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ORIGINAL ARTICLE

Evaluation of dietary *Lemna minor* supplementation on growth, digestive enzyme activity, and carcass composition of *Heteropneustes fossilis* (Bloch, 1794)

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Abstract
Finding affordable protein-rich replacements for fishmeal in fish feeds is important for sustainable aquaculture. *Lemna minor* is emerging as a promising high-protein yet low-cost alternative to traditional fishmeal. *Heteropneustes fossilis* is a valuable and highly nutritious food fish, but its production in India is low due to the high cost of feed production. In this study, the potential of incorporating *L. minor* into the diet of *H. fossilis* was assessed for its impact on growth, digestive enzyme activity, biochemical parameters, and carcass composition. Five isonitrogenous diets LM0 (0%), LM5 (5%), LM10 (10%), LM15 (15%) and LM20 (20%) with varying percentage inclusion of *L. minor* were fed to *H. fossilis* fries (0.51±0.01g, 4.1±0.03cm) for 60 days. LM15 diet-fed fish showed better Feed Conversion Ratio (0.93±0.05), Specific Growth Rate (2.60±0.06% day^{-1}), and Protein Efficiency Ratio (2.68±0.14) among all the groups. The inclusion of *L. minor* increased amylase, lipase, and pepsin activities, whereas chymotrypsin, trypsin and, total protease activities did not differ significantly (P>0.05). Arginine, histidine, methionine, valine and polyunsaturated fatty acids were significantly (P<0.05) elevated in LM15 diets fed fish. Biochemical parameters (thiobarbituric acid reactive substances, superoxide dismutase, and aminotranferases) showed no adverse effect of *L. minor* on the fish. Our results indicated that *L. minor* can be added to the fish diet up to 15% for optimum growth without adversely affecting fish health. The results of this study may be useful for the development of a cost-efficient and sustainable plant-based nutrient-rich feed for fish using freely available local resources.

Keywords: Aquafeed, Plant protein, Stinging catfish, Sustainable feed.

INTRODUCTION

With the rise in global population, the consumption of aquatic food has increased, escalating from 9.9kg per capita during the 1960s to 20.2kg per capita by 2020 (FAO 2022). Aquaculture holds the promise of fulfilling the nutritional needs of this expanding population. However, the advancement of the aquaculture sector hinges on accessible, economically viable, and nutritionally well-rounded diets. Moreover, the dependence on wild fish for aquafeed raises concerns regarding excessive harvesting and sustainability concerns (Naseem et al. 2021). This issue stems from the conventional employment of fishmeal, a key component in fish feed, which not only incurs high costs but is also environmentally unsustainable (Ali & Kaviraj 2021). Consequently, numerous investigations have been conducted to assess the potential of local feed resources to achieve both cost-effectiveness and sustainability in aquaculture (Dorothy et al. 2018).

In fish farming, fish feed becomes a prime factor in determining the cost and quality of fish (Mukherjee et al. 2010), as it constitutes 50-70% of the production cost of farmed fish (Iskandar et al. 2019). Therefore, several studies have evaluated alternative protein sources, especially plant proteins, by partial and complete substitution of fishmeal with plant protein to decrease the feed cost so that farmers can grow fish more economically on a large scale (Naseem et al. 2021). Aquatic macrophytes are regarded as a potential alternative plant protein for replacing fishmeal, among which *L. minor* is highly valued due to its rich content of protein, vitamins, carotenoids, essential amino acids, and availability (Kabir et al. 2009; Chakrabarti et al. 2018). Various studies have highlighted the potential benefits of *L. minor* diet in

Table 1. Analysis of experimental diet composition and proximate components (% dry matter).

Ingredients (%)	LM0	LM5	LM10	LM15	LM20
Wheat flour*	51.33	47.11	42.9	38.68	34.46
L. minor	0	5	10	15	20
Dry Fish Powder*	47.27	46.49	45.7	44.92	44.14
Vitamin-mineral premix$^{\phi}$	0.4	0.4	0.4	0.4	0.4
Cod Liver Oil$^{\Psi}$	1	1	1	1	1
Proximate analysis (%)					
Protein	38.43	38.93	37.47	38.99	39.76
Moisture	5.26	5.21	5.42	5.50	5.75
Ash	6.79	7.38	8.00	8.96	9.46
Fibre	1.94	1.77	2.43	3.58	3.73
Lipid	4.90	4.48	3.95	5.06	4.99
Carbohydrate	42.68	42.23	42.73	37.91	36.31
Energy (Kcal $100g^{-1}$)	368.54	364.96	356.35	353.14	349.19

*Local Market, Kokrajhar, Assam.
$^{\phi}$Supradyn, Bayer Consumer Care AG, Basel, Switzerland. Vitamins: 5000 IU Vitamin A, 500mcg Methylcobalamin, 400 IU Vitamin D3, 150mcg D – Biotin USP, 75mg Ascorbic acid, 50mg Vitamin B3, 25mg Tocopheryl Acetate, 10mg Calcium D-Pantothenate, 5mg Vitamin B2, 5mg Vitamin B1, 1.5mg Folic Acid, and 1.5mg Vitamin B6. Trace Elements: 2mg Copper Sulphate, 250mcg Chromium Picolinate, 70mcg Selenium, 25mcg Sodium Molybdate, 5mg Manganese Sulfate Monohydrate. Amino acid: 50mg L- glutamic acid
$^{\Psi}$SEACOD, Cod Liver Oil (Type B) BP Universal Medicare, Mumbai, India.

several fish species (Noor et al. 2000; Yilmaz et al. 2004; Devi et al. 2022; Goswami et al. 2022).

Heteropneustes fossilis (Bloch, 1794) is a high-value fish species widely distributed in India and Bangladesh (Rahman et al. 2019) that has high market value because of its low fat content, high flesh quality, high nutrient content, and medicinal value (Nushy et al. 2020). Although it has gained market popularity, the dependency of fish farmers on fishmeal for its production has resulted in a rise in the production cost of the fish (Hossain et al. 2023). Nevertheless, few studies have reported replacing fishmeal with locally available plant protein sources up to certain levels. Ali & Kaviraj (2021) successfully included fermented *Ipomea aquatica* in the diet of *H. fossilis*, replacing fishmeal up to 25-50% with better growth rates at 50% inclusion. Although, the optimal inclusion level for fermented mulberry leaf meal was reported to be 52.28% (Ali et al. 2019), sunflower meal was observed to replace up to 14.3% fishmeal in *H. fossilis* optimally (Hossain et al. 2023). Other plant proteins, such as soybean meal, have also been reported as potential replacements for fishmeal in the feed of the species without affecting growth, feed efficiency, and health status (Howlader et al. 2023). Nandi et al. (2023) observed better growth when fermented *Ipomoea aquatica* replaced fishmeal in *H. fossilis*. Additionally, the growth and feed conversion efficiency of *H. fossilis* were unaffected by adding up to 15% soybean meal to their diet (Siddiqui et al. 2013). These studies suggest that plant proteins may be incorporated up to a certain level into the diet of the species, and hence, the aquatic macrophyte *L. minor* may be a good option for inclusion in the aquafeed of the species. Thus, the current study aimed to assess the effects of *L. minor* incorporated diets at varying substitution levels on the growth, digestive enzymes, nutritional profile, and biochemical parameters of *H. fossilis*.

MATERIALS AND METHODS

Experimental diet: *L. minor* samples were collected from Kokrajhar, Assam, India. The plant samples were air-dried in a shed until the moisture level dropped to below 50%, then oven-dried at 50°C, crushed into a fine powder, and sieved through 1mm wire mesh. Five isonitrogenous diets (40% crude protein) with increasing percentages of *L. minor* inclusion were prepared and labelled LM0 (0%), LM5 (5%), LM10 (10%), LM15 (15%), and LM20 (20%),

respectively (Table 1). The incorporation levels of *Lemna minor* in the diet were determined based on studies showing positive growth effects in various fish species, including *Barbodes gonionotus* Bleeker (Noor et al. 2000), *Cyprinus carpio* (Yilmaz et al. 2004; Goswami et al. 2022), and *Oncorhynchus mykiss* (Fiordelmondo et al. 2022). All feed ingredients were gently blended with water to form a paste and passed through a 1mm mesh. The strands were manually cut and left to oven-dry at 50°C. For 60 consecutive days, the fish were fed satiation at 9:00 a.m. and 4:00 p.m. each day. Uneaten feeds were removed after one hour of feeding and oven-dried at 50°C to determine feed intake.

Feeding experimental unit: The trial was conducted at Bodoland University's wet laboratory facility in Kokrajhar, Assam, India for 60 days. A batch of 750 *H. fossilis* juveniles (average weight: 0.51±0.01g, average length: 4.1±0.03cm) was procured from the Bijni fish farm in Chirang, Assam. These were randomly distributed among 15 aquaria, each with a 50-litre capacity. For each treatment, 50 fish were stocked in triplicate in each aquarium. An inlet and outlet system were established in each aquarium to facilitate water aeration and renewal. Regular assessments of pH, dissolved oxygen, and water temperature were conducted, following the standardised procedures outlined in the APHA (2017) guidelines. Throughout the study period, the recorded values for temperature, dissolved oxygen and pH ranged from 25.2°C to 27.4°C, 6.40 to 7.36mg L^{-1} and 6.97 to 7.09, respectively.

Sampling and growth parameters: Fish length and weight were consistently measured weekly throughout the experimental period. After the completion of the 60-day trial, the fish were fasted for 24 hours. Subsequently, phenoxyethanol (0.5mL L^{-1}) was used for anesthetisation, and the final length and weight were noted. Several parameters, including Body Mass Gain (BMG), Specific Growth Rate (SGR), Feed Conversion Ratio (FCR), Survival Rate (SR), Feed Efficiency (FE), and Protein Efficiency Ratio (PER), were assessed using the following formulae:

BMG (%) = [(Final body mass in g – initial body mass in g)/Initial body mass in g] × 100

SGR (% day^{-1}) = [(ln final body mass in g – ln initial body mass in g)/number of trial days] × 100

FCR = Dry feed fed (g)/body mass gain (g)

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

FE (%) = 100 × (total final body weight – total initial body weight)/total dry feed intake

PER = BMG (g)/protein intake (g)

Proximate composition: Proximate composition analysis of the diet and dry muscle tissue of all the different treatment groups was carried out following the standard method specified in AOAC (2000). The Micro Kjeldahl technique was employed to assess the total nitrogen concentration and to obtain the crude protein percentage by multiplying the concentration of nitrogen by 6.25. Moisture content was determined by heating at 135°C for 2 hours in a hot-air oven. Crude lipids were analysed using petroleum ether extraction followed by Soxhlet extraction. Ash combustion in a muffle furnace for 16 hours at 550°C. Crude fibre was measured gravimetrically following chemical digestion and solubilisation of other components.

Digestive enzyme activity analysis: After a 60-day trial period, the whole digestive tract was collected from fish that underwent a 24-hour fast and anaesthesia. A total of 15 fish per diet were included by randomly selecting 5 fish from each aquarium. Dissections were performed in a chilled environment, followed by sample homogenisation (1:10 w/v, tissue: distilled water) using a mechanical tissue homogeniser. After centrifugation (10,000×g) at 4°C, the supernatants were stored at -20°C.

The amylase activity was assessed using starch as a substrate, following Bernfeld's (1955) method. To determine the lipase enzyme activity, the Winkler and Stuckman (1979) method was employed using *p*-nitrophenyl palmitate as a substrate. Pepsin activity was measured following Anson's (1938) method, using haemoglobin as a substrate. Total protease enzyme activity was determined using an azocaesin substrate according to the method described by

Garcia-Carreno (1992). Trypsin and chymotrypsin enzyme activities were determined spectrophotometrically using N-α-Benzoyl-L-arginine ethyl ester and N-Benzoyl-L-tyrosine ethyl ester substrates, respectively (Bergmeyer 1974). Total protein content was determined following Bradford (1976) method using Bovine Serum Albumin (BSA) as the standard.

Biochemical parameters: To examine the biochemical parameters, wet muscle tissue samples were obtained from all treatments in triplicate. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using buffered aspartate-alpha-ketoglutarate and buffered alanine-alpha-ketoglutarate substrates, respectively (Reitman & Frankel, 1957). Superoxide dismutase (SOD) activity was tested using Xanthine oxidase following Roy et al. (2020). The thiobarbituric acid reactive substances (TBARS) assay was performed following Ohkawa et al. (1979) method using 1,1,3,3-tetramethoxy propane as the standard.

Fatty acid analysis: Fatty acid analysis was performed in a Perkin Elmer (USA) GC-MS, Clarus 680 GC and Clarus 600C MS, controlled by Turbo Mass Ver. 6.4.2 software. A 60m x 0.25mm film Elite-5MS (0.25µm) capillary column containing 5% diphenyl 95% dimethyl polysiloxane was employed with a helium injection volume of 1µL (carrier gas, 1mL min^{-1}). EI+ mode at 70eV was employed for mass spectra, covering the m/z 50-600amu mass range. Compound identification was based on the NIST-2014 database comparison. Peaks were analysed using the data analysis software NIST-2014 to obtain insights into the names, molecular weights, and empirical formulas of components.

Amino acid analysis: Amino acid profiling procedure using LC-MS involves two extraction methods (Nimbalkar et al. 2012): one for free amino acids and another for bound amino acids via acid hydrolysis. Sample homogenisation with formic acid (0.1%) was done in methanol (20%) followed by centrifugation and filtration, to extract free amino acids. For bound amino acids, samples are hydrolysed with 6M hydrochloric acid, processed, and reconstituted before injection. LC-MS conditions included gradient composition, temperature control, and a PDA detector for amino acid monitoring. The mobile phases for the analysis included water with formic acid (0.1%) and a mixture of water and methanol (50:50) with formic acid (0.1%), utilising the Waters Acquity UPLC H (TQD MS/MS, USA) system.

Statistical analysis: The data is presented as mean±standard deviation. Prior to analysis, the normality (determined by the Shapiro-Wilk test) and homogeneity (determined by Levene's test) of the data were examined. Tukey post hoc analyses along with one-way ANOVA to compare group means. $P<0.05$ was used to define the significance level. To determine the optimal dietary level of *L. minor* incorporation, quadratic polynomial regression analysis was performed on FCR and SGR.

RESULTS

Growth performance: The growth performance of fish is presented in Table 2. No mortality was recorded for any of the treatments throughout the experiment. The LM15 diet-fed fish showed higher FW, BMG and SGR values. Additionally, they exhibited enhanced FE and PER, and a lower FCR compared to the control (LM0) group. According to the FCR and SGR regression analysis, the optimal dietary inclusion level of *L. minor* for *H. fossilis* was within the range of 11.89-12.30% (Fig. 1a, b).

Proximate composition: The proximate composition of the fish muscle samples is presented in Table 3. Ash content, lipid, and crude protein content in *H. fossilis* fed the diet LM15 were significantly higher ($P<0.05$), whereas moisture content was higher in the LM0 group than in the other diet groups. The fibre content in the fish fed different diets did not significantly differ ($P>0.05$).

Digestive enzyme activity: The digestive enzyme activities in the fish fed with different levels of *L. minor* are shown in Fig. 2 (a-f). Fish fed the LM15 diet exhibited significantly higher amylase activity ($P<0.05$) than those fed the LM0 diet. However, there was no notable difference ($P>0.05$) in amylase activity

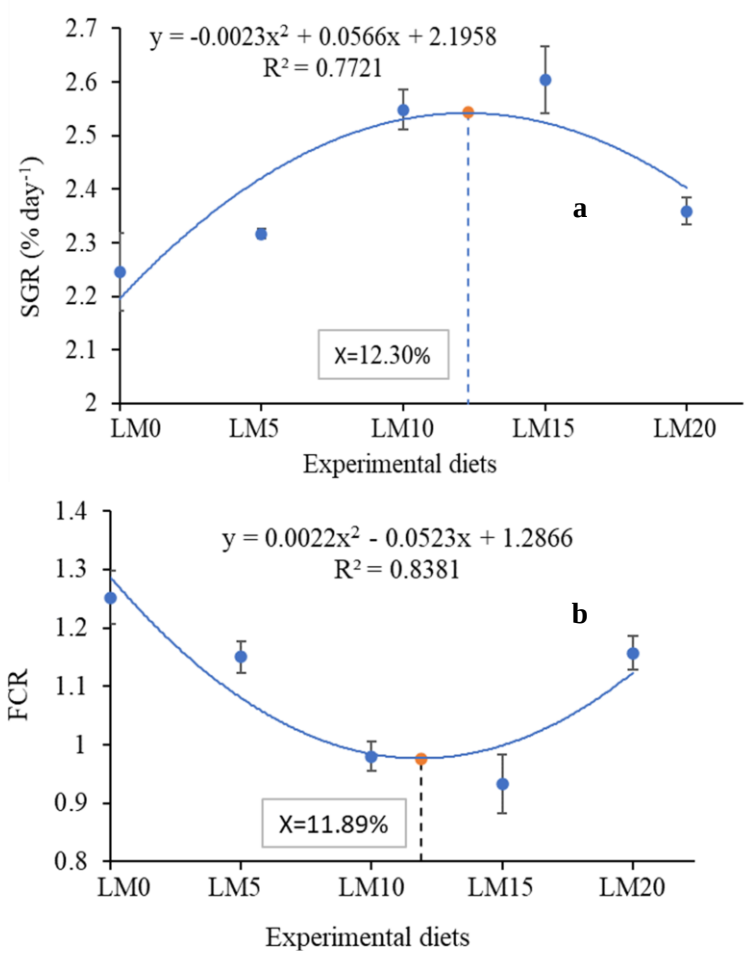

Fig.1. Optimizing *Lemna minor* dietary intake via polynomial regression analysis based on (a) SGR and (b) FCR.

between the LM10 and LM15 groups. Lipase activity was markedly increased in both LM10 and LM15 groups compared with the other treatments. Trypsin, total protease, and chymotrypsin activities did not differ significantly (P>0.05) between the treatments. Pepsin activity was highest in the plant-fed group, LM15 (P<0.05), while LM10, LM15 and LM20 did not vary significantly (P>0.05).

Fatty acid composition: The fatty acid composition of fish fed different levels of *L. minor* is presented in Table 4. In all treatments, C17:0 was the predominant saturated fatty acid (SFA), while C18:0 was consistently the least abundant. Monounsaturated fatty acids (MUFA) exhibited a decreasing trend, whereas polyunsaturated fatty acids (PUFA) increased with increasing *L. minor* in the diet. Increased levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found with increasing *L. minor* inclusion levels in the study. Notably, the LM15 group exhibited the highest PUFA, EPA+DHA, and PUFA/SFA ratio (P<0.05).

Amino acid composition: Among essential amino acids (EAA), arginine, valine, histidine and methionine were highest in LM15, whereas leucine and phenylalanine were highest in LM0 (Table 5). Lysine levels were highest in LM5, whereas the highest threonine and tryptophan content were observed in LM10. Alanine, glycine, and citrulline were among the non-essential amino acids (NEAA) found to be highest in LM20, whereas cysteine and serine were maximum in LM5. Additionally, proline, asparagine and tyrosine were recorded highest in the control among all the groups.

Biochemical parameters: SOD activity did not vary significantly (P>0.05) between any of the treatment groups (Table 6).

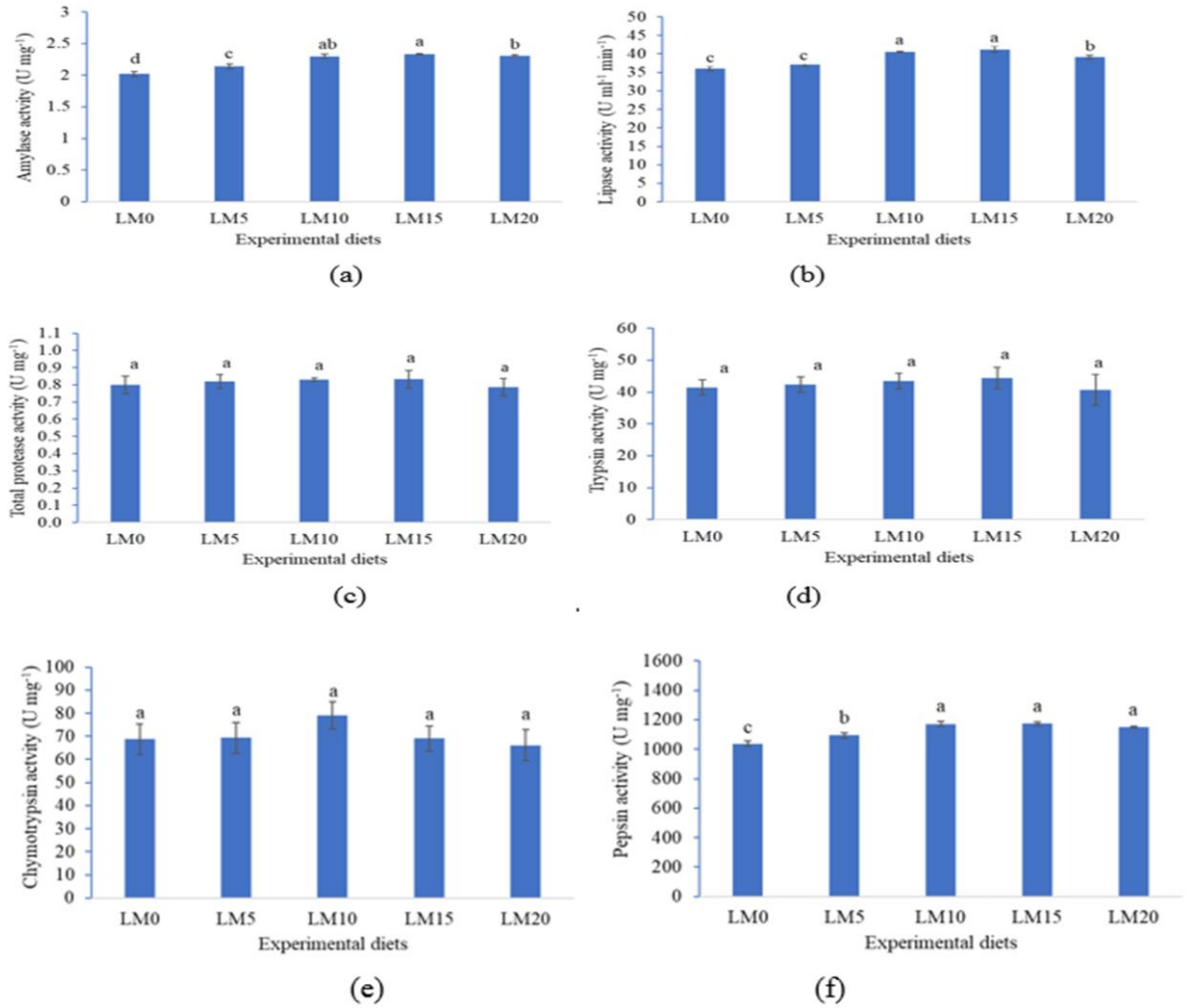

Fig.2. Digestive enzyme activity of *Heteropneustes fossilis* fed with different levels of *Lemna minor* in the diet for 60 days. Bars with different lower cases indicate significant differences (n= 3, P<0.05). (a) Amylase, (b) Lipase, (c) Total Protease, (d) Trypsin, (e) Chymotrypsin, and (f) Pepsin activity.

Lower AST and ALT activities were observed in LM10 compared with the other groups (P<0.05). TBARS levels decreased non-significantly among all plant-fed groups of fish compared with the control group.

DISCUSSION

Most studies on plant proteins as a replacement for fishmeal in aquafeed are restricted to fish such as cyprinids, salmonids, trout, etc., and limited studies are available on catfish and air-breathing species (Dorothy et al. 2018; Naseem et al. 2021). However, some studies have demonstrated the positive impacts of aquatic macrophyte incorporated diets on the growth performance of catfish (Kari et al. 2020; Naseem et al. 2021; Nandi et al. 2023). *L. minor*, known for its rich nutrient content (Chakrabarti et al. 2018), has been extensively tested in various fish species (Noor et al. 2000; Raj et al. 2001; Herawati et al. 2020; Fiordelmondo et al. 2022; Goswami et al. 2022; Irabor et al. 2022). This present study was conducted to assess the potential of *L. minor* as an alternative protein source for *H. fossilis* by examining its effects on growth performance, digestive enzyme activities, biochemical parameters, and the carcass composition of the fish. Our results showed better growth efficiency and nutrient utilisation in *H. fossilis*, when *L. minor* was incorporated in the diet up to a certain level, beyond which there seems to be no effect. This is evident from the increased FW, BMG, and SGR in the groups fed with dietary *L. minor* incorporated diet.

Table 2. Growth and nutrient efficiency of *Heteropneustes fossilis* fed varying levels of *Lemna minor* incorporated diet for 60 days.

Parameters	LM0	LM5	LM10	LM15	LM20	*P* value
IW (g)	0.51±0.01	0.51±0.00	0.51±0.01	0.51±0.01	0.50±0.01	0.068
FW (g)	1.95±0.10^{b}	2.05±0.02^{b}	2.34±0.05^{a}	2.44±0.08^{a}	2.06±0.03^{b}	<0.001
BMG (%)	284.76±16.69^{b}	301.58±2.34^{b}	361.50±10.13^{a}	377.16±18.07^{a}	311.94±6.29^{b}	<0.001
SGR (% day^{-1})	2.24±0.07^{b}	2.32±0.01^{b}	2.55±0.04^{a}	2.60±0.06^{a}	2.36±0.03^{b}	<0.001
FCR	1.25±0.05^{a}	1.15±0.03^{b}	0.98±0.02^{c}	0.93±0.05^{c}	1.16±0.03^{ab}	<0.001
Survival (%)	100	100	100	100	100	
FE (%)	79.93±2.88^{b}	86.96±2.08^{b}	102.03±2.52^{a}	107.29±5.61^{a}	86.47±2.15^{b}	<0.001
PER	2.00±0.07^{b}	2.17±0.05^{b}	2.55±0.06^{a}	2.68±0.14^{a}	2.16±0.05^{b}	<0.001

Table 3. Proximate analysis of *Heteropneustes fossilis* (% dry weight basis) fed varying levels of *Lemna minor* incorporated diet for 60 days.

Parameters	LM0	LM5	LM10	LM15	LM20	*P* value
Moisture	17.57±0.01^{a}	17.45±0.01^{c}	17.18±0.01^{d}	16.76±0.01^{e}	17.53±0.01^{b}	<0.001
Protein	63.54±0.02^{e}	63.66±0.03^{d}	63.84±0.03^{b}	63.95±0.05^{a}	63.69±0.02^{c}	<0.001
Lipid	7.97±0.05^{e}	8.07±0.02^{d}	8.27±0.05^{b}	8.44±0.06^{a}	8.13±0.01^{c}	<0.001
Ash	9.38±0.02^{d}	9.44±0.01^{c}	9.51±0.03^{b}	9.60±0.05^{a}	9.50±0.06^{b}	<0.001
Fibre	0.10±0.01^{a}	0.10±0.01^{a}	0.11±0.01^{a}	0.10±0.01^{a}	0.11±0.01^{a}	0.499
Carbohydrate	1.44±0.03^{a}	1.28±0.03^{b}	1.09±0.05^{cd}	1.15±0.05^{c}	1.04±0.04^{d}	<0.001

Note. Superscript letters indicate significant differences in a shared row (n= 3, P<0.05).

Table 4. Fatty acid composition (% of total fatty acid) in *Heteropneustes fossilis* fed varying levels of *Lemna minor* incorporated diet for 60 days.

Fatty acid	LM0	LM5	LM10	LM15	LM20	P value
C13:0	1.72±0.01^{b}	2.88±0.01^{a}	2.87±0.02^{a}	2.70±0.02^{c}	2.47±0.02^{d}	<0.001
C14:0	2.45±0.01^{a}	2.39±0.01^{b}	2.38±0.01^{b}	2.31±0.01^{c}	2.30±0.02^{c}	<0.001
C17:0	15.85±0.01^{b}	15.69±0.01^{c}	15.56±0.02^{d}	15.96±0.03^{a}	15.69±0.02^{c}	<0.001
C18:0	0.14±0.01^{a}	0.08±0.00^{b}	0.09±0.01^{b}	0.09±0.01^{b}	0.08±0.01^{b}	<0.001
C20:0	1.01±0.01^{b}	0.37±0.01^{e}	0.57±0.02^{c}	0.53±0.02^{d}	1.06±0.01^{a}	<0.001
C27:0	2.77±0.02^{a}	2.58±0.01^{bc}	2.55±0.02^{c}	2.55±0.02^{c}	2.62±0.01^{b}	<0.001
C34:0	2.07±0.01^{a}	1.97±0.01^{b}	1.96±0.01^{b}	1.72±0.01^{d}	1.86±0.02^{c}	<0.001
Σ SFA	26.01±0.05^{ab}	25.96±0.06^{ab}	25.96±0.03^{ab}	25.84±0.09^{b}	26.08±0.04^{a}	0.009
C16:1n-5	12.39±0.01^{a}	12.26±0.01^{b}	12.10±0.04^{c}	11.95±0.03^{e}	12.03±0.03^{d}	<0.001
C16:1n-7	3.07±0.01^{a}	2.75±0.01^{b}	2.65±0.01^{c}	2.63±0.02^{c}	2.67±0.02^{c}	<0.001
C18:1n-9	1.53±0.01^{a}	1.48±0.02^{b}	1.42±0.01^{c}	1.36±0.01^{d}	1.28±0.02^{e}	<0.001
C18:1n-16	14.45±0.02^{a}	14.32±0.02^{b}	14.26±0.02^{c}	14.18±0.02^{d}	14.25±0.01^{c}	<0.001
C20:1n-9	3.96±0.02^{a}	3.90±0.02^{d}	3.85±0.02^{c}	3.81±0.01^{a}	3.82±0.01^{b}	<0.001
C18:1n-5	13.30±0.01^{a}	13.29±0.01^{a}	13.24±0.03^{b}	13.30±0.01^{a}	13.27±0.01^{ab}	0.004
Σ MUFA	48.71±0.06^{a}	48.01±0.08^{b}	47.51±0.08^{c}	47.23±0.12^{d}	47.32±0.10^{cd}	<0.001
C18:3n-3	1.29±0.02^{c}	1.35±0.01^{b}	1.37±0.01^{b}	1.41±0.02^{a}	1.27±0.01^{c}	<0.001
C20:5n-3	2.56±0.01^{c}	2.62±0.02^{c}	2.77±0.02^{a}	2.72±0.01^{b}	2.70±0.02^{b}	<0.001
C20:3n-3	2.13±0.02^{c}	2.16±0.02^{c}	2.22±0.01^{b}	2.24±0.03^{b}	2.29±0.02^{a}	<0.001
C22:6n-3	1.40±0.02^{d}	1.46±0.01^{c}	1.55±0.02^{b}	1.64±0.03^{a}	1.64±0.03^{a}	<0.001
C20:4n-6	3.91±0.01^{d}	3.97±0.02^{c}	4.05±0.02^{b}	4.07±0.02^{ab}	4.11±0.02^{a}	<0.001
C20:2n-6	3.72±0.01^{d}	3.93±0.03^{c}	3.95±0.02^{bc}	3.99±0.02^{ab}	4.00±0.01^{a}	<0.001
C18:2n-6	8.24±0.02^{c}	8.38±0.02^{b}	8.45±0.02^{a}	8.45±0.02^{a}	8.46±0.01^{a}	<0.001
C22:4n-6	1.92±0.02^{c}	2.03±0.02^{b}	2.05±0.01^{b}	2.16±0.01^{a}	2.12±0.01^{a}	<0.001
Σ PUFA	25.17±0.01^{d}	25.90±0.13^{c}	26.41±0.09^{b}	26.69±0.11^{a}	26.59±0.06^{ab}	<0.001
PUFA/SFA	0.97±0.00^{d}	1.00±0.00 ^{c}	1.02±0.00^{b}	1.03±0.01^{a}	1.02±0.00^{b}	<0.001
ω6/ω3	2.41±0.02^{a}	2.41±0.01^{a}	2.34±0.01^{b}	2.33±0.02^{b}	2.36±0.02^{b}	<0.001
EPA+DHA	3.96±0.01^{c}	4.08±0.03^{b}	4.32±0.03^{a}	4.36±0.04^{a}	4.35±0.05^{a}	<0.001

Note. Superscript letters indicate significant differences in a shared row (n= 3, P<0.05).
SFA: Saturated fatty acid
MUFA: Monounsaturated fatty acid
PUFA: Polyunsaturated fatty acid
EPA: Eicosapentaenoic acid
DHA: Docosahexaenoic acid

Table 5. Amino acid composition of *Heteropneustes fossilis* fed varying levels of *Lemna minor* incorporated diet for 60 days.

Amino acids (mg/g)	LM0	LM5	LM10	LM15	LM20	*P* value
EAA						
Arginine	24.94±0.26^{e}	27.79±0.10^{d}	28.81±0.19^{c}	32.31±0.01^{a}	31.54±0.10^{b}	<0.001
Histidine	14.99±0.41^{d}	17.33±0.07^{c}	18.03±0.23^{b}	20.25±0.13^{a}	14.92±0.10^{d}	<0.001
Lysine	73.12±0.12^{d}	90.83±0.21^{a}	82.15±0.20^{b}	81.61±0.01^{c}	70.39±0.07^{e}	<0.001
Leucine	114.61±0.50^{a}	78.58±0.26^{c}	59.68±0.04^{e}	106.04±0.12^{b}	61.37±0.27^{d}	<0.001
Methionine	51.82±0.11^{b}	33.19±0.28^{c}	25.91±0.06^{e}	63.50±0.58^{a}	30.64±0.12^{d}	<0.001
Phenylalanine	70.86±0.93^{a}	57.27±0.66^{c}	35.14±0.11^{d}	59.70±0.25^{b}	27.69±0.40^{e}	<0.001
Threonine	25.60±0.28^{d}	27.67±0.09^{c}	46.66±0.10^{a}	27.69±0.36^{c}	44.86±0.40^{b}	<0.001
Tryptophan	5.51±0.04^{b}	5.34±0.03^{c}	6.60±0.01^{a}	3.85±0.01^{e}	4.85±0.01^{d}	<0.001
Valine	22.29±0.08^{ab}	16.20±0.70^{d}	20.58±0.07c	22.36±0.11^{a}	21.45±0.06^{b}	<0.001
Total	403.75±0.53^{b}	354.21±1.03^{c}	323.56±0.53^{d}	417.30±1.52^{a}	307.70±0.46^{e}	<0.001
NEAA						
Alanine	43.84±0.18^{d}	50.04±0.80^{c}	73.47±0.05^{b}	44.40±1.00^{d}	79.44±1.07^{a}	<0.001
Aspartic acid	73.10±1.13^{c}	59.25±1.29^{e}	92.89±0.82^{a}	64.57±0.76^{d}	89.54±0.47^{b}	<0.001
Cysteine	0.66±0.01^{a}	0.72±0.05^{a}	0.60±0.01^{b}	0.57±0.02^{b}	0.56±0.01^{b}	<0.001
Glycine	0.55±0.02^{b}	0.39±0.02^{c}	0.58±0.01^{b}	0.34±0.02^{d}	0.64±0.00^{a}	<0.001
Glutamic acid	45.56±0.11^{e}	74.73±0.46^{c}	110.46±0.87^{a}	47.43±0.69^{d}	99.60±0.81^{b}	<0.001
Proline	90.39±0.15^{a}	53.71±0.15^{e}	63.01±0.04^{d}	74.64±0.18^{c}	81.42±1.31^{b}	<0.001
Serine	52.20±0.23^{d}	96.91±2.01^{a}	93.57±1.12^{a}	57.00±1.57^{c}	86.50±1.58^{b}	<0.001
Tyrosine	22.64±0.05^{a}	11.29±0.11^{d}	13.67±0.03^{c}	18.14±0.24^{b}	8.40±0.02^{e}	<0.001
Citrulline	1.30±0.05^{d}	1.56±0.03^{c}	1.29±0.03^{d}	2.57±0.01^{b}	2.93±0.05^{a}	<0.001
Asparagine	1.40±0.01^{a}	0.80±0.00^{b}	0.08±0.00^{e}	0.58±0.00^{c}	0.45±0.00^{d}	<0.001
Beta 3-4 dihydroxy phenylalanine	0.07±0.01^{a}	0.03±0.01^{b}	0.01±0.00^{c}	0.03±0.00^{b}	0.02±0.00^{cb}	<0.001
Total	331.70±1.42^{c}	349.42±0.59^{b}	449.61±2.90^{a}	310.26±4.45^{d}	449.49±1.21^{a}	<0.001
Total amino acids	735.45±1.96^{c}	703.63±1.62^{d}	773.18±3.43^{a}	727.56±5.96^{c}	757.19±0.75^{b}	0.001

Note. Superscript letters indicate significant differences in a shared row (n= 3, P<0.05).
EAA: Essential Amino Acids
NEAA: Non-Essential Amino Acids

Table 6. Biochemical parameters of *Heteropneustes fossilis* fed with different levels of *Lemna minor* incorporated diet for 60 days.

Parameters	LM0	LM5	LM10	LM15	LM20	*P* value
SOD (U mg^{-1})	371.78±92.38^{a}	370.77±66.59^{a}	371.97±92.64^{a}	370.62±35.12^{a}	371.63±68.07^{a}	0.780
TBARS (U mg^{-1})	2.78±0.07^{ab}	2.74±0.09^{b}	2.77±0.12^{a}	2.75±0.03^{ab}	2.76±0.22^{a}	<0.001
AST (U mg^{-1})	2.20±0.09^{a}	2.13±0.05^{a}	2.11±0.08^{b}	2.16±0.15^{ab}	2.14±0.05^{b}	<0.001
ALT (U mg^{-1})	2.16±0.35^{ac}	2.07±0.09^{c}	2.06±0.14^{b}	2.15±0.13^{a}	2.14±0.08^{ab}	0.002

Note. Superscript letters indicate significant differences in a shared row (n= 3, P<0.05).
SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reactive substances, AST: Aspartate aminotransferase (AST), ALT: Alanine aminotransferase

Higher BMG and SGR in the *L. minor* incorporated diet fed fish indicated the acceptance, and subsequent digestion of the *L. minor* incorporated diet, which led to the improved growth performance. This may also indicate the improvement in feed utilisation due to proper digestion of the plant-incorporated diets. Our results align with Siddiqui et al. (2013), where the growth and feed conversion efficiency of *H. fossilis* were unaffected by a diet containing up to 15% soybean meal instead of fishmeal. Similar positive results in growth performance were also observed in various fish species when *L. minor* was included upto 10% in *Barbodes gonionotus* (Noor et al. 2000), 50% in *Channa striatus* (Raj et al. 2001), 20% in *Oncorhynchus mykiss* (Fiordelmondo et al. 2022), 20% in *Cyprinus carpio* (Goswami et al. 2022), and 40% in *Clarias gariepinus* (Irabor et al. 2022). Growth performance, however, did not exhibit a noteworthy distinction from the control group at the 20% incorporation level in this study. This implies that a higher proportion of plant protein might affect digestion and assimilation processes. Contrary to our findings, however, a higher percentage inclusion of some plant proteins gave better results in the same species. For instance, a complete replacement with soybean meal resulted in improved growth and feed

conversion efficiencies (Shukla et al. 2018). Similarly, water spinach meal replacing up to 50% fishmeal resulted in improved growth and health of *H. fossilis* (Nandi et al. 2023). Additionally, soybean meal also improved the health and growth performance of *H. fossilis* (Howlader et al. 2023).

The use of regression analysis is an effective technique for determining the optimal dose of feed additives (Yossa & Verdegem 2015). Based on SGR and FCR regression analysis, *L. minor* concentration is recommended at 11.89-12.30% in *H. fossilis* diets. This technique was used to find the optimum sunflower meal inclusion level (14.3%) for *H. fossilis* (Hossain et al. 2023). The ash, lipid and protein content of the fish were notably influenced by the increasing dietary inclusion of *L. minor*. Similar trends were reported in *Channa striatus* fed with *L. minor* (Raj et al. 2001; Fiordelmondo et al. 2022). This indicated that the fish can digest and successfully assimilate the nutrients present in the plant. However, few studies have also reported decreased carcass protein content, such as in *Clarias gariepinus* (Irabor et al. 2022) and in *Barbodes gonionotus,* Bleeker (Noor et al. 2000).

Assessing the activity of digestive enzymes yields insights into the overall digestive capacity and utilisation of nutrients of the fish through hydrolysis of protein, lipids and carbohydrates (Gawlicka et al. 2000; Johnston et al. 2004; Devi et al. 2022). Higher amylase activities may be correlated with the increase in plant content in the diet. Fish fed the LM10 and LM15 diets exhibited significantly elevated amylase and lipase activities, indicating efficient utilisation of carbohydrates and lipids in the diet incorporating *L. minor*. This finding aligns with earlier reports on *H. fossilis* fed 50% inclusion of mulberry leaf (Ali et al. 2019), and soybean meal (Khanom et al. 2022). Protein digestion in *H. fossilis* fed with *L. minor* improved as pepsin activity was significantly higher in LM10, LM15, and LM20. The chymotrypsin, trypsin and total protease activities were not significantly affected, although there was an increasing trend. Similar results were also observed in *Centropmus viridis* fed soybean meal (Arriaga-Hernández et al. 2021). Similarly, an increase in enzyme activity was observed in *Cyprinus carpio* when fed with *L. minor* (Goswami et al. 2022) and *Spirodela polyrhiza* supplemented feed (Shrivastav et al. 2022). The elevated enzymatic activities found in our study indicated that the plant-based diet did not negatively affect the digestion of protein, lipids and carbohydrates up to 15% *L. minor* incorporation. This led to efficient utilisation of the ingested feed, thereby, resulting in increased PER, decreased FCR and improved growth.

Fatty acids are pivotal in determining the nutritional quality of fish (Zhang et al. 2020). The fish nutritional content is markedly influenced by the composition of its diet (Ackman 1989). In this study, the fatty acid composition of *H. fossilis* was significantly affected by *L. minor* in its diet, leading to increased PUFA and decreased SFA in the fish with higher percentage of *L. minor*. Similar findings were reported in *Oreochromis niloticus* fed with fermented *L. minor* (Herawati et al. 2020). The ratios of n6/n3 (Simopoulos 2002) and PUFA/SFA (HMSO 1994) are important indicators of fat quality. Our study suggests that the observed ranges of these indices hold good for human health. This may be due to the enhanced fatty acid composition of *H. fossilis* when fed with *L. minor*, suggesting potential advantages for human health.

The efficient utilisation of amino acids in animal feed is essential for sustainable protein production (Kaushik et al. 2010). *L. minor* exhibits a rich source of amino acids, making it suitable for aquafeed (Chakrabarti et al. 2018). *H. fossilis* fed with LM15 diets exhibited significantly elevated levels of EAA, particularly arginine, histidine, methionine, and valine. This agrees with the results of Goswami et al. (2022), where *Cyprinus carpio* fed with *L. minor* exhibited elevated levels of EAA, including arginine, isoleucine, methionine, tryptophan, threonine and valine. Thus, feeding *H. fossilis* with *L. minor* incorporated diet may help enhance the composition of amino acids in the fish carcass.

The capacity of the fish to withstand environmental stress relies on the health of their immune systems (Adel et al. 2015). Plant-based proteins added to the fish diet impact fish development and immunological state (Dossou et al. 2018). Antioxidant defences and reactive oxygen species (ROS) in animal cells were highlighted by Tocher et al. (2002), where an imbalance in ROS causes oxidative stress. Sheikhzadeh et al. (2012) established a connection between fish antioxidant defences and nutrition. In response to toxicants, the generation of ROS increases, which is counteracted by an antioxidant enzyme system (Lushchak 2011). The first line of protection against ROS oxidative damage is provided by the antioxidant enzyme SOD (Fridovich 1995). This study found no significant difference in SOD activity in the plant-fed fish compared to the control. Similar results were also reported in *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix* fed with *L. minor* (Aslam et al. 2021). However, Wang et al. (2017) observed a noteworthy diminishing trend of SOD in *Larimichthys crocea* when fed with soy protein concentrate. The TBARS assay evaluates the peroxidative damage to lipids by generating free radicals to quantify oxidative stress (Oakes et al. 2003). In this study, the TBARS in *L. minor* fed fish was comparable with the control. However, *Ctenopharyngodon idella* and *Hypophthalmichthys molitrix* fed *L. minor* showed no significant difference (Aslam et al. 2021). AST and ALT are found in various tissues of the skeletal muscles, liver, erythrocytes, etc. (Hadi et al. 2009), and these are tested as biomarkers for liver dysfunction and fish health status. These enzymes provide specific information about organ dysfunction when present in blood serum or plasma; for instance, as an elevated ALT activity reflects liver disease (Shahsavani et al 2010). Lower activities of both the enzymes in *L. minor* fed groups than control in the present study indicates no negative impact on the liver. Similar results were also reported in *Lates calcarifer* fed with *L. minor* incorporated diet (Mustofa et al. 2022). However, increased activity of both these enzymes was also observed in the plasma of African catfish fed with fermented soy pulp (Kari et al. 2020).

In conclusion, the results of our study indicated that *L. minor* may be incorporated in the diet of *H. fossilis* up to 15% for better growth performance, with the optimal results observed at 11.89-12.35% *L. minor* supplementation in the diet. This dietary supplementation also increased digestive enzyme activity, improving feed utilisation and nutrient digestibility. Moreover, including *L. minor* enriched the fish nutritional profile of the fish by increasing protein, fatty acid, and amino acid content. The results of this study may be useful in the development of a cost effective and sustainable feed based on aquatic macrophytes, *L. minor* for enhanced production of *H. fossilis*.

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مقاله کامل

ارزیابی مکمل غذایی *Lemna minor* بر رشد، فعالیت آنزیم گوارشی و ترکیب لاشه *Heteropneustes fossilis* (Bloch, 1794)

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چکیده: یافتن جایگزین‌های غنی از پروتئین مقرون به صرفه برای پودر ماهی در خوراک ماهی برای آبزی‌پروری پایدار ضروری است. *Lemna minor* به‌عنوان یک جایگزین امیدوارکننده با پروتئین بالا و در عین حال کم هزینه برای پودر ماهی سنتی در حال گسترش است. *Heteropneustes fossilis* یک ماهی با ارزش غذایی و بسیار مغذی است، اما تولید آن در هند به‌دلیل هزینه بالای تولید خوراک کم است. در این مطالعه، پتانسیل ترکیب *L. minor* در رژیم غذایی *H. fossilis* در راستای تأثیر آن بر رشد، فعالیت آنزیم گوارشی، پارامترهای بیوشیمیایی، و ترکیب لاشه بررسی شد. پنج جیره با محتوای نیتروژنی همسان، شامل LM0 (۰٪)، LM5 (۵٪)، LM10 (۱۰٪)، LM15 (۱۵٪) و LM20 (۲۰٪) با درصد متفاوتی از *L. minor* برای تغذیه لاروهای *H. fossilis* (۰/۰۱±۰/۵۱ گرم و ۰/۰۳±۴/۱ سانتی‌متر) به‌مدت ۶۰ روز مورد استفاده قرار گرفت. ماهی‌های تغذیه شده با رژیم غذایی LM15، ضریب تبدیل غذایی (۰/۰۵±۰/۹۳)، نرخ رشد ویژه (۰/۰۶±۲/۶٪) در روز) و نسبت کارایی پروتئین (۰/۱۴±۲/۶۸) بهتری در بین همه گروه‌ها نشان دادند. گنجاندن *L. minor* باعث افزایش فعالیت‌های آمیلاز، لیپاز و پپسین شد، در حالی‌که فعالیت‌های کیموتریپسین، تریپسین و کل پروتئاز تفاوت معنی‌داری نداشتند ($P>0.05$). آرژنین، هیستیدین، متیونین، والین و اسیدهای چرب غیراشباع به‌طور قابل توجهی ($P<0.05$) در جیره LM15 تغذیه شده با ماهی افزایش یافت. پارامترهای بیوشیمیایی (مواد واکنش‌دهنده اسید تیوباربیتوریک، سوپراکسید دیسموتاز و آمینوترانفرازها) هیچ اثر نامطلوبی از *L. minor* بر روی ماهی نشان نداد. نتایج ما نشان داد که *L. minor* می‌تواند تا ۱۵ درصد به جیره ماهی اضافه شود که رشد مطلوبی داشته باشد بدون اینکه بر سلامت ماهی تأثیر منفی بگذارد. نتایج این مطالعه ممکن است برای توسعه یک خوراک غنی از مواد مغذی گیاهی مقرون به صرفه و پایدار برای ماهی‌ها با استفاده از منابع محلی به‌صورت آزاد در دسترس مفید باشد.

کلمات‌کلیدی: منابع غذایی آبی، پروتئین گیاهی، گربه‌ماهی نیش‌دار، خوراک پایدار.