Aquaculture Studies, 23(1), AQUAST859
https://doi.org/10.4194/AQUAST859

RESEARCH PAPER

Effects of Dietary Phenylalanine: Tyrosine Ratio on Growth, DNA/RNA, Serum Biochemistry, Digestive Enzyme Activities and Physiological Responses of *Heteropneustes fossilis*

Shabihul Fatma Sayed[1,*], Imtiaz Ahmed[2]

[1]College of Nursing, Jazan University, Farasan Campus, KSA, Saudi Arabia.
[2]Department of Zoology, University of Kashmir, Hazratbal, Srinagar, India.

How to Cite

Sayed, S.F., Ahmed, I. (2023). Effects of Dietary Phenylalanine: Tyrosine Ratio on Growth, DNA/RNA, Serum Biochemistry, Digestive Enzyme Activities and Physiological Responses of *Heteropneustes fossilis*. *Aquaculture Studies*, *23(1), AQUAST859*. https://doi.org/10.4194/AQUAST859

Article History
Received 25 April 2022
Accepted 03 July 2022
First Online 22 July 2022

Corresponding Author
Tel.: +966538024737
E-mail: ftm77@rediffmail.com
ssaid@jazanu.edu.sa

Keywords
Nutrition
Optimum ratio of phe:tyr
Protein deposition
Physiological response
Stinging catfish

Abstract

Stinging catfish (*Heteropneustes fossilis*) has high consumer demand due to its high iron and calcium contents. Also, being a lean fish, it is very suitable for people who do not prefer to consume animal fats. In view of its nutritional and therapeutic significance and lack of data on dietary phenylalanine: tyrosine ratio, a dose-response experiment was conducted to determine phe: tyr ratio for *H. fossilis* fry (4.1±0.3g). Six isonitrogenous (380 g/kg crude protein) and isocaloric (15.3 kJ/g digestible energy) amino acid test diets were prepared by adjusting 26.6 g/kg of total phenylalanine and tyrosine in varying ratios of phenylalanine/tyrosine (30:70, 40:60, 50:50, 60:40, 70:30, 80:20) on molar basis. Quadruplicate groups of fishes were fed with diets indicated for 12-weeks to apparent satiation, thrice daily. Mathematical analyses of Daily Growth Coefficient (DGC%), Specific Growth Rate (SGR%/day), Feed Conversion Ratio (FCR) and Erythrocyte Osmotic Fragility (EOF) as quadratic-broken line regression analyses, exhibited optimum dietary phenylalanine: tyrosine ratio of 64.94: 35.06 corresponding to 15.83 g/kg: 8.55 g/kg on equimolar basis. Data of this study would be of high significance to ensure that optimum phenylalanine and tyrosine ratio of fish is met while using a greater variety of cost-effective dietary protein feedstuffs for sustainable aquafarming of this fish.

Introduction

Fish consumption has more than doubled from 9.6 kg/capita in 1961 to 20.5 kg/capita in 2018 (FAO, 2020). In present pandemic situation, global appetite for fish is expected to increase further due to its superior nutritional profile of high biological value. With the fall in capture fisheries, aquaculture has been the only alternate to fill the gap between supply and demand of quality protein diet as fish.

Amino acids (AA) are vital molecules in the metabolism of all living organisms and are the building blocks of enzymes (Yaghoubi *et al.,* 2018). Essential amino acids are key molecules for building proteins, as well as important regulators of key metabolic pathways including cell signaling, appetite stimulation, growth and development, energy utilization, immunity, osmoregulation, ammonia detoxification, antioxidative defense, metamorphosis, pigmentation, gut and neuronal development, stress responses, reproduction, normal pancreatic and liver function, and for suppression of aggressive behavior in aquatic animals (Wu *et al.,* 2013, 2014**;** Yaghoubi *et al.,* 2018). Aromatic properties of fish meat partly depend up on amino acid distribution (Hall & Ahmad, 1992). Since most alternative protein sources are deficient in point of including all essential amino acid, it is crucial to establish essential amino acid requirements for various cultivable

fish species so that alternative protein feedstuffs could be supplemented appropriately with the deficient amino acid as per the requirement of candidate species.

All cultivable food fish species require the 10 essential amino acids in their diets for maximum growth (Wilson, 2002), therefore, information of dietary essential amino acid requirements of fish species is of high importance in formulating cost-effective feeds (Yun *et al.,* 2015).

Phenylalanine is an essential aromatic amino acid. Dietary phenylalanine has a great impact on feed intake, growth performance, immunity and survival of fish in natural environment (Li *et al.,* 2008; Pinto *et al.,* 2009). It is important for the synthesis of thyroid hormones that control metabolic processes, thereby influencing growth of different body structures; feed efficiency; oxygen consumption; synthesis and metabolism of proteins, carbohydrates and lipids; thermogenesis; and acclimation to environmental changes (Gropper & Smith, 2012). A reduction in plasma stress markers were reported in response to dietary supplementation with a high content of phenylalanine in Gilthead seabream, *Sparus aurata* and Meagre, *Argyrosomus regius* (Salamanca *et al.,* 2021). Phenylalanine deficiency results in an immediate reduction in feed intake followed by loss in weight gain and a negative nitrogen balance (Tacon, 1992). However, only few studies have evaluated the effect of phenylalanine supplementation in diets for aquatic animals (Chance *et al.,* 1964; Kim, 1993; Khan, & Abidi 2007; Ahmed, 2009; NRC, 2011; Kim *et al.,* 2012; Zehra & Khan, 2014; Castillo *et al.,* 2015; Li *et al.,* 2015; Ren *et al.,* 2015; Xiao *et al.,* 2020; Zehra & Khan, 2021). Although adequacy of essential amino acid supplies has received much attention, availability of semi-essential amino acids is also necessary for maintenance of physiological homeostasis and their *de novo* synthesis is important for survival. Tyrosine is among one such amino acid.

It is also converted into L-dopa, dopamine, norepinephrine and epinephrine, the three key neurotransmitters. Brain concentrations of these neurotransmitters depend upon intake of tyrosine. In animals, tyrosine-deficient diets have been reported to decrease DOPA accumulation (Fernstrom & Fernstrom, 1995; Allen, 2010).

Since phenylalanine requirement is influenced by the dietary adequacy of tyrosine, for determining true dietary requirement for phenylalanine, tyrosine should also be included appropriately. In case of inadequacy of tyrosine in body, dietary phenylalanine would get converted to tyrosine and will give a false exaggerated phenylalanine requirement. For this reason, varying ratios of phenylalanine and tyrosine have been taken into considerations so that phenylalanine could be spared from being converted to tyrosine.

Growth is the most widely used criterion when defining optimal nutrient levels and is a reliable indicator of the suitability of diets. The RNA/DNA ratio is sensitive to the levels of essential nutrients in the body and can be used as an index of fish growth (Bhat *et al.,* 2020). In present study, RNA/DNA ratio has been used as a sensitive tool to have precise information on nutritional status and growth of the fish.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are two most important amino acid metabolizing enzymes and their activities reflect the intensity of fish amino acids metabolism (Ballantyne, 2001). The ALT and AST are generally used as indicators of cellular damage both in mammals and in fishes (Olsen *et al.,* 2005). For this reason, serum biochemistry as ALT and AST activities has been analyzed to determine optimum phenylalanine/tyrosine ratio more precisely. Fish growth is based on protein and fat deposition, which is closely related to the digestion and absorption of nutrients (Zhao *et al.,* 2012; Rønnestad *et al.,* 2013) and hence estimation of digestive enzyme activities can serve as indicators of nutrient utilization by fish. Since enzyme secretion closely relates to the development of the pancreas and several studies reported that various essential amino acid can affect the development and structural integrity of pancreas in fish (Chen *et al.,* 2012; Tang *et al.,* 2013; Li *et al.,* 2015), and have significant role on digestive enzyme activities in fish (Chen *et al.,* 2012; Tang *et al.,* 2013; Li *et al.,* 2015). Protein utilization can also be affected by digestible enzymes (Tu *et al.,* 2015), therefore, acidic and alkaline proteases activities were considered to have more accurate information on that ratio of dietary phe: tyr which maximizes protein utilization.

Blood is one of the most dynamic body tissues, and it is capable of reflecting changes in physiological and nutritional health status (Kader *et al.,* 2012). It has also been proposed that nutritionally deficient diets cause decrease in haemoglobin concentration, reduced haematocrit and red blood cell counts (Saha & Ratha, 2007), and the erythrocytes counts are one variable that is most consistently affected by dietary nutrients. For this reason, physiological responses were taken into consideration to accurately determine phe:tyr ratio of *H. fossilis* fry.

Stinging catfish, Heteropneustes fossilis (Bloch) commonly known as Singhi is a *Silurid* fish, found abundantly in most of the tropical and sub-tropical regions and is a highly-prized fish in most of the Asian markets. The fish is an ideal species for aquaculture due to its being well adapted to derelict and stagnant, slow-flowing water bodies, agricultural fields or swamps and wetlands, and exceptional tolerance to high ammonia and low oxygen (Saha & Ratha, 2007). Being a lean fish, it is very suitable for people to whom animal fats are undesirable (Saha & Guha, 1939; Bhatt, 1968; Rahman *et al.,* 1982). These characteristics make this fish advantageous for aquaculture.

However, due to lack of information on most of its nutrient requirements, farmers mostly depend on personal experience and feeding standards of other catfish species which hinders yield of this medicinally important food fish. Information on complete ten

dietary essential amino acid needs are available only for a limited number of cultured species (NRC, 2011) and to our knowledge no published study is yet available on optimum dietary phe: tyr ratio for any stage of this fish concerning growth performance, changes in and metabolic and physiological variables, and digestible enzyme activities.

Data generated in this study would be of high significance in formulating cost-effective practical feeds with optimum ratio of phe: tyr, for fry stage of this fish.

Materials and Methods

Preparation of Amino Acid Test Diets

Casein-gelatin based basal diet (Table 1) was supplemented with varying levels of phenylalanine and tyrosine to prepare six isonitrogenous (380 g/kg crude protein; CP) and isoenergetic (17.7 kJ/g gross energy; GE, 15.3 kJ/g digestible energy; DE) amino acid test diets containing total phenylalanine + tyrosine in diets at 26.6 g/kg dry diet. Diets with various ratios of phenylalanine: tyrosine (30:70, 40:60, 50:50, 60:40, 70:30, 80:20) were then prepared to have phenylalanine and tyrosine as 7.3 g/kg+16.96 g/kg (A_1), 9.69 g/kg+14.54 g/kg (A_2), 12,12 g/kg+12.12 g/kg (A_3),14.54 g/kg+9.69 g/kg (A_4), 16.96 g/kg+7.3 g/kg (A_5) and 19.39 g/kg+4.85 g/kg (A_6) in each diets on a molar basis as per their calculated amounts. This was done to work out that specific combination of phe: tyr where tyrosine could maximally be included with phenylalanine without any adverse health effects on fish so as reduce the dependence on this expensive nutrient phenylalanine and save diet cost. Amount of dietary total phenylalanine and tyrosine were fixed on the basis of a preliminary trial on this fish conducted under almost similar conditions and also the information available on other warmwater catfish species (Robinson *et al.,* 1980; Unprasert, 1994; Ahmed, 2009; Farhat, 2014; Ren *et al.,* 2015; Castillo *et al.,* 2015). Crystalline L-amino acid mixtures were prepared taking into account the amount of amino acids contributed by casein and gelatin. Total phenylalanine and tyrosine contents contributed by the intact protein sources casein and gelatin were 4.91 g/kg and 4.61 g/kg of the dry diet, respectively. The overall composition of amino acids in the test diets was simulated to that of 380 g/kg whole chicken egg protein excluding the test amino acids phenylalanine and tyrosine. To make the diets isonitrogenous, amount of glycine on protein to protein basis was replaced by the incremental levels of phenylalanine in the diets. Glycine was used as dietary isonitrogenous control because glycine has dietary attractant properties, gives natural smell to the diet and hence stimulates the olfactory bulb of fish (Polat & Beklevik, 1999). Since amino acid test diets are not very tasty for fish, glycine was used to increase and promote quicker feed intake hence minimize the time feed remains in water and thereby to minimize nutrient leaching.

Final diet with bread dough consistency (320 g/kg moisture) was poured into a Teflon-coated pan, cut in the form of small cubes and stored at -20°C until fed. Since *H. fossilis* has high acceptance for diets as semi-moist cakes as has been exhibited by preliminary trials, the diets were prepared in semi-moist form. The ingredients and proximate composition and analyzed amino acid composition of diets have been given in Table 1 and Table 2, respectively.

Table 1. Ingredients used to prepare amino acid test diets

Ingredients (g/kg)	(A_1)	(A_2)	(A_3)	(A_4)	(A_5)	(A_6)	(A_1)
Casein*	90	90	90	90	90	90	90
Gelatin†	22.5	22.5	22.5	22.5	22.5	22.5	22.5
Amino acid mix (g/kg dry diet)**	205.29	205.29	205.29	205.29	205.29	205.29	205.29
Total phenylalanine (g/kg dry diet)**	2.4	4.97	7.22	9.64	12.06	14.49	2.4
Total tyrosine (g/kg dry diet)**	12.35	9.93	7.51	5.08	2.69	2.69	12.35
Total glycine (g/kg dry diet)**	84.50	84.40	84.40	84.30	84.45	84.32	84.50
Dextrin	336.5	336.6	336.7	336.9	337.0	334.9	336.5
Corn oil	50	50	50	50	50	50	50
Cod liver oil	20	20	20	20	20	20	20
Mineral mix§‖	40	40	40	40	40	40	40
Vitamin mix¶‖	30	30	30	30	30	30	30
α- Cellulose	6.5	6.4	6.4	6.3	6.3	6.0	6.5
Carboxymethyl cellulose	100	100	100	100	100	100	100
Total	1000	1000	1000	1000	1000	1000	1000
Total crude protein	380	380	380	380	380	380	380
Gross energy (kJ/g; GE)	17.7	17.7	17.7	17.7	17.7	17.7	17.7
Digestible energy (kJ/g; DE)	15.2	15.2	15.2	15.2	15.2	15.2	15.2

*Crude Protein (760 g/kg).

†Crude Protein (960 g/kg).

§Mineral mixture (40 g/kg of diet) calcium biphosphate Ca (H_2PO_4)$_2$. 4H_2O 5.43; calcium lactate ([$CH_3CH(OH)COO]_2Ca$) 13.1; ferric citrate 1.19; magnesium sulphate ($MgSO_4.7H_2O$) 5.28; potassium phosphate (dibasic) 9.59; sodium biphosphate 3.48; sodium chloride 1.74; aluminium chloride. 6H_2O0.006; potassium iodide 0.006; cupric chloride 0.004; manganese sulphate monohydrate ($MnSO_4$. H_2O) 0.032; cobalt chloride. 6H_2O0.04; zinc sulphate ($ZnSO_4$. 7H_2O) 0.12; Loba Chemie, Mumbai, India.Halver (2002).

¶Vitamin mixture (30 g/kg of diet) (10g vitamin mix+20g œ-cellulose) choline chloride 5.00; inositol 2.00; ascorbic acid 1.00; niacin 0.75; calcium pantothenate 0.5; riboflavin 0.2; menadione 0.04; pyridoxine hydrochloride 0.05; thiamin hydrochloride 0.05; folic acid 0.015; biotin 0.005; alpha-tocopherol 0.4; vitamin B_{12} 0.0001; Loba Chemie, Mumbai, India.

**L-crystalline amino acids supplemented to intact protein sources casein and gelatin to make the desired doses of the test amino acids

Water Stability of Amino Acid Test Diets

Water stability of amino acid test diets was assessed with slight modification of Fagbenro & Jauncey (1995). Replicate samples of the diets (4g) were taken in an empty tea bag and suspended randomly in experimental troughs (70-litre, water volume 55-litre) at 26°C. The samples were remained suspended in water for 30 minutes, then taken out, allowed to drain for 1 minute, oven-dried at 105°C for 2 hours, cooled in a desiccator and reweighed. Water stability of the experimental diets was calculated as the percentage difference in sample weight after re-weighing (Farhat, 2014) and was found to be 97%. The dried samples after immersion in water were subjected to amino acid analyses to check the leaching loss. Amino acid analysis revealed no significant change in amino acid composition of the test diets after 30 minutes of stability test (Table 3).

Fish Rearing and Acclimation

Induced bred fry of *Heteropneustes fossilis* were obtained from the Ghazipur fish hatchery (New Delhi, India), transported to the wet laboratory (Department of Zoology, Kashmir University, J&K, India), given a prophylactic dip treatment in mild $KMnO_4$ solution (1:3000; Loba Chemie, Mumbai, India) and stocked in indoor plastic syntax circular tanks (water volume 600liter; Plastic Crafts Corp, Mumbai, India) so that the fish get acclimatized to laboratory conditions. Throughout this period, their feeding behavior and feed intake were carefully observed. Apparent satiety was ensured simply by visual observation and the fish were carefully observed during feeding to ensure satiety without overfeeding (Farhat, 2014). The diet was fed till the fish actively consumed at each feeding. Fish were fed thrice daily at 08:00, 12:00 and 17:00 hours on casein-gelatin based (380 g/kg crude protein) H-440 (2002) diet as soft cake for two weeks.

Experimental Design and Weekly Measurements

A completely randomized design with six treatments and four replicates/treatment were used (4.1±0.3g, n=4,) in which each tank represented as one experimental unit. Fish were stocked at the rate of 20 fish per trough (70-liter circular polyvinyl troughs, water volume 55 liter, flow-through rate (1-1.5 l/min) system for each dietary treatment level. Fishes were fed test diets in the form of semi-moist balls (320 g/kg moisture; 5 mm in diameter) to apparent satiation thrice daily at 08:00, 12:00 and 17:00 hours. During feeding, consumption of the diet was particularly watched. Tanks were cleaned daily by siphon before dispensing the feed and after every active feeding whereas weekly cleaning of tanks were done during the experimental periods. Feed allocation was done till ad libitum and hence, left-over feed was not found in any of culture tank. The experiment has lasted for 12 weeks. On the day of

Table 2. Amino acid composition of reference protein and the amino acid test diets

Proximate composition of the amino acid test diets (g/kg)							
		(A_1)	(A_2)	(A_3)	(A_4)	(A_5)	(A_6)
Protein		380.3	380.5	380.4	380.2	380.1	379.8
Crude fat		6.99	6.95	6.98	6.99	7.11	7.19
Dry matter		76.4	76.9	76.5	76.3	76.2	76.1
Gross energy (kJ/g)		17.75	17.71	17.74	17.77	17.79	17.76
Essential Amino Acid Profile of the Test diets							
***EAA (g/kg)**	**[$]380 g/kg WCE protein**	(A_1)	(A_2)	(A_3)	(A_4)	(A_5)	(A_6)
Arginine	24.45	24.41	24.43	24.39	24.45	24.43	24.44
Histidine	7.99	7.97	7.99	7.96	7.98	7.99	7.99
Isoleucine	30.4	30.4	30.1	30.5	30.4	30.2	30.3
Leucine	34.95	34.91	34.99	34.97	34.95	34.96	34.94
Lysine	27.35	27.33	27.34	27.35	27.34	27.32	27.33
Methionine	15.66	15.68	15.65	15.63	15.61	15.65	15.63
**Phenylalanine	23.94	7.31	9.68	12.14	14.55	16.99	19.37
Threonine	16.43	16.45	16.47	16.45	16.48	16.45	16.44
Tryptophan	5.7	5.72	5.71	5.71	5.73	5.71	5.72
Valine	27.74	27.74	27.73	27.75	27.74	27.76	27.73
[#]NEAA							
Cystine	9.3	8.1	8.9	8.5	9.1	8.9	8.8
**Tyrosine	17.3	16.97	14.56	12.12	9.71	7.32	4.87
Alanine	21.85	21.86	21.84	21.87	21.81	21.85	21.83
Aspartic acid	13.11	13.11	13.11	13.12	13.11	13.13	13.15
Glutamic acid	26.86	26.88	26.89	26.87	26.83	26.85	26.88
Proline	31.7	31.8	31.6	31.7	31.8	31.9	31.8
Serine	6.53	6.6	6.7	6.9	6.4	6.5	6.7
Glycine	44.67	84.55	84.49	84.43	84.29	84.35	84.41

*Essential amino acids

**Dietary amino acids were mimicked as per the amino acid profile of whole chicken egg protein excepting phenylalanine and tyrosine which were fixed as per their calculated doses

[#]Non-essential amino acids

[$]Whole-chicken egg protein

weekly measurements, fish were fasted for 24 hours to empty their guts and their bulk weight recorded on a top-loading balance (Sartorus CPA- 224S 0.1 mg sensitivity, Goettingen, Germany) for calculating growth parameters. To avoid stress, above measurements were taken under moderate anesthesia (100 mg/L MS-222, Sigma, St Louis, MO, USA). Fish were treated with mild $KMnO_4$ solution (1:3000; Loba Chemie, Mumbai, India) before each weighing to prevent handling infection.

Water Quality Analysis

Water was sampled from each trough to determine water temperature, dissolved oxygen, free carbon dioxide, total alkalinity, TAN, nitrite and pH based on daily measurements as per the standard methods (APHA, 1992). The pH was determined by using digital pH meter (pH ep-HI 98107, USA). Fish were kept under a natural photoperiod of 12 hours light:12 hours dark. The water temperature was maintained at 26°C which was reported optimum for this fish by Fatma & Ahmed (2020).

Collection of Fish Sample

At the beginning and end of the 12 week feeding trial, fish were anaesthetized by moderate dose of 100 mg/L MS-222 (Flecknell, 2016), Sigma, St. Louis, MO, USA and killed by freezing at -20°C for whole body proximate analysis. Pooled sample of 30 fishes was taken from the initial stock and analyzed for initial body composition. There were 4 tanks per treatments. At the end of the experiment, six fish were taken from each tank, group pulverized and then 3 sub-samples from this ground up group of 6 fish were taken (n=4×3) for body composition analyses (Abidi & Khan, 2007; Abidi & Khan, 2012; Fatma & Ahmed, 2020). Before every sampling fish were starved for 24 hours.

Proximate and Gross Energy Density Analysis of Test Diets and Fish

Proximate composition of casein, gelatin, experimental diets, and initial and final whole body was estimated using standard methods (AOAC, 1995). Dry matter of the samples was determined by oven drying at 105±1ºC for 24 hours, crude protein using macro-kjeldhal method (N×6.25). Dried samples were digested in sulphuric acid (12 ml) at high temperature (420°C) in the presence of potassium sulphate and copper sulphate as catalysts (Kjeltec 8400, FOSS Denmark), crude lipid by solvent extraction with petroleum ether (B.P. 40-60ºC) for 2-4 hours (FOSS Avanti automatic 2050, Sweden) and the ash content was determined by incinerating 2 g of dried samples in a Muffle Furnace at 650ºC for 2-3 hours.

Gross energy content of the amino acid test diets was calculated on the basis of fuel values 23, 20.19, 24.24, 16 and 37.6 kJ/g for casein, gelatin, amino acids, dextrin and fat anddetermined using a bomb calorimeter (USA) in a professional laboratory at Delhi, India. The analysis revealed a close agreement with the calculated values of the gross energy (Table 1).

Amino Acid Analysis of Experimental Diets and Fish Samples

Samples of amino acid test diets, intact protein sources, reference protein (380 g/kg whole chicken egg protein) and initial and final fish body were prepared as

Table 3. Amino Acid Analyses of experimental diets after leaching test.

***EAA (g/kg)**	(A_1)	(A_2)	(A_3)	(A_4)	(A_5)	(A_6)
Arginine	24.11	24.41	24.34	24.44	24.45	24.41
Histidine	7.65	7.95	7.95	7.95	7.96	7.94
Isoleucine	30.23	30.0	30.49	30.31	30.23	30.29
Leucine	34.92	34.95	34.93	34.93	34.95	34.93
Lysine	27.32	27.32	27.34	27.31	27.31	27.32
Methionine	15.63	15.63	15.61	15.57	15.65	15.62
Phenylalanine	7.30	9.63	12.11	14.53	16.97	19.34
Threonine	16.44	16.44	16.43	16.47	16.47	16.41
Tryptophan	5.71	5.67	5.69	5.71	5.73	5.71
Valine	27.71	27.71	27.74	27.72	27.73	27.72
NEAA						
Cystine	8.0	8.89	8.49	9.2	8.92	8.78
Tyrosine	16.94	14.54	12.11	9.73	7.31	4.82
Alanine	21.83	21.82	21.84	21.82	21.84	21.81
Aspartic acid	13.10	13.01	13.11	13.08	13.11	13.14
Glutamic acid	26.81	26.82	26.85	26.81	26.83	26.83
Proline	31.79	31.59	31.69	31.76	31.87	31.78
Serine	6.4	6.69	6.87	6.34	6.49	6.69
Glycine	84.47	84.44	84.39	84.33	84.39	84.29

*Essential amino acids

**Dietary amino acids were mimicked as per the amino acid profile of whole chicken egg protein excepting phenylalanine and tyrosine which were fixed as per their calculated doses

#Non-essential amino acids

$Whole-chicken egg protein

per Abidi, & Khan (2012) for analyses of amino acid contents. After preparation, the samples were sent to a professional lab for the analyses. Briefly, 0.4mg sample was hydrolyzed in 1ml of 6N HCl for nearly 22 hours at 110°C. Samples thus obtained were diluted in HCl (0.02N). The hydrolyzed samples were filtered using microfilter (0.45 micron, Cellulose acetate membrane, Corning, Tokyo, Japan). For analysis of tryptophan, recovery hydrolysis was performed in 4N methanesulfonic acid followed by decomposition at 110°C for 22 hours. Samples were then diluted in 4N NaOH to adjust pH at 2 followed by another dilution in 0.02N HCl. Recovery hydrolysis of methionine and cystine was done in 2ml of performic acid for 4-24 hours. The samples thus prepared were then sent to a professional laboratory for amino acid analyses. The results of the amino acid analyses of whole chicken egg protein and amino acid test diets are given in Table 2.

Estimation of Nucleic Acids

After the termination of feeding trial, 5 fish from each replicate of the treatment groups (n=20) were randomly euthanized with MS-222 (100 mg/L) and white muscle tissues were removed. Quadruplicate tissue samples from each replicate of the treatment group (n=4) were taken for the determination of RNA and DNA. Nucleic acids and RNA/DNA ratio were determined as per Schmidt & Thannhauser (1945). The amount of RNA and DNA were expressed as mg/g.

RNA

RNA concentrations were determined using orcinol method as per Mejbaum (1939). White muscle tissue (250 mg) from each replicate was placed in centrifuge tubes followed by addition of 4 ml cold 0.22 M per-chloric acid (PCA). The contents were then stored at 4ºC for 15 minutes and centrifuged at 3000 *g* (4ºC) for 15 minutes. Supernatant was discarded and precipitate was washed twice with PCA as per above procedure. Resulting precipitate was dissolved in 4 ml potassium hydroxide (0.3 N) while incubating in hot waterbath for 2 hours at 38ºC. Standard using purified yeast RNA (Sigma Aldrich) was prepared in similar manner. Samples and standard were then cooled, 1 ml PCA added and centrifuged. RNA was determined by addition of 2 ml orcinol reagent to 1 ml supernatant, the mixture were then incubated in a hot water bath at 98ºC for 20 minutes. Intensity of the color developed was read at 665 nm after adjusting the blank (Spectrophotometer, Genesys 10-UV, Thermo Spectronic, Madison, WI, USA). The quantity was expressed as µg/100mg.

DNA

DNA concentrations were determined using a dual wavelength method of Wilder & Stanley (1983). Samples of white muscle tissue from each replicate weighing 50 mg and standard calf thymus DNA (Sigma Aldrich) were placed in centrifuge tubes to which 5 ml PCA (0.5N) were added; tubes were then heated at 70°C for 30 minutes. After cooling and centrifugation at 3000 g for 15 minutes, absorbance of the samples was measured at 232 and 260 nm. Quantity of DNA was expressed as µg/100mg.

Collection of Blood and Serum

At the end of the 12-week feeding trial, fish were fasted for 24 h immediately prior to blood sampling. Five fish from each replicate of the treatment were anesthetized with tricaine methanesulfonate (MS-222, 40 mg/L), and blood was collected from the caudal puncture. At the end of the feeding trial, fish were starved for 24 hours and were randomly tranquilized with 50 mg/L solution of MS-222 for collection of blood. Before taking blood, the caudal region was wiped with blotting paper to avoid handling contamination with mucus. Blood samples were collected by tail vein puncture using heparinized syringes. The samples were placed in microcentrifuge tubes for centrifuging (1000 *g*, 10 min at room temperature). The collected blood was divided into two part of Eppendorf tubes. One set of blood was transferred in vials containing heparin and shaken gently to allow thorough mixing of its contents which was used for analyses of blood parameters as erythrocyte counts, Heamoglobin contents (Hb), Heamatocrit (Hct%), Erythrocytes sedimentation rate (ESR) and Erythrocyte osmotic fragility (EOF). The second set, without an anticoagulant, was left to clot at 4°C and centrifuged at 5,000 × g for 15 min at room temperature. The collected sera were stored at −20°C for further assays.

Serum Biochemistry

Activities of alanine aminotransferase (ALT; IU/L) and aspartate aminotransferase (AST; IU/L) in serum were analyzed using an automatic biochemical analyzer (VetScan VS2, Abaxis, Union City, CA) as per manufacturer's protocol. Briefly, immediately after collection of sample in heparinized micropipette, 100 µL blood was dispensed into the reagent rotor and then the equipment after automatic analysis generates the results. Enzyme activity was expressed as IU/L. All analyses were performed in quadruplicate.

Digestive Enzymatic Activities

To analyze the digestive enzymes, alimentary tract was dissected to remove adherent adipose and connective tissues. Digestive tracts including stomach and intestines of 4 fish per replicate of the treatment group (n = 16) were then weighed, and homogenized in buffer solution containing phosphate (10mM) and Tris (20mM), pH 7.0 in a homogenizer. The homogenates

were centrifuged at 1000 × g for 10 min and the supernatant (crude extract) was used as enzyme source for all enzymatic assays. Acidic protease activity was measured in the stomach using casein as substrate according to the method of Hidalgo *et al.* (1999). The assay was performed using 0.2 M KCl buffer at pH 1.8, and samples were incubated at 30°C for 40 minutes. The reaction was terminated with 15% TCA, and absorbance was recorded at 280 nm. The activity of alkaline protease was determined in the intestine as per Hummel (1959). Tyrosine was used as a standard. The enzymatic activity was defined as the amount of enzyme needed to catalyze the formation of 1 mg tyrosine/min. Intestinal amylase activity was determined spectrophotometrically at 660 nm as per Silva *et al.,* (2020).

Liver Glycogen and Blood Glucose

Determination of liver glycogen was performed according Bidinotto *et al.,* (1997), with spectrophotometer readings at 480 nm absorbance. Blood glucose was recorded using a digital glucometer.

Physiological Assay

Physiological responses such as erythrocyte counts× 10^9/ml, hemoglobin content (Hb g/dl), hematocrit value (Hct %) and the erythrocyte sedimentation rate (ESR mm/hour) were determined as has been done earlier (Fatma & Ahmed, 2020). Briefly, erythrocyte counts was determined by an improved Neubauer hematocytometer with Yokoyama (1977) solution as the diluting medium, haemoglobin (Hb g/dL) was estimated colorimetrically following Wong's (1928) method and Haematocrit value (Hct%) was measured by spinning the micro-wintrobe tube containing well mixed blood for about 5 min at 12000 *g*. For the determination of erythrocyte sedimentation rate (ESR), 2 mL blood was drawn into a Westurgen tube. The tubes were placed vertically and left undisturbed for 60 min, after which time, the level of the column of sediment was noted as ESR. Erythrocyte osmotic fragility was assessed in terms of percentage haemolysis using gradient hypotonic salt solution as per Ezell *et al.,* (1969). Percentage haemolysis was calculated by reading the optical density of supernatant against salt concentrations at 540 nm using spectrophotometer (Thermo Spectronic, Genesys-10S, Rochester, New York, USA). All hematological determinations were carried out in quadruplicates with 5 fish per replicate (n=5x4=20) for each treatment. The study was approved by Animal Ethical Committee registered under R.No. 801/Go/RE/S/2003/CPCSEA.

Calculations

Following growth parameters were evaluated to assess the performance of test diets:

Daily growth coefficient (DGC%)= (Final wt)$^{1/3}$-(Initial wt)$^{1/3}$/No of days x 100

Specific Growth rate (SGR%/day) = (ln) final body wt.-(ln) initial body wt./No. of days x 100

Feed conversion ratio (FCR; Dry basis) = Feed consumption/weight gain

Survival Rate (SR %) = Final number of fish/Initial number of fish × 100

Feed intake (g/fish) = Total dry feed consumes/No. of fish

Protein deposition (PD %) = Protein gain (g)/Protein fed (g) × 100

Statistical Analysis

All growth data were subjected to one-way analysis of variance (Sokal & Rohlf, 1981). Optimum dietary phenylalanine and tyrosine ratio was determined according to the method of Baker *et al.,* (2002) and Parr *et al.,* (2003) in which the broken-line and quadratic analyses are superimposed and the requirement is determined by establishing the point where the quadratic curve first intersected the plateau of broken-line. Differences among the treatment means were determined by Tukey's significant difference test at a $P<0.05$ level of significance (Tukey, 1953). Statistical analysis was done using Origin (version 8.1; Origin Software, San Clemente, CA). All the analyses were done in quadruplicates (n=4).

Results

Growth Performance

Growth performance of fish fed varying ratios of dietary phenylalanine and tyrosine are summarized in Table 4 and presented as Figure 1 and 2. Fish showed almost uniform growth in all the diets during the first week of feeding trial. The weight gain differences become significantly apparent ($P<0.05$) after the first week of the feeding trial. Fish fed varying ratios of phenylalanine: tyrosine in diets A_1, A_2 and A_3 exhibited reduced growth and anorexia. No other obvious deficiency signs or any external pathology were noted in groups fed below 60:40 ratio of dietary phenylalanine: tyrosine. At the end of the experiment, fish receiving phenylalanine and tyrosine at a ratio of 60:40 in diet A_4 had significantly higher ($P<0.05$) daily growth Coefficient% (DGC 5.18%), specific growth rate (SGR; 1.92%/day), best feed conversion ratio (FCR; 1.59) and protein deposition (PD; 28.6%). However, a significant decline ($P<0.05$) in above parameters was recorded at still higher phe: tyr ratio in diets A_5 (70:30) and A_6 (80:20). Reduction in feed intake were apparent in

groups fed diets A_5 and A_6 indicating that fingerling *H. fossilis* could not go easy with higher levels of dietary phenylalanine in diets with higher ratios of phenylalanine: tyrosine.

The results clearly indicated thatdietary phenylalanine and tyrosine together in a preferred combination at 60:40 ratio is necessary.

RNA/DNA Ratio

RNA/DNA ratio also increased significantly (P<0.05) with the increase in dietary phenylalanine: tyrosine ratio up to 60:40 (14.54 g/kg phe: 9.69 g/kg tyr dry diet) indicating maximum protein synthesis at this dietary ratio. However, those fed phenylalanine below and beyond 60:40 ratio had significantly lower values for RNA/DNA ratio and hence decreased growth rate. Data pertaining to this have been provided in Table 5.

Whole Body Composition

Body protein significantly improved and best values were obtained for the groups fed with phenylalanine and tyrosine at 60:40 ratio (A_4), however, a significant decline (P<0.05) in the body protein was noted for the groups receiving diets beyond this ratio at 70:30 (A_5) and 80:20 (A_6) indicating that protein synthesis rate remained at highest levelsat60:40 ratio of phe:tyr. Body moisture content remained almost unchanged (P<0.05) with the increasing dietary

Table 4 Growth, conversion efficiencies and protein deposition of fry *H. fossilis*.

Dietary phenylalanine: tyrosine ratio (g/kg dry diet)						
	30 :70 (A_1)	40 :60 (A_2)	50 :50 (A_3)	60 :40 (A_4)	70 :30 (A_5)	80 :20 (A_6)
Average initial wt. (g)	3.9^{a}±0.2	3.8^{a}±0.1	4.0^{a}±0.1	3.8^{a}±0.2	3.9^{a}±0.3	4.1^{a}±0.2
Average final wt. (g)	8.8^{e}±0.3	11.3^{d}±0.2	13.9^{c}±0.3	15.1^{a}±0.1	14.5^{b}±0.2	13.1^{c}±0.1
DGC%	2.26±0.05	3.43^{e}±0.12	4.58^{c}±0.02	5.18^{a}±0.11	4.91^{b}±0.03	4.17^{d}±0.11
SGR%/day	1.14±0.05	1.51^{e}±0.02	1.72^{c}±0.03	1.92^{a}±0.02	1.82^{b}±0.04	1.61^{d}±0.03
FCR[1,2]	2.64^{a}±0.01	2.23^{b}±0.02	1.88^{c}±0.01	1.59^{f}±0.01	1.65^{e}±0.02	1.69^{d}±0.03
FI (g/fish) [1,2]	12.94^{f}±0.02	16.73^{d}±0.04	18.61^{c}±0.03	17.98^{a}±0.05	17.49^{b}±0.03	15.21^{e}±0.04
PD%[1,2]	8.8^{e}±0.3	12.9^{d}±0.2	18.3^{c}±0.1	28.6^{a}±0.4	23.3^{b}±0.3	18.9^{c}±0.2

[1,2]Mean values of 4 replicates ±SEM. Mean values with the same superscripts in a row are insignificantly different (P>0.05).
DGC% = Daily growth coefficient%
SGR%/day = Specific growth rate
FI (g/fish) = Feed intake
FCR = Feed conversion ratio
PD% = Protein deposition

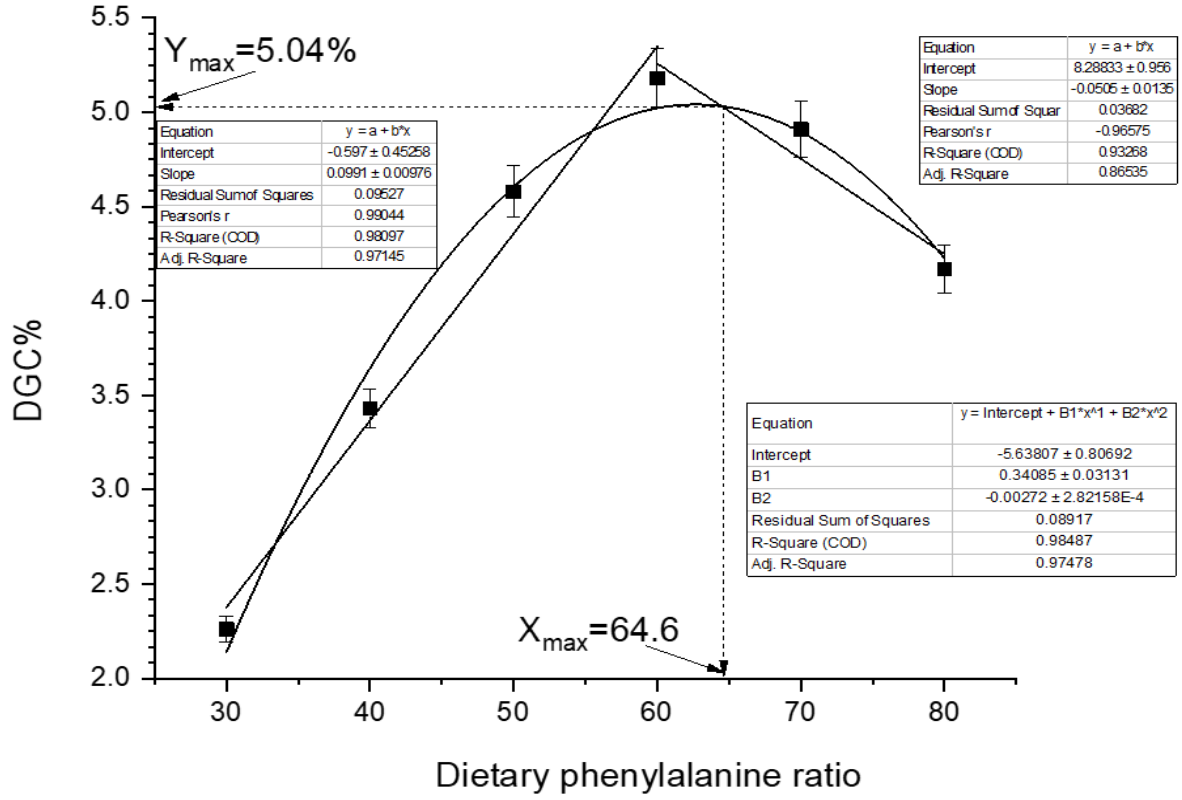

Figure 1. Fitted plot of DGC% vs. dietary phenylalanine ratios. Each point represents the means of four replicates per treatment (n=4) with 20 fishes per replicate.

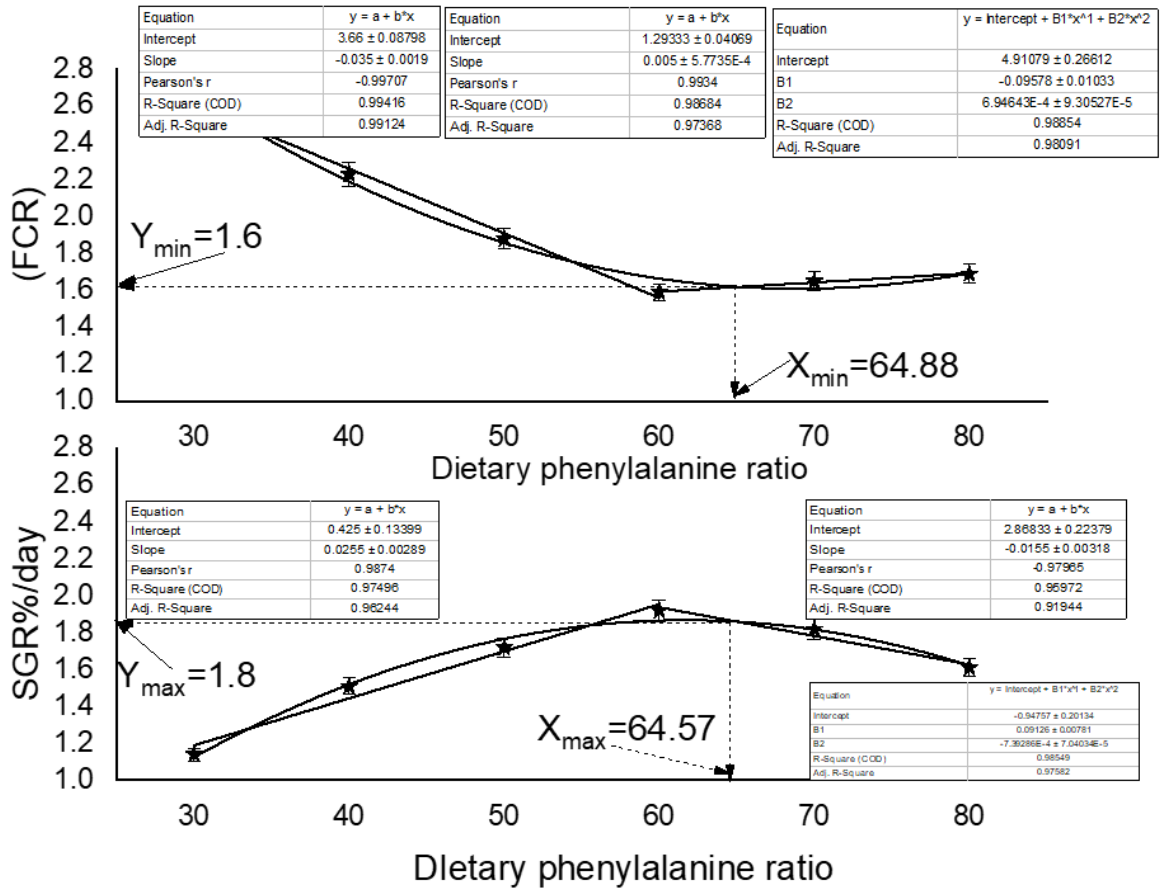

Figure 2. Fitted plot of SGR%/day and FCR vs. dietary phenylalanine ratios. Each point represents the means of four replicates per treatment (n=4) with 20 fishes per replicate.

Table 5. Body composition, nucleic acid indices digestive enzyme activities, liver glycogen and blood glucose of *H. fossilis* fry.

		Dietary phenylalanine: tyrosine ratio (g/kg dry diet)					
g/kg (wet wt. basis)	Initial	30 :70 (A_1)	40 :60 (A_2)	50 :50 (A_3)	60 :40 (A_4)	70 :30 (A_5)	80 :20 (A_6)
Moisture	762.1±0.4	767.1^{a}±0.2	766.3^{a}±0.1	764.7^{a}±0.1	763.1^{a}±0.3	754.9^{b}±0.2	750.9^{c}±0.4
Protein	157.4±1	119.1^{f}±2	128.0^{e}±3	139.0^{c}±2	169.1^{a}±1	149.2^{b}±3	133.1^{d}±1
Fat	31.1±0.2	32.3^{e}±3	34.7^{d}±4	35.9^{c}±2	36.3^{c}±3	38.9^{b}±2	41.9^{a}±3
Ash	29.9±0.4	32.5^{c}±0.2	31.8^{d}±0.2	32.1^{c}±0.3	31.4^{c}±0.1	33.7^{b}±0.4	35.1^{a}±0.2
RNA (mg/g)	-	1.4^{e}±3	1.7^{d}±2	2.0^{b}±3	2.3^{a}±4	2.1^{b}±3	1.8^{c}±2
DNA (mg/g)	-	0.45^{a}±2	0.43^{b}±3	0.41^{c}±4	0.43^{b}±3	0.44^{a}±1	0.41^{c}±4
RNA/DNA ratio	-	3.13^{f}±0.11	3.96^{e}±0.13	4.86^{b}±0.02	5.23^{a}±0.11	4.79^{c}±0.12	4.46^{d}±0.22
Amylase*	-	5.02^{e}±0.13	5.33^{d}±0.11	5.55^{c}±0.13	5.62^{c}±0.14	5.96^{b}±0.11	6.19^{a}±0.11
Acidic protease**	-	14.2±0.1	20.4±0.3	31.9±0.1	43.5±0.2	36.7±0.3	30.4±0.3
Alkaline protease***	-	5.08^{f}±0.21	5.41^{e}±0.51	5.58^{b}±0.31	5.71^{a}±0.21	5.49^{c}±0.41	5.31^{d}±0.21
Glycogen	-	14.44^{d}±0.22	14.59^{c}±0.14	14.61^{c}±0.13	14.59^{c}±0.14	17.66^{b}±0.12	18.25^{d}±0.23
Glucose	-	65.81^{e}±0.01	69.14^{d}±0.03	75.09^{c}±0.08	79.95^{c}±0.05	94.91^{b}±0.01	98.89^{a}±0.04

Mean values of 4 replicates ±SEM. Mean values with the same superscripts in a row are insignificantly different (P>0.05). Liver glycogen is expressed as µ moles glucose g/tissue; Blood glucose is expressed as mg/dL,*1µmol glucose/min/mg protein,**mg casein hydrolyzed/min/mg protein, ***1 mg tyrosine/min/mg protein

phenylalanine: tyrosine ratio up to 50:50 (A_4) and, thereafter (70:30; A_5 and 80:20; A_6), a significant decrease (P<0.05) in the moisture content was noted. Body fat a positive correlation with the increasing ratio of dietaryphenylalanine: tyrosine. However, the body ash showed insignificant (P>0.05) change in fish fed diets containing different ratio of phenylalanine and tyrosine (Table 5).

Serum Biochemistry (ALT and AST activities)

Data of serum biochemistry have been detailed in Table 6. Fish fed phe:tyr ratio below 60:40 in diet A_4 had significant reduction in serum ALT and AST activities which may probably be due to balanced dietary proportion of phe and tyrosine at 60:40 ratio indicating that the metabolism geared up for sparing the amino

acid to be used as an energy source. The mathematical analysis of AST and ALT activities confirmed that at 60:40 ratio of phenylalanine: tyrosine, amino acids were more being utilized for anabolic purposes (Table 6).

Digestive Enzyme Activities

Activities of acidic and alkaline proteases were significantly ($P<0.05$) lower in the A_1, A_2 and A_3 groups, indicating role of phenylalanine in synthesis and secretion of these enzymes in fish. The activity of acidic protease enzyme increased in groups fed phe:tyr ratio up to 60:40 in diet A_4 where phenylalanine and tyrosinewere at 15.9 g/kg and 10.6 g/kg of the diets indicating that balance proportion of phenylalanine: tyrosine is necessary for synthesis and secretion of these enzymes.

Higher ratios of phenylalanine in diets A_5 and A_6 (16.96 g/kg and 19.39 g/kg diet) did not result a proportional increase in the activity of this enzyme. Amylase activity in fish of diets A_5 and A_6 was higher ($P<0.05$) than those fed other diets (Table 5). It is well known that increase in substrate concentrations, increases enzyme activities. Higher amylase activities in fish fed diets A_5 and A_6 may probably be due to the reasons that fish fed these diets converted excess phenylalanine to glucose which were available as substrate for increase in amylase activities in fish fed dietary phenylalanine: tyrosine at ratios higher than 60:40

Blood Glucose and Liver Glycogen

A significant effect ($P<0.05$) of dietary ratios of phenylalanine and tyrosine inclusion was recorded on liver glycogen and blood glucose in groups fed ratios of phe: tyr beyond 60:40 in diets A_5 and A_6. Fish fed these diets with higher proportions of phenylalanine had significantly higher liver glycogen and blood glucose levels than those fed other diets (Table 5).

Effects of Dietary Phe: Tyr on Physiological Responses

Theerythrocytescounts (Table 6) and Hb contents were significantly ($P<0.05$) lower for the groups fed diets A_1 and A_2 where phe: tyr ratios were 30:70 and 40:60indicating that these diets become deficient in phenylalanine at higher ratios of tyrosine. However, above hematological parameters tended to be at their best values in groups fed phe: tyr at ratio of 60:40 indicating that phenylalanine: tyrosine ratio up to 60:40 is feasible for this fish. However further increase in ratio of phenylalanine: tyrosine in diets A_5 and A_6 resulted in sharp decline in these physiological responses.

Erythrocyte Osmotic Fragility (EOF)

Erythrocyte osmotic fragility in terms of % haemolysis was found to increase from diet A_1 to A_4. Groups fed diets A_5 and A_6 exhibited significant decrease ($P<0.05$) in erythrocyte fragility in hypotonic salt solution (Table 6, Figure 3).

Water Quality Parameters

The water quality parameters were measured three times daily at 07:00, 12:00 and 18:00 hours. The water temperature, dissolved oxygen, free carbon dioxide, total NH_3-N, pH, and total alkalinity were 26.2 ± 0.1 (C), 6.7 ± 0.4 (mg/mL), 6.3 ± 0.5 (mg/mL), 0.03 ± 0.01 (mg/mL), 7.2 ± 0.2 and 75.6 ± 0.4 (mg/mL), respectively. All water quality parameters were within the acceptable limits for fingerling *H. fossilis* during the length of the experiments.

Optimum Dietary Phenylalanine: Tyrosine Ratio

To generate precise information, DGC%, SGR%/day, FCR, and erythrocyte osmotic fragility% data were subjected to quadratic-broken-line regression analyses. The two models were superimposed and optimum dietary phe:tyr ratio was determined by establishing the point where the quadratic curve first intersected the plateau of broken-line and the average value was calculated to recommend the optimum phe:tyr ratio. The abscissa of intersection of the straight broken-line with a quadratic regression curve revealed optimum phe: tyr ratio for fry *H. fossilis* to be at64.94:35.06 corresponding to 15.83 g/kg: 8.55 g/kg on equimolar basis.

Discussion

A balanced dietary amino acid profile increases the amino acid retention and may improve growth and protein utilization (Aragão *et al.,* 2004). This study also showed that dietary phenylalanine: tyrosine ratio if not as per requirement, restricts growth and significantly influences carcass quality parameters in *H. fossilis*.

If phenylalanine is fed at optimal level in the diet but with inadequate level of dietary tyrosine, a portion of the phenylalanine gets converted to tyrosine thus becomes less than the optimum and hence adversely affects the growth performance and body composition. The conversion of phenylalanine to tyrosine in the absence or at low levels of dietary tyrosine is possible through the process of hydroxylation but the reverse reaction does not occur in animals (Martin *et al.,* 1993). Nose (1979) reported that the phenylalanine requirement of common carp was 65 g/kg in the absence of tyrosine but was reduced to 34% in the presence of 26% tyrosine. Growth retardation in terms of DGC, FCR, SGR and PD at phenylalanine and tyrosine ratios of 70:30 and 80:20 in diets A_5 and A_6 is due to the fact that in these diets phenylalanine was at excess (16.96 g/kg dry diet in A_5 and 19.39 g/kg dry diet in A_6) thus causing a significant growth retardation and inferior body composition due to combined effects of phenylalanine excesses and inadequate tyrosine

content in fish fed these diets. The reduction in growth at phe:tyr ratio of 80:20 (diet A_6) with still high level of dietary phenylalanine intake in fish fed diet A_6 could also be attributed to extra energy expenditure in nitrogenous excretion, because excess amino acids are deaminated and eliminated in the form of ammonia (Campbell, 1991) which requires energy and hence the diversions of energy from anabolic to catabolic processes at higher dietary phenylalanine intake with inadequate ratio of tyrosine might have resulted in growth retardation in this group. Therefore, optimum balance of both phenylalanine and tyrosine is needed in the diet. These results therefore, clearly indicate that dietary inclusion of phenylalanine and tyrosine in adequate combination (60:40) is necessary and that ratio of phenylalanine and tyrosine at 60:40 was permissible and hence best growth was recorded at this dietary combination of phenylalanine and tyrosine.

Carcass quality is the most important issue for aquaculturists as it influences the quality of the product and its acceptance by the consumer (Klanian & Alonso, 2015; Hamandishe *et al.,* 2018). Hence, efforts have to be made on means by which quality of fish may be improved. Since a major component in fish tissue is protein, priority has been given to increase the protein content of the fish tissue to improve the quality of the product. In this study, stinging catfish fed dietary phenylalanine and tyrosine at a ratio of 60:40 exhibited

Table 6. Serum biochemistry and heamatological parameters of *H. fossilis* fry.

g/kg (wet wt. basis)	Dietary phenylalanine: tyrosine ratio					
	30 :70 (A_1)	40 :60 (A_2)	50 :50 (A_3)	60 :40 (A_4)	70 :30 (A_5)	80 :20 (A_6)
AST (IU/L)	39^{b}±3	37^{c}±1	34^{e}±3	30^{f}±2	36^{d}±3	44^{a}±4
ALT (IU/L)	51^{b}±1	49^{c}±4	47^{e}±2	44^{f}±1	48^{d}±4	52^{a}±3
Erythrocyte counts (× 10^{9}/ml)	2.69^{f}±0.12	2.93^{e}±0.11	3.48^{b}±0.14	3.79^{a}±0.12	3.41^{c}±0.13	3.17^{d}±0.11
Hb (g/dL)	10.8^{d}±0.1	11.9^{c}±0.4	12.4^{b}±0.2	13.7^{a}±0.2	12.9^{b}±0.3	10.4^{d}±0.1
Hct%	26.4^{f}±0.3	28.3^{d}±0.2	30.2^{b}±0.1	32.5^{a}±0.2	29.1^{c}±0.4	27.9^{e}±0.3
ESR (mm/hour)	3.29^{a}±0.02	3.17^{b}±0.04	2.89^{d}±0.01	2.29^{e}±0.03	2.99^{c}±0.04	3.17^{b}±0.02
EOF (% Heamolysis)	78^{a}±	62^{b}±2	53^{c}±1	41^{e}±2	54^{c}±4	59^{d}±3

Mean values of 4 replicates ±SEM. Mean values with the same superscripts in a row are insignificantly different (P>0.05). Hb=Heamoglobin; Hct=Hematocrit; ESR=Erythrocyte sedimentation rate; EOF=Erythrocyte osmotic fragility

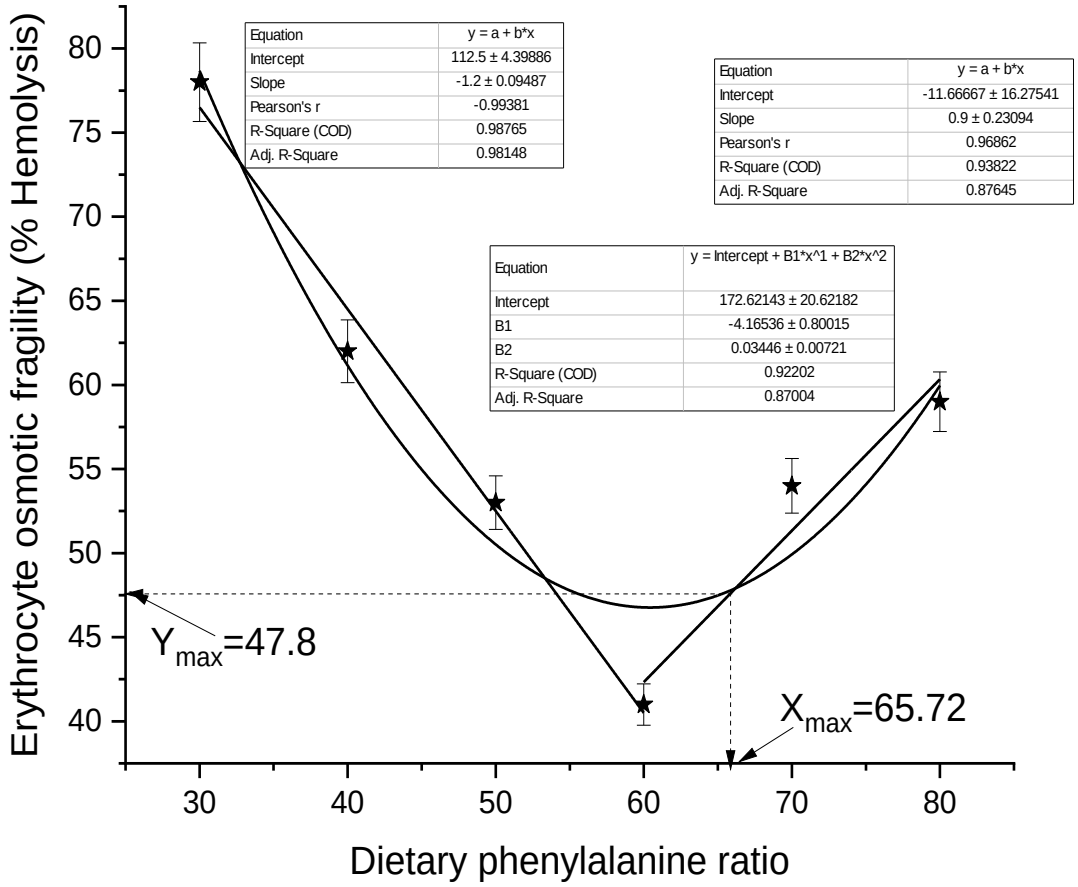

Figure 3. Fitted plot of Erythrocyte Osmotic Fragility% vs. dietary phenylalanine ratios. Each point represents the means of four replicates per treatment (n=4) with 20 fishes per replicate.

excellent carcass quality in terms of carcass protein content. Body fat content of fish fed diets A_5 and A_6 exhibited significant linear increase, probably due to the fact that phenylalanine is both, glucogenic and ketogenic amino acid and excess dietary phenylalanine in fish fed at 70:30 and 80:20 ratios might have converted to fat through the process of gluconeogenesis leading to higher body fat content in fish fed theses diets.

The serum biochemistryas ALT and AST activities reveals health and nutritional status. Any physiological or pathological changes in serum biochemical parameters of ALT and AST activities in fish may be due to external factors, stress, or due to changes in dietary amino acids (Lin *et al.,* 2015) and high ALT and AST activities indicates a damage of normal liver function (Habte-Tsion *et al.,* 2015), specifically related to an excess or imbalanced dietary amino acid contents.

Fish fed dietary phenylalanine: tyrosine at 60:40 ratio (14.54 g/kg: 9.69 g/kg of the diet) exhibited normal physiological range of serum ALT and AST activities indicating normal hepatic functions at this ratio. In this study, groups fed phenylalanine and tyrosine ratios at 70:30 (A_5) and 80:20 (A_6) had comparatively higher values for aspartate and alanine transferases indicating that higher ratios of phenylalanine in these diets have affected liver and hence the body metabolism (Ballantyne, 2000; Olsen *et al.,* 2005; Zhao *et al.,* 2012). Increased serum ALT activities in groups fed diets A_5 and A_6 also suggest catabolism of amino acids at high dietary intake of phenylalanine in these diets (16.96 and 19.39 g/kg phenylalanine). Serum AST activity also increased probably to cope with the increasing energy demands at higher dietary phenylalanine intake at phe: tyr ratios of 70:30 and 80:20 which is to be fulfilled through the catabolism of excess phenylalanine.

Digestive enzyme secretion was lower in A_1, A_2 and A_3 groups, indicating main role of amino acids in synthesis and secretion of these enzymes in fish as amino acids are vital molecules in the metabolism of all living organisms and are the building blocks of enzymes (Yaghoubi *et al.,* 2018). The relatively low pepsin activity in fish fed diet A_1 might also be due to the lower availability of amino acid for protein synthesis and hence lower availability of protein as substrate for protease activity in fish fed these diets. The activity of acidic protease enzyme increased in groups fed phe:tyr ratio up to 60:40 in diet A_4 where phe and tyr were used at 14.54g/kg and 9.69 g/kg of the diets. The higher ratio of dietary phe in diets A_5 and A_6 (16.96 g/kg and 19.39 g/kg diet) did not result a proportional increase in the activity of this enzyme, indicating the saturation of enzymatic action mechanisms, wherein the free enzyme was completely bound to the substrate (phenylalanine). Alkaline protease also exhibited almost same trend. Noticeably, the amylase activity in fish of diets A_5 and A_6 was higher than those fed other diets indicating the conversion of excess phenylalanine to glucose through gluconeogenesis in these diets. Higher amylase activities in fish fed diets A_5 and A_6 may probably be due to the reasons that fish fed these diets converted excess phenylalanine to glucose which were available as substrate for increase in amylase activities in fish fed dietary phenylalanine: tyrosine at ratios higher than 60:40

Growth performance of the fry may also have been influenced by changes in metabolic variables, where an increase of blood glucose, plasma triglycerides and hepatic glycogen levels occurred. Phenylalanine is a glucogenic amino acid (Redei, 2008) that may be converted to glucose at dietary excesses and higher levels of glucose cause hyperglycaemia, raise the concentrations plasma triglycerides and reduced food intake (Blasco *et al.,* 2001; Tran-Duy *et al.,* 2008). Excess phenylalanine at higher dietary phe:tyr ratios in diets A_5 and A_6 might have entered to gluconeogenesis and resulted in hyperglycemic response that resulted in increased liver glycogen and blood glucose for the groups fed diets A_5 and A_6. Feed intake showed a linear positive correlation up to diet A_4. However, a consistent decline was recorded for the groups fed phenylalanine at 16.96 and 19.39g/kg in diets A_5 and A_6 where the proportion of phenylalanine was high (70:30 and 80:20). In fish, hyperglycaemia causes reduced food intake and physiological alterations related to changes in brain glucose sensing markers (Polakof *et al.,* 2011). Excess glucose is stored as glycogen, in the liver and muscle, or can be converted to lipids (Polakof *et al.,* 2012; Song *et al.,* 2018). Liver glycogen corresponded positively with the phe: tyr ratio at 70:30 and 80:20 in diets A_5 and A_6 that led to hyperglycaemia which was probably the reason for reduced feed intake and inferior growth performance of groups fed diets A_5 and A_6.

The physiological responses of farmed fish are an integral part for evaluating their health status. Dietary state and stress have been known to alter blood values (Barnhart, 1969; McCarthy *et al.,* 1973). Significant dose-dependent increase in erythrocytes $\times 10^9$/ml, Hb g/dL and Hct% values in fish fed diets up to A_4 indicate that this diet was having optimum amount of phenylalanine with an adequate ratio of tyrosine. Since diets A_1, A_2 and A_3 were deficient in phenylalanine, this might have resulted anemia in these groups. Erythrocytes sedimentation rate (ESR) is a non-specific hematological parameter that generally increases in case of stress, general infections or nutritional pathologies, acute infections and heavy metal poisoning (Blaxhall & Daisley, 1973). The ESR in fish fed phenylalanine up to A_4 diet decreased and then increased in fish fed diets A_5 and A_6. Significant decline in physiological responses such as erythrocytes, Hb and Hct% in fish fed A_5 and A_6 diets indicate that the fry fed these diets might have suffered nutritional toxicity of excess phenylalanine at phe:tyr ratios of 70:30 and 80:20. The smaller volume of the erythrocytes, as well as the smaller concentration of hemoglobin in fish fed A_5 and A_6 compared to those fed diets A_1, A_2, A_3 and A_4 was probably due to the lesser availability of

phenylalanine in these diets for the synthesis of the proteins that make the above blood parameters.

As a protective system against hyperglycaemia in fish, erythrocytes are rather impermeable to glucose, thus possibly delaying the formation of glycosylated proteins (Polakof *et al.,* 2012). Therefore, fish fed with high concentrations of the phenylalanine in A_5 and A_6 diets, decrease in oxygen transport capacity of erythrocytes might have led to reduction in the metabolism required for higher growth performance (De Souza *et al.,* 2019).

Erythrocytes break down was reported to be faster in case of improper membrane function (Robbins *et al.,* 1984). In this study, ratios of phe:tyr significantly affected the strength of erythrocyte membranes. Erythrocytes of fish fed dietary phe:tyr ratio at 14.54 g phenylalanine/kg diet with 9.69g/kg tyrosine in diet A_4 exhibited lower susceptibility to haemolysis when placed in a hypotonic salt solution indicating that erythrocytes were more resistant to haemolysis. This might be due to the fact that cell wall strength was maximum at optimum level of dietary phenylalanine and tyrosine. The erythrocytes were more susceptible to osmotic lysis in fish fed diets with either below (A_1, A_2, A_3) or beyond this combination of dietary phenylalanine and tyrosine in diets A_5 and A_6.

Nutrient requirements in fish can be estimated by either broken-line regression analysis (Robbins *et al.,* 1979) or quadratic regression analysis (Zeitoun *et al.,* 1976). Parr *et al.,* (2003) concluded that straight broken-line model is likely to underestimate the nutrient requirement whereas the quadratic regression analysis may overestimate the requirement (Baker, 1986) because requirement is taken at the point where the response of fitted line plateaus. Therefore, an objective approach as proposed by Baker *et al.,* (2002) and Parr *et al.,* (2003) has been taken in this study to determine the optimum dietary phenylalanine/tyrosine ratio for *H. fossilis* fry. In this method, the broken line and quadratic analyses are superimposed and requirement is determined as the point where the quadratic curve first intersected the plateau of broken-line. Moreover, these researchers observed that growth response to lower levels of a limiting nutrient was curvilinear and they suggested that the abscissa of the intersection of straight broken-line with a quadratic regression curve is, therefore, a more accurate estimate (Robbins *et al.,* 2006).

Based on above mathematical analyses of growth and nutrient utilization data it is recommended that dietary phenylalanine: tyrosine ratio at 64.94:34.06 corresponding to 15.83 g/kg: 8.55 g/kg on an equimolar basis is optimum for *H.fossilis* fry. The ratio further indicates that *H. fossilis fry* has moderate efficiency of utilizing more of supplemental tyrosine. The requirement determined in this study is comparable to the requirements determined by other workers (Chance *et al.,* 1964; Kim, 1992, Kim *et al.,* 2012; Zehra & Khan 2014; Ren *et al.,* 2015; Xiao *et al.,* 2020) as have been presented in Table 7.

Conclusion

Data of this study has high relevance as the information can be widely used to ensure that optimum phenylalanine and tyrosine ratio of the fish is met while using a greater variety of cost-effective dietary protein feedstuffs used during fishmeal replacement formulations in sustainable aquafarming of this fish. The results of the current study could serve as a basis for establishing the optimum phenylalanine and tyrosine ratio in diet for *H. fossilis* fry to increase aquaculture productivity while preventing nitrogenous waste and environmental damage caused by imbalanced dietary essential nutrients.

Ethical Statement

Not applicable

Funding Information

This research received no specific grant from any funding agency.

Author Contribution

Dr. Shabihul Fatma Sayed has conducted the experiments, data analyses and manuscript writing and Dr. Imtiaz Ahmed has provided the laboratory facilities for feeding trials and sample analyses. All authors have read and approved the final manuscript.

Table 7. Comparison of various studies

Authors and Year of publications	Fish species	Phe: tyr ratio (g/kg dry diet)
Present study	*H. fossilis fry*	15.83: 8.55
Ahmed (2009)	*Cirrhinus mrigala*	13: 8
Khan & Abidi (2007)	*Labeo rohita*	11.6: 10
Zehra & Khan (2014)	*Catla catla*	10.1: 6.8
Zehra & Khan (2021)	*Oreochromis niloticus*	12.1: 8.5
Castillo et al. (2015)	*Sciaenops ocellatus*	16.9: 4.1
Ren et al. (2015)	*Megalobrama amblycephala*	10.1: 10.3
Xiao et al. (2020)	*Oreochromis niloticus× O. aureus*	8.78: 0
Farhat (2014)	*H. fossilis (fingerling Unpublished)*	16.6: 9.9

Conflict of Interest

There is no conflict of interest between third parties.

Acknowledgements

The authors are highly grateful to the honourable Dean of Farasan University College, Jazan University, Dr. Afaf Mohammad Babeir for providing necessary facilities for electronic submission of the manuscript and for providing a highly conducive work environment. The authors are also grateful to the Head, Department of Zoology, Central University of Kashmir, Hazratbal, Srinagar, India for providing the laboratory facilities to carry out the experimental work and to Mr. Mufti Buhran, University Chief Executive Engineer for helping the construction of new Feed Technology Laboratory (Wet-Laboratory) in the Department of Zoology.

References

Abidi, S.F., & Khan, M.A. (2007). Dietary leucine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton). Aquaculture Research, 38, 478-486. https://doi.org/10.1111/j.1365-2109.2007.01687.x

Abidi, S.F., & Khan, M.A. (2012). Dietary arginine requirement of fingerling Indian major carp, *Labeo rohita* (Hamilton) based on growth, nutrient retention efficiencies, RNA/DNA ratio and body composition. Journal of Applied Ichthyology, 25, 707-714. https://doi.org/10.1111/j.1439-0426.2009.01270.x

Ahmed, I. (2009). Dietary total aromatic amino acid requirement and tyrosine replacement value for phenylalanine in Indian major carp: *Cirrhinus mrigala* (Hamilton) fingerlings. Journal of Applied Ichthyology, 25, 719-727. https://doi.org/10.1111/j.1439-0426.2009.01284.x

Allen, G.F.G. (2010). The neurochemical consequences of aromatic L-amino acid decarboxylase deficiency. Department of Molecular Neuroscience UCL Institute of Neurology Queen Square, London, Submitted November 2010 Funded by the AADC Research Trust, UK, Thesis submitted for the degree of Doctor of Philosophy, University College London (UCL).

AOAC (1995). Official Methods of Analysis of the Association of Official Analytical Chemists, Association of Official Analytical Chemists, 16th ed. [Patricia Cunniff, editor], Arlington: VA.

APHA (1992). Standard methods for the examination of water and wastewater, American Public Health Association, 18th ed., Washington, DC, 1268 pp.

Redei, G.P. (2008). Glucogenic Amino Acids. Encyclopedia of Genetics, Genomics, Proteomics and Informatics. Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-6754-9_6938

Aragão, C., Luís, E., Conceição, C., *et al.* (2004). A balanced dietary amino acid profile improves amino acid retention in post-larval Senegalese sole (*Solea senegalensis*). Aquaculture, 233, 293-304. https://doi.org/10.1016/j.aquaculture.2003.08.007,

Baker, D.H. (1986). Problems and pitfalls in animal experiments designed to establish dietary requirements for essential nutrient. Journal of Nutrition 116, 2339-2349. https://doi.org/10.1093/jn/116.12.2339.

Baker, D.H., Batal, A.B., Parr, T.M., *et al.,*. (2002). Ideal ratio (relative to lysine) of tryptophan, threonine, isoleucine and valine for chicks during the second and third weeks post hatch. Poultry Science, 81, 485-494. https://doi.org/10.1093/ps/81.4.485

Ballantyne, J.S. (2001). Amino acid metabolism P. Wright, P. Anderson (Eds.), Fish physiology, Academic Press, New York, pp. 77-107.

Barnhart, R.A. (1969). Effects of certain variables on haematological characteristics of rainbow trout, *Salmo gairdneri* (Richardon). Transaction of American Fisheries Society 98, 411–418. https://doi.org/10.1577/1548-8659(1969)98[411:EOCVOH]2.0.CO;2

Bhatt, V.S. (1968). Studies on the biology of some fresh water fishes. Part-Vll. *Heteropneustes fossilis* (Bloch). Indian Journal of Fisheries, 15, 99–115.

Bhat, H., Ahmad, I., & Asimi, O. (2020). RNA: DNA ratio as an indicator of growth, nutritional status and condition of fish: A review. Journal of Entomology and Zoology Studies, 8(6), 654-658

Bidinotto, P.M., Bidinotto, G., & Moraes, R.H.S. (1997). Hepatic glycogen and glucose in fifth tropical freshwater teleost fish: a procedure for field determinations of micro samples. B.Téc. CEPTA, 10, 53-60.

Blasco, J., Marimon, I., Viaplana, I., *et al.,* (2001). Fate of plasma glucose in tissues of brown trout in vivo: effects of fasting and glucose loading. Fish Physiology & Biochemistry, 24, 247-258. https://doi.org/10.1023/A:1014084313207

Blaxhall, P.C., & Daisley, K.W. (1973). Routine haematological methods for use with fish blood. Journal of Fish Biology, 5, 771-781. http://dx.doi.org/10.1111/j.1095-8649.1973.tb04510.x

Campbell, J.W. (1991). "Excretory nitrogen metabolism" in Environmental and Metabolic Animal Physiology. Comparative Animal Physiology, 4th Edn., Prosser, C.L. (Ed.), New York: Wiley-Interscience, 277-324.

Castillo, S., Halligan, S., & Gatlin, 3rd D.M. (2015). Growth Responses of Juvenile Red Drum *Sciaenops ocellatus* to Dietary Phenylalanine and Tyrosine Can Be Used to Calculate the Total Aromatic Amino Acid Requirement. Journal of Nutrition, 145, 2341-6. https://doi.org/10.3945/jn.115.215848.

Calheiros, A.C., Reis, R.P., Diana, B.C., *et al.,* (2019). *Ulva* spp. as a natural source of phenylalanine and tryptophan to be used as anxiolytics in fish farming. Aquaculture, 509, 171-177. https://doi.org/10.1016/j.aquaculture.2019.05.028

Chance, R.E., Mertz, E.T., & Halver, J.E. (1964). Nutrition of Salmonoid Fishes: XII. Isoleucine, Leucine, Valine and Phenylalanine Requirements of Chinook Salmon and Interrelations between Isoleucine and Leucine for Growth. The Journal of Nutrition, 83 (3), 177–185, https://doi.org/10.1093/jn/83.3.177

Chen, G., Feng, L., Kuang, S., *et al.,* (2012). Effect of dietary arginine on growth, intestinal enzyme activities and gene expression in muscle, hepatopancreas and intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). British Journal of Nutrition, 108, 195-207. https://doi.org/10.1017/S0007114511005459.

De Souza, R.C., De Souza, E.M., & Da Costa, M.M., *et al.,* (2019). Dietary addition of the essential oil from *Lippia alba* to Nile tilapia and its effect after inoculation with *Aeromonas* spp. Aquaculture Nutrition, 25, 39-45. https://doi.org/10.1111/anu.12827

Driedzic, W.R., & Clow, K.A. (2013). Glucose uptake and metabolism by red blood cells from fish with different extracellular glucose levels. Journal of Experimental Biology, 216, 437-446. https://doi.org/10.1242/jeb.079178

Ezell, G.H., Sulya, L.L., & Dodgen, C.L., 1969. The osmotic fragility of some fish erythrocytes in hypotonic saline. Comparative Biochemistry and Physiology, 28, 409-415. https://doi.org/10.1016/0010-406x(69)91354-1

Fagbenro, O.A., & Jauncey, K. (1995). Growth and Protein Utilization by Juvenile Catfish (*Clarias gariepinus*) Fed Dry Diets Containing Co-Dried Lactic Acid - Fermented Fish Silage and Protein Feedstuffs. Bioresource Technology, 51, 29-35. https://doi.org/10.1016/0960-8524(94)00064-8.

FAO (2020). The State of World Fisheries and Aquaculture 2020, #244 p. FAO, Rome, Italy.

Farhat (2014). Essential amino acid requirements of fingerling stinging catfish *Heteropneustes fossilis* (Bloch). Chapter 6, pp 139-176, Ph.D Thesis 320pp.

Fatma, S., & Ahmed, I. (2020). Effect of water temperature on protein requirement of *Heteropneustes fossilis* (Bloch) fry as determined by nutrient deposition, hemato-biochemical parameters and stress resistance response. Fisheries & Aquatic Sciences, 23, 1-14. https://doi.org/10.1186/s41240-020-0147-y

Fernstrom, M.H., & Fernstrom, J.D. (1995). Acute tyrosine depletion reduces tyrosine hydroxylation rate in rat central nervous system. Life Sciences, 57, 97–102.

Flecknell, P. (2016). Laboratory Animal Anaesthesia (Fourth Edition), Chapter 5 - Anaesthesia of Common Laboratory Species: Special Considerations. Pages 193-256, Academic Press https://doi.org/10.1016/B978-0-12-800036-6.00005-3

Gropper, S.S., & Smith, J.L. (2012). Advanced Nutrition and Human Metabolism. Sixth Edition, Cengage Learning; pp. 186-233.

Habte-Tsion, H.M., Ge X., & Liu, Bo., *et al.* (2015). A deficiency or an excess of dietary threonine level affects weight gain, enzyme activity, immune response and immune-related gene expression in juvenile blunt snout bream (*Megalobrama amblycephala*). Fish Shellfish Immunology, 42, 439-446. https://doi.org/10.1016/j.fsi.2014.11.021

Hall, G.M., & Ahmad, N.H. (1992). Functional Properties of Fish Protein Hydrolysates. Fish Processing Technology. New York, Ny: VCH Publishers, inc. 249-274.

Halver, J.E. (2002). The vitamins. in: *Fish Nutrition*, 3rdHalver, J.E., Hardy, R.W. (Eds), Academic Press, San Diego: CA., pp. 61-141.

Hamandishe, V.R., Saidi, P.T., Chikosi, I., *et al.* (2018). A Comparative Evaluation of Carcass Quality, Nutritional Value, and Consumer Preference of *Oreochromis niloticus* from Two Impoundments with Different Pollution Levels in Zimbabwe. International Journal of Food Science, 7862971. https://doi.org/10.1155/2018/7862971

Hidalgo, M.C., Urea, E., & Sanz, A. (1999). Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture, 170, 267-283. https://doi.org/10.1016/S0044-8486(98)00413-X

Hummel, B.C.W. (1959). A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. Canadian Journal of Biochemistry & Physiology, 37, 1393-1399. https://doi.org/10.1139/o59-157

Jauncey, K. (1982). The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapias (*Sarotherodon mossambicus*). Aquaculture 27, 43–54. https://doi.org/10.1016/0044-8486(82)90108-9

Kader, M.D.A, Bulbul, M., & Koshio, *et al.* (2012). Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, *Pagrus major.* Aquaculture, 350–353, 109-116. https://doi.org/10.1016/j.aquaculture.2012.04.009

Khan, M.A., & Abidi, S.F. (2007). Total aromatic amino acid requirement of Indian major carp *Labeo rohita* (Hamilton) fry. Aquaculture, 267, 111-118. https://doi.org/10.1016/j.aquaculture.2007.02.025.

Kim, K.I. (1993). Requirement for phenylalanine and replacement value of tyrosine for phenylalanine in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 113(3), 243-250.

Kim, Sung-Sam., Rahimnejad, S., & Song, Jin-Woo. (2012). Comparison of Growth Performance and Whole-body Amino Acid Composition in Red Seabream (*Pagrus major*) Fed Free or Dipeptide Form of Phenylalanine. Asian Australian Journal of Animal Sciences, 25(8), 1138–1144. https://doi.org/10.5713/ajas.2012.12054

Klanian M. G., & Alonso M. G. (2015). Sensory characteristics and nutritional value of red drum Sciaenops ocellatus reared in freshwater and seawater conditions. Aquaculture Research, 46(7), 1550–1561. https://doi.org/10.1111/are.12307.

Kyu-Il (1993). Requirement for phenylalanine and replacement value of tyrosine for phenylalanine in rainbow trout (*Oncorhynchus mykiss*). Aquaculture, 113 (3), 243-250. https://doi.org/10.1016/0044-8486(93)90477-G.

Li, P., Mai, K., & Trushenski, J., *et al.,* (2008). New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids, 37, 43-53. https://doi.org/10.1007/s00726-008-0171-1.

Li, W., Feng, L., Liu, Y., *et al.,* (2015). Effects of dietary phenylalanine on growth, digestive and brush border enzyme activities and antioxidant capacity in the hepatopancreas and intestine of young grass carp (*Ctenopharyngodon idella*). Aquaculture Nutrition, 21, 913-925. https://doi.org/10.1111/anu.12223

Lin, H.Z., Tan, X.H., Zhou, C.P., *et al.,* (2015). Effect of dietary arginine levels on the growth performance, feed utilization, non-specific immune response and disease resistance of juvenile golden pompano *Trachinotus ovatus*. Aquaculture, 437, 382-389. https://doi.org/10.1016/j.aninu.2016.07.001.

Mambrini, M., & Kaushik, S.J. (1994). Partial replacement of dietary protein nitrogen with dispensable amino acids in diets of Nile tilapia, *Oreochromis niloticus*. Comparative Biochemistry & Physiology, 109A, 469-477. https://doi.org/10.1016/0300-9629(94)90152-X

Martin, N.B., Houlihan, D.F., & Talbot, C., *et al.,* (1993). Protein metabolism during sexual maturation in female Atlantic salmon (*Salmo salar* L.). Fish Physiology & Biochemistry 12, 131-141. https://doi.org/10.1007/BF00004378

Mejbaum, W., (1939) Z. physio. Chem., 268, 117.

McCarthy, D.H., Stevenson, J.P., & Roberts, M.S. (1973). Some blood parameters of the rainbow trout (*Salmo gairdneri* Richardson). II. The Shasta variety. Journal of Fish Biology, 5, 1-8. https://doi.org/10.1111/j.1095-8649.1973.tb04425.x

Nose, T. (1979). Summary report on the requirement of essential amino acids for carp, in: Halver, J.E., Tiews, K. (Eds.), Proceedings of the World Symposium on Finfish Nutrition and Finfish Technology, vol. 1, 20–23 June 1978, Hamburg, Heenemann, Berlin, pp. 145-156.

NRC (2011). Nutrient requirements of fish and shrimp. The National Academies Press, Washington, D.C978-0-309-16338-5 (pp. 376 + XVI).

Olsen, R.E., Sundell, K.M., & Mayhew, T., *et al.,* (2005). Acute stress alters intestinal function of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquaculture, 250, 480-495. https://doi.org/10.1016/j.aquaculture.2005.03.014

Parr, T.M., Kerr, B.J., & Baker, D.H. (2003). Isoleucine requirement of growing (25 to 45 kg) pigs. Journal of Animal Science, 81, 745-752. https://doi.org/10.2527/2003.813745x

Perez-Jimenez, A., Abellan, E., & Arizcun, M., *et al.* (2015). Nutritional and metabolic responses in common dentex (*Dentex dentex*) fed on different types and levels of carbohydrates. Comparative Biochemistry & Physiology A, Molecular & Integrative Physiology 184, 56-64. https://doi.org/10.1016/j.cbpa.2015.02.002

Pinto, W., Figueira, L., Dinis, M.T., *et al.,* (2009). How does fish metamorphosis affect aromatic amino acid metabolism? Amino Acids, 36, 177-183. https://doi.org/10.1007/s00726-008-0045-6.

Polakof, S., & Mommsen, T.P., Soengas, J.L. (2011). Glucosensing and glucose homeostasis: from fish to mammals. Comparative Biochemistry & Physiology B, 160, 123-149.

Polakof, S., Panserat, S., Soengas, J.L., *et al.,* (2012). Glucose metabolism in fish: a review. Journal of Comparative Biochemistry & Physiology B, Biochemical, Systemic, and Environmental Physiology, 182, 1015–1045. https://doi.org/10.1007/s00360-012-0658-7.

Polat A., & Beklevik, G. (1999). The importance of betaine and some attractive substances as fish feed additives. In: Brufau J. (ed.), Tacon A. (ed.). Feed manufacturing in the Mediterranean region: Recent advances in research and technology. Zaragoza: CIHEAM, 1999. p. 217-220 (Cahiers Options Méditerranéennes; n. 37)

Rahman, M.A., Gheyasuddin, H., & Rasid, M.H., *et al.,* (1982). Proximate composition and native quality of freshwater Zeol fishes of Bangladesh. Bangladesh Journal of Fisheries, 25, 37-43.

Ren, M., Habte-Michael, B., & Habte-Tsion, *et al.,* (2015). Dietary phenylalanine requirement and tyrosine replacement value for phenylalanine of juvenile blunt snout bream, *Megalobrama amblycephala*. Aquaculture**,** 442, 51-57. https://doi.org/10.1016/j.aquaculture.2015.02.029

Robbins, K.R., Norton, H.W., & Baker, D.H. (1979). Estimation of nutrient requirements from growth data. Journal of Nutrition, 109, 1710–1714. https://doi.org/10.1093/jn/109.10.1710.

Robbins, K.R., Saxton, A.M., & Southern, L.L. (2006). Estimation of nutrient requirements using broken-line regression analysis. Journal of Animal Science, 84, E155–E165. https://doi.org/10.2527/2006.8413_supple155x.

Robbins, S.L., Cotran, R.S., & Kumar, V. (1984). Pathologic Basis of Disease. Saunders, Philadelphia, PA.1467 pp.

Robinson, E.H., Wilson, R.P., & Poe, W.E. (1980). Total aromatic amino acid requirement, phenylalanine requirement and tyrosine replacement value for fingerling channel catfish. Journal of Nutrition, 110, 1805–1812. https://doi.org/10.1093/jn/110.9.1805.

Rønnestad, I., Manuel, Y., Ueberschar, B., *et al.,* (2013). Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Reviews in Aquaculture, 5, 59-98. https://doi.org/10.1111/raq.12010.

Saha, K.C., & Guha, B.C. (1939). Nutritional investigation on Bengal fish. Indian Journal of Medical Research, 26, 921-927.

Saha, N., & Ratha, B.K. (2007). Functional ureogenesis and adaptation to ammonia metabolism in Indian freshwater air-breathing catfishes. (Review). Fish Physiology & Biochemistry, 33, 283–295. https://doi.org/10.1007/s10695-007-9172-3

Schneider, W.C. (1957). Determination of nucleic acids in tissues by pentose analysis, Volume 3.in: Wchneiger, W.C. (Ed.), Methods in Enzymology. New York, New York, USA: Academic Press; 1957. p. 1154.

Schmidt, G., & Thannhauser, S.J. (1945). A method for the determination of deoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissue. Journal of Biological Chemistry, 161, 83-89.

Salamanca, N., Giráldez, I., Morales, E., et al. (2021). Phenylalanine and Tyrosine as Feed Additives for Reducing Stress and Enhancing Welfare in Gilthead Seabream and Meagre. Animals, 11(1), 45. https://doi.org/10.3390/ani11010045

Siddiqui, T.Q., & Khan, M.A. (2009). Effects of dietary protein levels on growth, feed utilization, protein retention efficiency and body composition of young *Heteropneustes fossilis* (Bloch). Fish Physiology & Biochemistry, 35, 479-488. https://doi.org/10.1007/s10695-008-9273-7

Silva, A.F., Copatti, C.E., & Oliveira, E.P., *et al.,* (2020). Effects of whole banana meal inclusion as replacement for corn meal on digestibility, growth performance, haematological and biochemical variables in practical diets for tambaqui juveniles (*Colossoma macropomum*). 17, https://doi.org/10.1016/j.aqrep.2020.100307

Sokal, R.R., & Rohlf, F.J. (1981). Biometry. W.H. Freeman and Company New York, pp. 859.

Song, X., Marandel, L., & Dupont-Nivet, M., *et al.,* (2018). Hepatic glucose metabolic responses to digestible dietary carbohydrates in two isogenic lines of rainbow trout. Biology Open, 7, 1–11. https://doi.org/10.1242/bio.032896.

Tacon, A.G.J. (1992). Nutritional fish pathology: morphological signs of nutrient deficiency and toxicity in farmed fish. FAO Fisheries Technical Paper No.330, FAO, 75 pp., Rome.

Tang, L., Feng, L., Sun, C., *et al.,* (2013). Effect of tryptophan on growth, intestinal enzyme activities and TOR gene expression in juvenile Jian carp (*Cyprinus carpio* var. Jian): studies in vivo and in vitro. Aquaculture, 412-413, 23-33. https://doi.org/10.1016/j.aquaculture.2013.07.002.

Tran-Duy, A., Smit, B., van Dam, A.A., & Schrama, J.W. *et al.,* (2008). Effects of dietary starch and energy levels on maximum feed intake, growth and metabolism of Nile tilapia, *Oreochromis niloticus*. Aquaculture, 277, 213-219. https://doi.org/10.1016/j.aquaculture.2008.03.004.

Tu, Y.Q., Xie, S.Q., & Han, D. *et al.,* (2015). Growth performance, digestive enzyme, transaminase and GH-IGF-I axis gene responsiveness to different dietary protein levels in brood stock allogenogynetic gibelcarp (*Carassius auratus gibelio*) CAS III. Aquaculture, 446, 290-297.

https://doi.org/10.1016/j.aquaculture.2015.05.003.

Tukey, JW. (1953). The problem of multiple comparisons. in: Turkey, T.H. (Ed.), The Collected Works of John H. Tukey VIII. Multiple Comparisons: 1948–1983. Chapman and Hall, New York, pp. 1–300.

Unprasert, N.G. (1994). "An evaluation of the use of 'Ideal' protein concept to estimate essential amino acid requirements of the *Clarias* Hybrid (*Clarias macro cephalus × Clarias gariepinus*)," Ph.D. dissertation. Mississippi State University, Mississippi State.

Walker, A.M., Copatti, C.E., & Melo, F.V.S.T., *et al.,* (2020). Metabolic and physiological responses to intraperitoneal injection of chromium oxide in hyperglycaemic Nile tilapia juveniles. Aquaculture, 517, *A*rticle734821. https://doi.org/10.1016/j.aquaculture.2019.734821.

Wilder, I.B., & Stanley, J. G. (1983). RNA-DNA ratio as an index to growth in salmonid fishes in the laboratory and in streams contaminated by carbaryl. 22 (2), 165-172. https://doi.org/10.1111/j.1095-8649.1983.tb04737.x

Wilson, R.P. (1989). Fish Nutrition, 2nd ed. In: Halver, J.E. (Ed.), Academic press, San Diego, CA, 111 pp.

Wilson, R.P. (2002). Amino acids and protein. Fish Nutrition3rd ed. in: Halver, J.E., Hardy, R.W. (Eds.), Academic Press, San Diego, CA, USA, pp. 143–179.

Wilson, R.P., Harding, Jr. O.W., & Garling, Jr. D.L. (1977). Effects of dietary pH on amino acid utilization and the lysine requirement of fingerling channel catfish. *Journal of Nutrition 107:* 166–170. https://doi.org/10.1093/jn/107.1.166.

Wong, S.Y. (1928). Colorimetric determination of iron and haemoglobin in blood. Journal of Biological Chemistry, 77, 409-412.

Wu, G., Wu, Z., & Dai, Z., *et al.,* (2013). Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. Amino Acids, 44, 1107-1113. HTTPS://DOİ.ORG/10.1007/s00726-012-1444-2.

Wu, G., Bazer, F., Dai, Z, Li D, Wang J, & Wu Z. (2014). Amino acid nutrition in animals: protein synthesis and beyond. Annual Review of Animal Biosciences, 2, 387–417. https://doi.org/10.1146/annurev-animal-022513-114113.

Xiao, W., Zou, Z, & Li, D., *et al.,* (2020). Effect of dietary phenylalanine level on growth performance, body composition, and biochemical parameters in plasma of juvenile hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus*. Journal of the World Aquaculture Society, 51, 437-451. https://doi.org/10.1111/jwas.12641

Yaghoubi M., Mozanzadeh M.T., Safari O., *et al.,* (2018). Gastrointestinal and hepatic enzyme activities in juvenile silvery-black porgy (*Sparidentex hasta*) fed essential amino acid-deficient diets. Fish Physiology & Biochemistry, 44, 853-868. https://doi.org/10.1007/s10695-018-0475-3.

Yokoyama, H.O. (1977). Studies on the origin, development and seasonal variations in the blood cells of the perch, *Perca* J. Eavescens. Ph.D. Thesis. Univ. Wisconsin, Madison, Wis. 144 pp, 1974.

Yun, H., Park, G., Ok, I., *et al.,* (2015). Evaluation of Optimum Dietary Threonine Requirement by Plasma Free Threonine and Ammonia Concentrations in Surgically Modified Rainbow Trout, *Oncorhynchus mykiss*. Asian-Australasian Journal of Animal Sciences (AJAS), 28, 551-558. https://doi.org/10.5713/ajas.14.0495

Zehra, S., & Khan, M.A. (2014). Dietary phenylalanine requirement and tyrosine replacement value for phenylalanine for fingerling *Catla catla* (Hamilton). Aquaculture, 433, 256-265. https://doi.org/10.1016/j.aquaculture.2014.06.023

Zehra, S., & Yousif, R.A. (2021). Dietary total aromatic amino acid requirement and tyrosine replacement value for phenylalanine for fingerling *Oreochromis niloticus*. Aquaculture Nutrition, https://doi.org/10.1111/anu.13242

Zeitoun, I.H., Ullrey, D.E., &Magee, W.T., *et al.,* (1976). Quantifying nutrient requirements of fish. Journal of Fisheries Research Board of Canada, 33, 167–172. https://doi.org/10.1139/f76-019

Zhao, J., Liu, Y., & Jiang, J. (2012). Effects of dietary isoleucine on growth, the digestion and absorption capacity and gene expression in hepatopancreas and intestine of juvenile Jian carp (*Cyprinus carpio* var. Jian). Aquaculture, 368-369, 117-128.

Zhou, F., Shao, J., & Xu, R., *et al.,* (2010). Quantitative L-lysine requirement of juvenile black sea bream (*Sparus macrocephalus*). Aquaculture Nutrition, 16, 194-204.