass carp fed with experiment diets for 42 days[a].

	SBM diet	*FM diet*	*NEAA diet*
ADCdry matter(%)[b]	64.6±1.28	65.1±1.81	67.8±0.64
ADCprotein(%)[c]	88.5±0.61	88.1±0.78	90.8±1.42
Protein Retention (%)[d]	32.0±0.66	40.3±0.16	36.5±4.48
Lipid Retention (%)[d]	323.1±24.8	256.0±24.3	274.8±32.9

a Means ±SEM of three replicates and values within the same column have no significant difference ($P>0.05$).
b ADC of dry matter (%) = 100 × [1 − (dietary Y_2O_3) / fecal Y_2O_3].
c ADCs of nutrients or energy (%) = 100 × [1 − (F / D × DY / FY)], where F is the percent of nutrients or energy in feces, D is the percent of nutrients or energy in diet, DY is the percent of Y_2O_3 in diet, and FY is the percent of Y_2O_3 in feces
d retentions of nutrients= 100 × retained nutrients (g) / lipid nutrients (g). PR, LR stand for protein retentions and lipid retentions respectively.

Amino acid composition of whole body. The amino acid pattern of whole body in grass carp is shown in Table 5. The essential amino acid content was similar among the different groups ($P>0.05$); however non-essential amino acid content showed that in the SBM diet group, whole body alanine content was higher than other groups, but the whole body glutamic acid and serine content was lower.

Table 5 Amino acid composition of whole body in grass carp fed with experimental diets for 42 days (g/kg dry body weight) [a].

	SBM diet	*FM diet*	*NEAA diet*
Essential amino acids			
Arginine	28.36±0.30	29.6±0.31	29.23±0.26
Histidine	11.36±0.08	11.3±0.24	11.02±0.09
Isoleucine	22.9±0.41	24.1±0.18	23.75±0.25
Leucine	37.02±0.06	37.4±0.46	37.7±0.61
Lysine	39.97±0.17	40.7±0.17	40.28±0.17
Methionine	13.18±0.02	13.5±0.06	13.34±0.04
Phenylalanine	19.54±0.20	20.3±0.10	20±0.03
Threonine	18.27±0.18	18.4±0.75	18.86±0.15
Valine	24.38±0.15	25.2±0.05	24.77±0.14
Non-essential amino acids			
Aspartic acid	47.92±0.56	49.3±0.12	47.18±0.80
Glutamic acid	70.62±0.49	74±0.11	72.15±0.46
Serine	14.48±0.47	15.7±0.11	15.65±0.18
Proline	21.87±0.39	22.5±0.46	21.77±0.26
Glycine	41.35±2.97	40.7±0.16	39.49±0.44
Alanine	39.83±7.8	33.2±0.36	32.19±0.14
Tyrosine	11.24±0.21	12±0.11	11.85±0.20
Cystine	2.26±0.01	2.29±0.00	2.29±0.01

a Means ±SEM of three replicates there was no significant difference ($P>0.05$) between the values within the same column.

Serum biochemical parameters. Serum AST, ALT, ALP activities and urea content were highest in the SBM diet (Table 6). Serum AST, ALT, ALP activities of fish fed FM diet were significantly lower than SBM diet ($P<0.05$) but similar to NEAA diet ($P>0.05$). Serum urea content of NEAA diet was significantly lower than FM and SBM diets ($P<0.05$). Serum TG content of SBM diet was significantly lower than FM and NEAA diets ($P<0.05$). No significant differences ($P>0.05$) were found between treatments for TP and CHO content.

Table 6 Serum biochemical parameters of grass carp fed with experimental diets for 42 days[a].

	SBM diet	*FM diet*	*NEAA diet*
AST(U/L)	23.73±1.02[b]	21.20±1.71[a]	21.40±0.66[ab]
ALT(U/L)	3.53±0.25[b]	2.87±0.45[a]	2.93±0.15[ab]
ALP(U/L)	183.0±4.00[b]	155.3±12.01[a]	154.0±6.08[a]
TP(g/L)	25.03±1.62	24.90±1.35	25.80±1.15
CHO(mmol/L)	5.43±0.09	5.54±0.03	5.91±0.68
TG(mmol/L)	2.62±0.06[a]	2.89±0.11[b]	2.83±0.07[b]
UREA(mmol/L)	1.90±0.10[b]	1.77±0.06[b]	1.53±0.06[a]

a Means ±SEM of three replicates and values within the same row with different letters are significantly different ($P<0.05$) .

Discussion

Several articles have shown that soybean meal (SBM) is a suitable alternative for the partial replacement of fish meal (FM) and it is utilized in commercial fish feeds. However, studies in carp species such as common carp *Cyprinus carpio* L., (Wang et al., 2009), juvenile allogynogenetic silver crucian carp *Carassius auratus gibelio* Bloch, (Cai et al., 2011) and Chinese sucker *Myxocyprinus asiaticus,* and Indian major carp (Patnaik, 2005; Yu et al. 2013) have shown that SBM had a negative effect on growth rate, FCR, and gut length. Similar results were observed in the present study. WG and SGR in the SBM diet group was significantly lower than FM diet group, while FCR was higher than the FM diet group. One reason for the poor results with SBM diet is its low palatability. Results of the present study have shown a reduction in voluntary feed intake of juvenile grass carp. The food intake of SBM diet group decreased 7.6% compared with the FM diet group. The reduction of voluntary feed intake with plant protein-based diets has also been reported in rainbow trout (Gomes et al., 1992) and in European sea bass (Dias et al., 1997). In addition the proportion of amino acids in the SBM diet was not optimal. Efficient protein synthesis occurs if all amino acids are present in the precursor pool at the correct proportion (Carter et al., 2000). Some nonessential amino acids have been proven to be important regulators of key metabolic pathways necessary for maintenance, growth, feed intake, nutrient utilization in various aquatic animals (Li et al., 2009). In this study, the serum urea content in fish fed the NEAA diet was significantly lower than fish fed the FM and SBM diets, indicating higher nutrient utilization.

Data from the present study showed that protein retention was lowest in the SBM diet group and lipid retention was highest in the SBM diet group. However in the NEAA diet group, protein retention increased 14.1% and lipid retention decreased 15.0% compared to the SBM diet group, while WG, and SGR, of the NEAA group improved 22.1% and 13.4% respectively compared to the SBM group. In addition, the SBM diet group had the highest muscle lipid content, which was significantly higher than both the FM and NEAA diet groups. Dietary glycine has been found to decrease adipose tissue content and serum lipid in mice fed normal or high-fat diets, and can also prevent hepatic steatosis (Liu et al. 2005). Dietary L-leucine and L-alanine supplementation have been found to have similar acute effects in the prevention of high-fat diet-induced obesity (Freudenberg et al. 2013). This may due to feed efficiency improvement, caused by supplemented glycine and alanine in the diets. FCR in the NEAA diet group decreased 15.3% compared to the SBM diet group. Early studies also found that the food intake of *P. japanicus* (Deshimaru and Yone 1978) and *P. monodon* (Murai et al., 1981) were stimulated by the addition of glycine. Nucleotide, alanine and glycine supplementation can significantly enhance weight gain in rainbow trout (Hunt et al., 2014), special growth rate, and feeding rate of *Litopenaeus vannamei* (Hu et al 2010).

Results presented in table 4 indicate that fish fed the NEAA diet had highest ADC of protein and dry matter, indicating that the NEAA diet improved nutrient absorption efficiency.

Alanine is an important substrate for hepatic gluconeogenesis and is one of the main amino acids released by skeletal muscle. It is also an important source of energy for fish. Dietary alanine is largely extracted by the splanchnic bed (Felig, 1973), but the question about the effect of adding alanine in diets is controversial. No positive effect of b-alanine supplementation on growth performance of Japanese flounder *Paralichthys olivaceus*, has been observed (Kim et al. 2003). In our trial, serum AST, ALT, ALP activities of fish fed the NEAA diet were significantly lower ($P<0.05$), than those fed the SBM diet however, the physiological significance of this finding has not been elucidated.

In conclusion, we may infer that dietary supplementation with nonessential amino acids such as alanine and glycine may be beneficial for food intake and growth performance of juvenile grass carp.

Acknowledgements

The research was supported by National natural science foundation of china (No.31172419/C190401).

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