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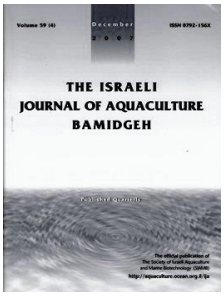

Lysine Supplementation of the Protein Concentrate of Crinkle Grass *Rhizoclonium riparium var implexum* as an Ingredient in the Diet of Nile Tilapia Fry

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Keywords: crystalline lysine; seaweed; protein concentrate; protein quality; Nile tilapia; non-conventional feed ingredient.

Abstract

A study was conducted on 1000 fish, to evaluate the effect of supplementing crystalline lysine (Lys) to a diet containing protein concentrate from seaweed *Rhizoclonium riparium var implexum* (RPC) on the performance of Nile tilapia fry. A previous finding showed that protein concentrate was deficient in lysine. Four experimental diets were prepared: Diet 1 - no RPC and no Lys; Diet 2 - 8.4% RPC+0.5% Lys; Diet 3 - 8.4% RPC+1.0% Lys; and Diet 4 - 12.6% RPC+1.5% Lys. After a 60 day feeding trial, results showed that final average body weight, weight gain, protein accumulation, and whole body biochemical composition were not affected by the dietary treatment. Specific growth rate, food conversion ratio, and protein efficiency ratio were all significantly higher in fish fed the test diets (i.e. Diets 2, 3 and 4) than in those fed the control diet. There were no significant differences in protein and energy retention; lipid retention was lowest in Diet 2 group and statistically similar to Diet 3 and 4 groups. In conclusion, most parameters improved significantly in fish fed diets with RPC and Lys supplementation compared to fish in the control group. The most cost effective dietary treatment was Diet 2 containing 8.4% *Rhizoclonium* protein concentrate supplemented with 0.5% crystalline lysine.

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Introduction

Aquaculture, which requires a good quality protein source, has grown rapidly, resulting in depleting supplies of fishmeal supply. The most practical protein sources are agricultural, aquatic, and farm byproducts however each one is deficient in one or more essential amino acids (EAA). This is exacerbated by the processing method (Nunes et al., 2014). Growth, defined as accumulation of body protein, is largely dependent on protein synthesized from amino acids assimilated from the diet. However, plant protein sources are usually deficient in lysine and methionine. Thus, the imbalance and bioavailability of protein from these sources can affect protein synthesis and/or catabolism in muscle tissue subsequently affecting fish growth and carcass yield.

Seaweed, especially some red and green ones are potential ingredients in aquafeeds as protein comprises 10-30% of their dry matter (Ramos et al., 2000). They are natural immunostimulants, specifically their sulfated polysaccharide content (Persson et al 2011), and they also promote growth in some fish species (Hashim and Mat Saat, 1992). *Rhizoclonium riparium* is a fast growing (2.1-10.4%/day) green seaweed, (Chao et al., 2005) with vast salinity tolerance (0.1-34 parts per thousand (ppt) (Imai et al., 1997). This makes it a good candidate for inclusion in fish diets.

The amino acid composition of *R. riparium* varies with location; the ratio of essential amino acids (EAA) to nonessential amino acids (NEAA) was found to range from 0.8:1-1.1:1. EAA index (EAAI) of *R. riparium* meal for Nile tilapia ranged from 1.0-1.2, indicating that it contained a balanced amount of EAA (Bunda et al. 2015). However, the chemical score (CS) of the unprocessed seaweed meal for the Nile tilapia was 62 with methionine as the first limiting AA while the concentrated form of the seaweed had a CS of 52-68 with lysine (Lys) as the first limiting AA (Bunda et al., 2015). This indicated that the process of concentrating protein from *R. riparium* using both pH shift and heat treatment destroyed the amino acid Lys.

Lysine is the most limited amino acid ingredient used to prepare fish and shrimp feeds, particularly those formulated with high levels of plant protein ingredients (Small and Soares, 2000) or with protein ingredients processed under harsh conditions (NRC, 2011). Growth rate may improve as Lys content can reduce oxidation of other amino acids by improving the use of other EAAs (Kerr & Easter 1985)..

Amino acid supplementation in diets increases the performance of some aquaculture fish species (Odum and Ejike, 1991; Murthy and Varghese, 1997). Thus, there is a need to enhance the protein quality of *Rhizoclonium riparium var implexum* protein concentrate by adding crystalline lysine to the Nile tilapia fry diet.

Materials and Methods

Growth trial experiments were carried out at the National Institute of Molecular Biology and Biotechnology (NIMBB) Laboratory, University of the Philippines Visayas, Miag-ao, Iloilo from August until October 2015. One thousand fish were used (400 in the feeding trial and 600 were used for poximate analysis at the onset).

Preparation of Rhizoclonium sp. protein concentrate (RPC). The alga was collected from Leganes, Iloilo, Philippines. It was washed, and cleaned by hand, shade-dried, and then oven-dried for 24 h at 60°C. The preparation of *Rhizoclonium* sp. protein concentrate (RPC) was carried out according to the method of Agbede et al. (2008) with an additional step of acidification. A kilo of dried algae was added to 1 L of distilled water, and the algal juice was extracted using an industrial mechanical juicer. The juice was acidified with HCl to pH 3.0 and the filtrate was extracted by squeezing the slurry through muslin cloth. The juice was heated for 15 min at 80-90°C and the precipitate was refiltered through muslin cloth. The thick slurry was oven-dried to about 10% moisture and then kept at -20°C until diet preparation.

Diet preparation. Four practical diets were formulated for this experiment. The diets contained 35–37% crude protein and 6-7% fat, satisfying the requirements of young tilapia (Santiago et al 1982)(Table 1). Experimental diets contained RPC at the following inclusion levels and Lys supplementation rates: 0% RPC+0% Lys (Control diet, Diet 1)), 8.4% RPC+0.5% Lys (Diet 2), 8.4% RPC+1.0% Lys (Diet 3) and 12.6% RPC+1.5% Lys (Diet 4).

The minimum RPC inclusion level of 8.4% was chosen based on a previous study which determined the optimum inclusion rate of RPC for Nile tilapia fry (Serrano, unpubl). Ingredients were powdered and sieved at 150 µm prior to mixing; all dried ingredients were thoroughly mixed then liquid ingredients were added. Gelatinized cornstarch was also added as a final step before pelletizing. The moistened mixture was pelleted in a meat grinder and oven-dried at 60°C to about 10% moisture. Diets were then crumbled into appropriate sizes, sealed in plastic bags, and stored at -20°C until use.

Table 1. Feed formulation of experimental diets for *Rhizoclonium* sp. protein concentrate as partial replacement to soybean meal

Ingredients	*Control (0% RPC+0%*	*8.4%*	*8.4% RPC+*	*12.6% RPC+*
Fish meal (65% CP-anchovy)	200.0	200.0	200.0	200.0
Squid Meal	80.0	80.0	80.0	80.0
Shrimp Meal	80.0	80.0	80.0	80.0
Copra (coconut) meal	100.0	100.0	100.0	100.0
Soybean meal	280.0	280.0	280.0	280.0
Rice bran	120.7	120.7	120.7	120.7
Cornstarch	50.0	50.0	50.0	50.0
Soybean oil	30.0	30.0	30.0	30.0
[a]Vitamin premix	21.7	21.7	21.7	21.7
[b]Trace mineral premix	21.6	21.6	21.6	21.6
CMC	16.0	16.0	16.0	16.0
Rhizoclonium protein conc.	0.0	84.0	84.0	126.0
Lysine	0.0	5.0	10.0	15.0
Total	1000.0	1000	1000.0	1000.0
Proximate Analysis (%)				
Moisture	8.95	8.15	9.67	8.14
Crude Protein	37.21	36.23	35.75	35.45
Crude Fat	6.73	7.44	6.39	6.86
Crude Fiber	4.40	4.32	4.10	3.99
NFE	28.30	26.03	26.73	26.19
Ash	14.41	17.83	17.36	19.37
[c]Gross Energy (kcal/g)	3.22	3.16	3.07	3.08

[a] Vitamin mix: Vitamin A, 1,200,000 IU/kg; Vitamin D3, 200,000 IU/kg; Vitamin E, 20, 000 mg/kg; Vitamin B_1, 8000 mg/kg; Vitamin B_2, 8000 mg/kg; Vitamin B_6, 5000 mg/kg; Vitamin B_{12}1%, 2000 mcg kg^{-1}; Niacin, 40,000 mg/kg; Calcium Pantothenate, 20, 000 mg/kg; Biotin, 40 mg/kg; Folic Acid, 1,800 mg/kg; Ethoxyquin , 500 mg/kg

[b] Mineral mix: Fe, 40,000 mg/kg; Mn, 10,000 mg/kg; Zn, 40,000 mg/kg; Cu, 4000 mg/kg; I, 1,800 mg/kg; Co, 20 mg/kg; Se, 200 mg/kg

[c] Gross energy estimated according to 23.6 KJ/g protein, 39.5 KJ/g lipid, and 17.0 KJ/g NFE(Ergun et al 2009)

Experimental tilapia and set up. Nile tilapia (*Oreochromis niloticus*) fry were purchased from SEAFDEC-AQD, Tigbauan, Iloilo, Philippines and acclimatized to laboratory conditions and also to the control diet. Four hundred Nile tilapia fry were randomly stocked in twenty 60 L tanks (20 fry per tank) in a closed recirculating system. The fish were fed the experimental diets twice daily with 5 replications of each diet. Fish were bulk-weighed at the start of the experiment, and every 15 days thereafter; quantity of feed for the following 15 days was adjusted until termination of the experiment at the 8th week. About 70% of the water in the system was replaced and filters cleaned every other day. Uneaten feed and feces were siphoned-off every morning before the first feeding. Clean water to replace the used water was treated with chlorine (100 ppm NaClO) and dechlorinated by strong aeration for 3 days before replacement. Water temperature and pH were measured twice a day (0800 and 1600 h), dissolved oxygen (DO) twice a week, and nitrite and total ammonia, weekly (using commercially available kits).

Growth performance parameters. Growth performance and feed utilization were estimated using the following formulae:

Weight gain, WG (g) = FABW – IABW

Where: FABW = final average boy weight(g) and

IABW = initial average boy weight (g)

Specific Growth Rate (SGR, %/day) = 100*(lnFABW–lnIABW)/(T2– T1)]
T_2 = Final time (in days) T_1 = Initial time (in days)

FCR = FI(g) /WG (g)
Where FI= total individual feed intake (g)

PG (g) = (FABW*final carcass CP in decimal-IABW*initial carcass CP in decimal)

PER = WG (g) / FI*dietary CP in decimal

Protein Retention = 100*PG (g)/(FI*dietary CP in decimal)

Lipid Retention = 100*(final carcass lipid in decimal x FABW (g) – (initial carcass lipid in decimal*IABW (g))/FI (g)*dietary lipid intake in decimal

Energy Retention = 100*(Energy gained i.e. carcass %CP, %CL and %NFE multiplied by physiological fuel values of each category/ (energy intake i.e. feed %CP, %CL and %NFE multiplied by the same physiological fuel values of each category)
Fuel values used were 4 kcal/g protein, 4 kcal/g carbohydrate and 9 kcal/g fat

Survival (%) = 100*(Survived fish / Initial fish stocked)

Feed and carcass analysis. Feed and fish body were subjected to proximate analysis. At least 50 g of each of the experimental diets was analyzed. At the onset of the experiment, 600 fish carcasses were frozen and kept at -20°C until analysis. At the end of the experiment all surviving fish from each replicate tank were sacrificed (20 fish per tank, i.e. replicate, except for Diet 2 which had only 96% survival rate). Samples were oven-dried at 60°C and pulverized, prior to the proximate analysis. Moisture was measured using a thermo-balance (Mettler Toledo HB43 halogen moisture analyzer). Ash content was determined after incineration in a muffle furnace at 550°C for 12 h. Crude protein was measured after block digestion and steam distillation using Foss Tecator™ digestion system and Foss Kjeltec™ 8200 auto-distillation unit. Crude fat was extracted using Foss Soxtec™ 2050 automatic system and fiber was determined using Foss Fibertec™ 2010 system.

Statistical analysis. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 23 software. Data were presented as mean ± standard error of the mean (SEM). Data were tested for normality using Shapiro-Wilk test and variance homogeneity using Levene's test. The one-way analysis of variance (ANOVA) was used on data which passed the tests while those that did not were subjected to transformation until they passed the tests, after which one-way ANOVA tests were used. Growth indices (FABW, WG, SGR), feed utilization parameters (FCR, protein, lipid and energy retention rates), body composition and survival rates were analyzed using ANOVA at α=0.1, Tukey HSD test was performed to rank the mean values of the performance parameters. In comparing the means, p values were given and the following categories of significance were used according to Le, (2003): when $p>0.10$ the result was not significant; $0.05<p<0.10$, was marginally significant; $0.01<p<0.05$ was significant and $p<0.01$ was highly significant.

Results

Growth performance, feed utilization, and survival of sex-reversed *Oreochromis niloticus* fry fed with experimental diets containing various inclusion/replacement levels of RCP is shown in Table 2.

Table 2. Growth performance and survival of juvenile *Oreochromis niloticus* fed with diets containing increasing replacement levels of soybean meal with *Rhizoclonium* protein concentrate (RPC)
Values in the same row with different superscript letters are significantly different ($P \leq 0.05$). Values are mean ±

Diet		*IABW (g)*	*FABW (g)*	*WG (g)*	*FI (g)*	*SGR(%/day)*	*FCR*	*PG*	*PER*	*Survival (%)*
Diet 1	0% RPC +0% Lys	0.15 ± 0.01	3.99 ± 0.41^{a}	3.87 ± 0.41^{a}	7.81 ± 0.39^{a}	4.90 ± 0.08^{a}	1.65 ± 0.07^{a}	2.40 ± 0.27^{a}	1.50 ± 0.08^{a}	100 ± 0.00
Diet 2	8.4% RPC +0.5% Lys	0.14 ± 0.01	4.36 ± 0.31^{a}	4.25 ± 0.31^{a}	7.73 ± 0.47^{a}	5.12 ± 0.05^{ab}	1.46 ± 0.04^{b}	2.65 ± 0.19^{a}	1.74 ± 0.04^{b}	96 ± 4.00
Diet 3	8.4% RPC +1.0% Lys	0.15 ± 0.01	4.67 ± 0.40^{a}	4.56 ± 0.40^{a}	8.23 ± 0.45^{a}	5.16 ± 0.09^{b}	1.46 ± 0.05^{b}	2.83 ± 0.24^{a}	1.76 ± 0.07^{b}	100 ± 0.00
Diet 4	12.6% RPC +1.5% Lys	0.14 ± 0.00	4.01 ± 0.18^{a}	3.90 ± 0.18^{a}	7.59 ± 0.07^{a}	5.01 ± 0.04^{ab}	1.57 ± 0.05^{ab}	2.41 ± 2.62^{a}	1.67 ± 0.06^{ab}	100 ± 0.00

SEM. ABW, final average body weight; WG, weight gain; FI, feed intake; SGR, specific growth rate; FCE, feed conversion efficiency; FCR, feed conversion efficiency; PG, protein gained; PER, protein efficiency ratio.

For all fish groups, IABW were statistically similar (p=0.13) as were the results for the FABW and WG values, (p=0.45 and p=0.44, respectively). FI values were proportional to the changes in body weight, and values were statistically similar for all dietary treatments (p=0.68). SGR values were marginally significantly different (p=0.08), the highest value was observed in fish fed Diet 3 (8.4% RPC+1.0% Lys). Results were similar in fish fed Diets 2 and 4. The control group (Diet 1) had the lowest SGR value, which was statistically similar to fish fed Diets 2 and 4.

For feed efficiency indices, FCR values exhibited marginal significant differences (p=0.09); the lowest values were observed in the Diet 1 group. This was statistically similar to the Diet 4 group; Diet 2 and 3 groups showed better FCR values the Diet 1 and 4 groups. PER values were significantly different (p=0.04); values from Diet2 and 3 groups were significantly higher than the control diet (Diet 1) group, which was not significantly different from Diet 4 group. PG values were statistically similar for all the treatment groups (p=0.43).

For nutrient retention rates, PR values were excellent in all dietary groups and exhibited significant differences (p=0.04) compared to fish fed Diets 2 and 3 exhibiting significantly higher retention rates (108.5% and 105.7%, respectively) but were not significantly different from those of fish fed Diet 4 (96.4%)(Table 3). PR was significantly the lowest in fish fed Diet 1 (92.98%) but was statistically similar to fish fed Diet 4. Lipid retention (LR) rates were all beyond 100% (range of 150.7%-174.5%) and were not significantly different among experimental groups (p=0.30). Energy retention(ER) values of all dietary treatments exhibited marginally significant differences (p=0.08) among fish fed the experimental diets, with a range of 74.39% - 79.7%.

Table 3. Nutrient and energy retention (%) of juvenile *Oreochromis niloticus* fed with *Rhizoclonium riparium* as partial replacement to soybean meal

Treatment		*Protein Retention*	*Lipid Retention*	*Energy Retention*
Diet 1	0% RPC+0% Lys	92.98 ± 5.19^{a}	152.15 ± 12.22^{b}	74.39 ± 3.74^{a}
Diet 2	8.4% RPC+ 0.5% Lys	108.45 ± 3.01^{a}	149.57 ± 2.20^{a}	85.45 ± 1.93^{a}
Diet 3	8.4% RPC+ 1.0% Lys	109.66 ± 4.24^{a}	172.62 ± 13.59^{ab}	85.35 ± 3.78^{a}
Diet 4	12.6% RPC + 1.5% Lys	103.03 ± 3.49^{a}	151.07 ± 3.50^{ab}	79.7 ± 2.89^{a}

Final body composition of the fish did not show significant variation among fish groups (Table 4).

Table 4. Whole-body composition (g/kg fresh weight) of juvenile *Oreochromis niloticus* after 8 weeks offeeding with the experimental diets * (n = 5).

Treatment		*Protein*	*Lipid*	*Moisture*	*Ash*
Diet 1	0% RPC+0% Lys	278.5±27.4	74.0±1.5	780.5±2.3	66.9±2.7
Diet 2	8.4% RPC+0.5% Lys	321.3±23.9	75.2±1.8	784.5±3.6	73.0±1.8
Diet 3	8.4% RPC+1.0% Lys	321.7±25.0	70.4±2.7	780.4±4.4	70.2±1.9
Diet 4	12.6% RPC+1.5% Lys	275.6±12.6	70.4±1.8	781.6±4.0	67.3±1.4

Values are means ± standard error of the mean (SEM). Mean values are not significantly different from each other (α>0.1).

Discussion

Certain amino acids, when ingested by animals in excessive or disproportionate amounts, can produce adverse reactions that range in severity from reduced growth and feed intake, to pathological lesions and death (Harper et al., 1970). In the present study, no such adverse effects were observed. Growth inhibition may occur in Nile tilapia fed diets containing unbalanced dietary arginine and lysine levels (Santiago and Lovell 1988). In our study, the levels of lysine supplementation which varied in relation to the RPC level did not result in excessive or disproportionate amounts of Lys. Our calculation of Lys supplementation was based on our previous findings on chemical score (CS) and essential

amino acid index (EAAI) of *Rhizoclonium* protein concentrate (Serrano, unpubl.). Although the EAAI of RPC was 1.05 implying a well-balanced EAA, the CS was only 64 with Lys as the first limiting EAA. This indicated that RPC could only supply Lys at 64% of Nile tilapia requirement. The rest was presumably supplemented in the present study. Reported values of Lys requirement in aquatic animals range from 3.8% to 5.8% of dietary protein (Nunez et al 2014) while supplemented Lys in the present study ranged from 1.38% to 4.23% of the dietary protein. Since this was only a supplementation, it is possible that 4.23% Lys together with Lys from other protein sources may have led to a slight excess but no adverse effects were observed even compared to the control group.

Supplementing Lys in the present study with two levels of dietary inclusion of RPC (i.e. 8.45 and 12.6%) did not affect growth performance of Nile tilapia fry however the SGR values differed marginally between the dietary groups. SGR was higher in fish fed RPC supplemented diets than the control diet. The SGR of the Nile tilapia fed diets with the protein concentrate (PC) of *Ulva intestinalis* was reduced with inclusion level of 7.8% PC (Serrano Jr. and Aquino, 2014). In the present study, this did not occur with 8.4% and even at 12.6% inclusion (with 0.5 or 1.0%, and 1.5% Lys supplementation, respectively). In the present study it was unclear whether the full effect of Lys supplementation was realized. This could be due to the problems of amino acid absorption and assimilation in the gut. AA utilization is problematic for some aquatic species, such as common carp, channel catfish, Japanese prawn, (*Penaeus japonicus*) which appear to utilize free AAs less efficiently than protein-bound AAs (Zhou et al., 2007). Free AAs pass from the stomach more rapidly than protein-bound AAs suggesting that crystalline AAs would be more readily absorbed than protein-bound AAs. All diet groups with RPC exhibited better growth performance than the control group. The inferior performance of the control diet may be caused by deficiency in Lys content since soy protein-based diet typically is low in lysine (Yuan et al., 2011).

White leg shrimp *Litopenaeus vannamei* and other species fed a Lys deficient diet had lower feed protein efficiency and PER (Xie et al., 2012). This was attributed to amino acid imbalance and loss of appetite caused by the deficiency (Persson et al., 2011, Zhang et al., 2008, Zhou et al., 2007). FCR and PER values of fish fed diets with RPC were better than the control group in the present study. We can assume that there was no deficiency or imbalance when it came to Lys levels, or that RPC promoted feed efficiency. Soy protein-based fish diets are also low in Lys and Met therefore the absence of Lys supplementation may explain why FCR was higher, and PER values were lower, in fish fed the control diet. Feed utilization is also affected by excessive dietary Lys (Xie et al., 2012). In our study there were no significant differences in feed utilization between Diets 3 and 4. These results were similar to those reported by Forster and Ogata (1998) and Zhou et al. (2007).

In conclusion, performance indices were better with supplementation of 0.5% or 1.0% Lys + 8.4% dietary RPC, or 1.5% Lys + 12.6% RPC, than no supplementation at all. Under these circumstances the most cost effective treatment, which was supplementation of 0.5% +8.4% RPC (Diet 2) would be recommended.

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