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Effect of Azolla meal-based diet on growth performance, haematological profile and proximate composition of common carp (*Cyprinus carpio*) fry

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Abstract

Optimising the cost of nutrients in fish diets is crucial for aquaculture production, as feed accounts for 50% of operational expenses in intensive aquaculture. The rising demand for mustard oil cake (MOC), a commonly used feed ingredient in Indian aquaculture, has led to a significant increase in its price due to its diverse applications. Azolla *(Azolla pinnata),* a free-floating freshwater plant, has attracted researchers in fish feed formulation due to its rich nutrient profile. The present study was conducted to evaluate the effects of replacement of MOC with Azolla meal in the diet of common carp (*Cyprinus carpio*) fry. Pelleted feed was formulated with Azolla meal replacing MOC at levels of 0, 10, 20, 30 and 40% and fed to fish. Growth performance, feed conversion ratio (FCR), haematological parameters and proximate composition in fish carcass were assessed after 90 days of experimental feeding. Simultaneously, a control feed without supplementing of Azolla meal was also prepared. Fish were fed daily with the experimental diets at 8, 6 and 4% of their body weight during the first, second and third months respectively, in the morning hours (10:00 hrs). Principal component analysis (PCA) was performed to assess the effect of formulated diets on fish carcasses composition and haematological parameters. The results showed that replacing 30% of MOC with Azolla meal in the diet, led to significantly ($p \leq 0.05$) higher specific growth rate (SGR) and percentage weight gain and significantly ($p \leq 0.05$) lower feed conversion ratio (FCR) at this concentration. Fish survival rate ranged from 80.00 to 93.33% among different treatments. Further, significant ($p \leq 0.05$) differences were observed in both haematological parameters of fish and the proximate composition of fish carcass. among the different treatments. These findings suggest that MOC can be effectively replaced with 30% Azolla meal in the diet for rearing common carp fry to fingerling stage, without compromising the growth performance.

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Introduction

Aquaculture offers nutritional security, livelihood, trade and employment to millions of people in developing countries. Formulating a nutritionally balanced, low-cost diet for cultured fish species is essential to uplift fish production. Optimising the nutrients in fish diets to control the input expenditure of aquaculture is paramount because feeding constitutes more than 50% of the operating cost in intensive aquaculture (Ahmed *et al.,* 2023). The health of fish is improved when dietary nutrients are balanced and protein is considered to be the most expensive nutrient in the fish diet which is responsible for muscle building, enzymatic activities, supply of energy and qualitative health (Wang *et al.,* 2017; Ahmed and Ahmad, 2020; Ahmed *et al.,* 2023). Mustard oil cake (MOC) is most extensively used by Indian fish farmers as protein source as it contains around 35-40% protein (Singh *et al.,* 2017; Mishra *et al.,* 2018; Sharma *et al.,*

2022; Banerjee *et al.,* 2023), but cost of MOC is increasing sharply in recent times as it is now widely used as feed ingredient for cattle, poultry and aquatic animals; organic fertilises, bio-herbicide, fermentation medium, biopolymer and natural antioxidant (Swati *et al.,* 2015; Jose *et al.,* 2022; Banerjee *et al.,* 2023). If fishes are fed only with MOC as the source of protein, the expenditure towards production of quality feed will increase significantly. Therefore, to reduce aquaculture operational costs and to enhance the marginal profitability of farmers, the MOC of the fish diet must be replaced with a cheaper plant protein source (Datta, 2011). Azolla *(Azolla pinnata)* is a freshwater free-floating plant that is widely distributed in lakes, wetlands, paddy fields, ponds, rivers and canals in India (Gangadhar *et al.*, 2015). Recently, Azolla has attracted considerable attention as a dietary protein source for aquaculture due to its nutrient composition. It contains upto 30% crude protein with a high quantity of vitamins, minerals, biopolymers, and probiotics (Das *et al.*, 2004; Maity and Patra, 2008; Datta, 2011; Gangadhar *et al.,* 2015; Magouz *et al.*, 2020; Kouberi Nath *et al*., 2022; Ahmed *et al.*, 2023) and also anti-nutritional factors (Gopan *et al.,* 2020). The inclusion of Azolla in the fish diet promotes growth in rohu (Datta, 2011), Nile tilapia (Ebrahim *et al*., 2007; Magouz *et al*., 2020), Common carp (Ahmed *et al.*, 2023), *Labeo fimbriatus* (Gangadhar *et al*., 2015) and grass carp (Majhi *et al.,* 2006). Common carp is the third-most important freshwater-farmed fish in the world, with more than 90% cultured in Asia (Rahman, 2015) due to its ability to tolerate various environmental conditions and significant economic value (Ahmed *et al*., 2023). Common carp is an omnivorous fish that primarily feeds on benthic macroinvertebrates, zooplankton, and detritus (Rahman, 2015). An inadequate diet can induce stress, cause diseases, and impair haematological parameters in cultured fish (Mansour and Esteban, 2017). As there is paucity of information on the effect of the Azolla supplementation on overall performance of common carp, the present study was undertaken, with the objective to evaluate the performance of Azolla meal supplementation on growth performance, feed utilisation, biochemical composition and haematological parameters in common carp fry.

Materials and methods

Experimental design

The experiment was executed in 15 Fiberglass-reinforced plastic (FRP) tanks of 500 l capacity at the wet Laboratory of the ICAR-Research Complex for Eastern Region (ICAR-RCER), Patna, India. All the tanks were thoroughly washed with 10 ppm of $KMnO_4$ solution and then dried under the sunlight. The experimental was set up in completely randomised design with continuous aeration in outdoor condition. After setting the experimental tanks, 10 fishes (length 34.33±1.61 mm and weight 2.50±0.05 g) were stocked in each tank hoding 400 l de-chlorinated freshwater (Bharti *et al.,* 2016). Azolla was cultured at ICAR-RCER, Patna, in a 500 l circular FRP tank. Azolla was harvested and sun-dried to reduce the bulk weight, followed by drying at 40°C in a hot air oven for 24 h. The dried Azolla were ground in an electric grinder and passed through a 0.5 mm mesh size sieve to prepare Azolla meal. All feed ingredients used in this study except Azolla meal were obtained from commercial suppliers. Five fish feed treatments with 0, 10, 20, 30 and 40% Azolla meal were prepared, namely T0, T1, T2, T3 and T4, respectively (Gangadhar *et al.*, 2015) as shown in Table 1. To prepare fish diets, all feed ingredients were dried first, ground to a small particle size in an electric grinder and sieved through a 0.5 mm mesh size. The ingredients weighed, thoroughly mixed manually, and a stiff dough was made by slowly adding water. The wet mixture was steamed at high flame in a pressure cooker for 15 min and after cooling the dough, mustard oil, vitamins and mineral mixture were added. The cooked dough was then passed through a hand pelletizer having 2.0 mm mesh dia, to get uniform fish pellets. The pellets were then dried overnight at 55°C in a hot air oven. After drying, the diets were broken into small pellet sizes, packed in plastic bags and stored in a dry and cool place to feed the experimental fish. The experiment was conducted in triplicate for 90 days from July to October 2022. The feeding of fishes with prepared diet was done in the morning hours daily (10:00 hrs) at 8, 6 and 4% of the body weight in the first, second and third months, respectively. Water quality parameters in the experimental tanks were monitored at monthly intervals, in the morning hours, and subsequently in the afternoon fishes were transferred to plastic buckets holding freshwater for length-weight measurements. During this time, water was completely siphoned out from the tanks, left over feed and faecal matter from each tank were removed, thoroughly cleaned, then refilled with clean freshwater and fishes were back to their respective tanks.

Table 1. Feed formulation for common carp fry

Ingredients (g)	Treatments				
	T0	T1	T2	T3	T4
Azolla meal	0	100	200	300	400
Mustard oil cake	400	300	200	100	0
Maize flour	100	100	100	100	50
Rice bran	200	200	200	200	200
Wheat flour	170	170	170	170	170
Maida	80	80	80	80	130
Vitamins and mineral mixture (Agrimin Forte, Virbac)	15	15	15	15	15
Mustard oil	30	30	30	30	30
Salt (Food grade commercial salt)	5	5	5	5	5
Total	1000	1000	1000	1000	1000

Water quality analysis

Samplings for water quality parameters were carried out in morning hours at monthly intervals. Water temperature was measured using a thermometer (G H Zeal Ltd., London, England). Water pH was estimated using a portable digital pH meter (Hana, China). Water samples were titrated against standard EDTA solution in the presence of ammonium chloride and ammonium hydroxide as a buffer and Eriochrom Black-T indicator for the hardness estimation. The alkalinity of the water sample was determined titrimetrically using standard sulphuric acid in the presence of phenolphthalein and methyl orange as indicators. Dissolved oxygen (DO) in water samples was estimated using Winkler's method. The estimation of ammonium-N and nitrite-N was carried out spectrophotometrically (Thermospectronic, UV Cambridge, UK) using the phenate method and Griess diazotisation reaction (sulphanilic acid and N-alpha-naphthyl ethylenediamine) method, respectively (APHA, 2005).

Growth parameters

All fish from each experimental tank were sampled every month to analyse the growth parameters in terms of percentage weight gain and specific growth rate (SGR). The feed conversion ratio (FCR) was also calculated. For estimation of feed consumption, total feed given to the fishes were taken into consideration. The length and weight of fish was measured using graduated scale and electronic balance (accuracy of 0.01 g), respectively. The number of live fish in each tank was counted to estimate the survival rate. The following formulae were used to analyse fish growth parameters:

Weight gain (%) = [(Final weight (g) - Initial weight (g)]/Initial weight (g)] ×100

SGR (%/day) = [(ln Final weight (g) - ln Initial weight (g)]/No. of days] ×100

FCR (%) = Total feed consumed (Dry weight)/Total body weight gain (Wet weight)

Survival rate (%) = [No. of fish harvested/No. of fish stocked] ×100

Length-weight relationship

The length and weight relationship was established for cultured fish using the formula $W = aL^b$, where "W" denotes the weight (g) of fish and "L" indicates the total length (mm) of fish, and "a" and "b" are parameters.

Haematological parameters

Blood was collected in EDTA-coated blood collection tubes by puncturing the caudal vein using a tuberculin medical syringe. Fish was anesthetised with clove oil (MERCK, Germany) @ 50 µl per litre of water before taking blood. Haemocytometer was used for red blood cells (RBC) and white blood cell (WBC) counts. The haemoglobin (Hb) level of blood was analysed by the cyanomethemoglobin method using Drabkin's Fluid (Qualigens). The absorbance was measured using a spectrophotometer at a wavelength of 540 nm. The final concentration was calculated by comparing it with the standard cyanomethemoglobin (Qualigens Diagnostics). The haematological indices as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated using standard formulae (Kumar and Banerjee, 2016).

Proximate composition analysis

The proximate composition of fish carcasses was analysed at the end of the experiment using standard methods (AOAC, 1995). Initially for moisture content and also for estimating other parameters, samples were dried in a hot air oven at 60˚C until constant weight. Nitrogen content in dried samples was estimated by digestion and distillation in a Micro-Kjeldahl unit (KEL Plus Classic DX VA, Pelican Equipment, India), followed by titration. Crude protein was calculated by multiplication of the nitrogen content with a factor of 6.25. Fat content in samples was estimated by Soxhlet apparatus using petroleum ether (boiling point 40-60˚C) as the solvent. Percentage ash content (dry weight basis) for each treatment were analysed in a muffle furnace separately at 550˚C for 16 h in silica crucibles (Liu, 2019).

Data analysis

The growth, proximate composition and haematological data were compared using one-way analysis of variance (ANOVA) at 5% level of significance. Treatment mean difference was analysed by Duncan Multiple Range Test (DMRT) using 'agricolae' package in R(R-4.3.1). Length-weight relationship and growth analysis were performed using R software. Principal component analysis (PCA) was performed to find out the variation in the proximate composition in fish feed and fish carcass, and the blood parameters using 'factoextra' package in R (Sumi *et al.*, 2023).

Results and discussion

Water quality parameters

In the present study, water temperature (29.41 to 29.75°C), pH (7.57 to 7.82), dissolved oxygen (5.56 to 7.64 mg l^{-1}), total alkalinity (198.83 to 228.17 mg l^{-1}), hardness (224.50 to 246.25 mg l^{-1}), ammonium-N (0.10 to 0.22 mg l^{-1}) and nitrite-N (0.08 to 0.13 mg l^{-1}) were estimated from each treatment (Table 2). There was no significant difference ($p \leq 0.05$) in temperature, pH, dissolved oxygen (DO), alkalinity, hardness, ammonium ion and nitrite in the water during the study period and all the parameters were within the conducive range for the growth of common carp (Gangadhar *et al.*, 2015; Das *et al.*, 2021). Though in the present study, a higher range of alkalinity (198.83-228.17 mg l^{-1}) and hardness (224.50-246.25 mg l^{-1}) were observed. As per the report of Bhatnagar and Devi (2019), both alkalinity and hardness above 300 mg l^{-1} is generally lethal to fish except for some euryhaline species. Similarly, Iffat *et al*. (2020) reported that common carp can tolerate high range of hardness (420-3250 mg l^{-1}).

Table 2. Average water quality parameters estimated from different treatments

Parameters	Treatments (%)				
	T0	T1	T2	T3	T4
Temperature (°C)	29.48±0.21^a	29.75±0.43^a	29.41±0.26^a	29.56±0.40^a	29.59±0.39^a
pH	7.57±0.20^a	7.82±0.17^a	7.72±0.17^a	7.65±0.15^a	7.65±0.14^a
Alkalinity (mg l^{-1})	215.42±27.5^a	218.33±22.7^a	198.83±27.3^a	228.17±29.7^a	212.33±24.1^a
Hardness (mg l^{-1})	224.50±14.3^a	231.33±11.4^a	230.67±8.94^a	246.25±13.5^a	246.08±13.9^a
DO (mg l^{-1})	5.78±0.47^a	6.27±0.71^a	6.62±0.41^a	5.56±0.79^a	7.64±0.10^a
Ammonium-N (mg l^{-1})	0.10±0.04^a	0.12±0.04^a	0.22±0.07^a	0.17±0.06^a	0.19±0.04^a
Nitrite-N (mg l^{-1})	0.09±0.02^a	0.08±0.01^a	0.08±0.01^a	0.08±0.01^a	0.10±0.04^a

Data are expressed as mean±SE. Different superscripts in the same row indicate significant difference at 5% level.

Growth parameters

In the present study, the results indicated that increased Azolla meal in diet significantly enhanced percentage weight gain as well as SGR and reached the highest level at 30% Azolla incorporation in diet (Table 3). Analysis of periodic growth of fish indicates that fishes at T4 treatment grow slowly up to the second month of rearing and in the third month, fishes showed much faster growth compared to first and second months of rearing (Fig. 1). However, in comparison to the present study, Gangadhar *et al.* (2017) reported better utilisation of Azolla meal by common carp after two months of the culture with 20% Azolla supplementation. Rahman (2015) stated that common carp shows seasonal change and ontogenetic dietary shifts in its feeding habits. Similar to the present study, Das *et al.* (2004) also reported that Azolla can effectively substitute ground nut oil cake in the conventional diet of Indian major carps by about 30%, without any adverse effects on their survival, growth rate and muscle protein content. This results also support the previous findings that up to 40% level of Azolla inclusion significantly increased growth of carps (Das *et al.*, 2004; Kouberi Nath *et al*., 2022). Similarly, the current study also indicated that variation of Azolla meal in the diet did not affect ($p \leq 0.05$) survival rate (80.00-93.33%) which was also previously reported by many researchers (Panigrahi *et al*., 2014; Gangadhar *et al.*, 2015). Similar to this result, a high growth performance, maximum survival rate and low FCR in *Labeo fimbriatus*, *Labeo rohita*, and *Cirrhinus mrigala* were reported by Datta (2011), Gangadhar *et al.* (2014) and Panigrahi *et al.* (2014) at 40% Azolla meal diets. According to Magouz *et al.* (2020), Azolla meal has beneficial effects on the digestive enzymes. For the length-weight relationship, the "b" value expressed a negative allometric growth ($b<3$) as the "b" value varied between 2.1 (T1) and 1.6 (T3) (Fig. 2) as previously observed by Datta (2011) and Datta *et al.* (2013). However, the deviation of growth pattern from cube law is caused by biological factors such as degree of stomach fullness, gonad maturity, sex, health, food availability and fatness of the species, as well as physicochemical factors of culture environment (Hossain *et al*., 2016, Andrabi *et al*., 2021). In this study clearly common carp fry exhibited negative allometric growth up to 40% Azolla meal in the diet.

Haematological parameters

The results indicated that the Hb, MCV, PCV and RBC of common carp significantly ($p \leq 0.05$) decreased with increase in dietary Azolla meal up to 30% level, but increased with rise in Azolla meal in the diet (40%). At the same time, WBC levels increased with increasing levels of Azolla meal in the diet up to 30% and then decreased

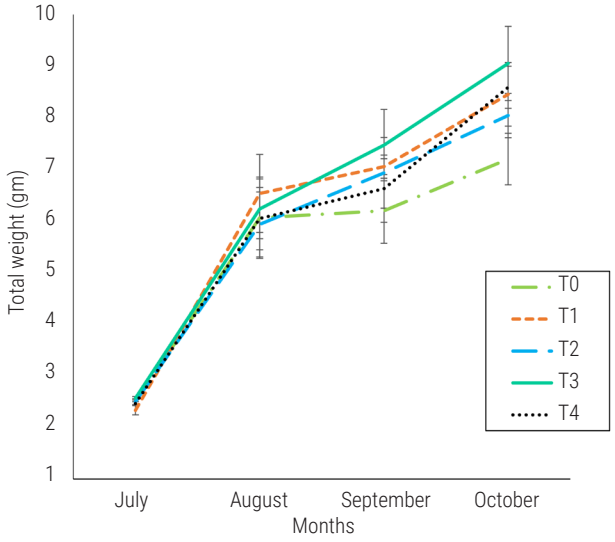

Fig. 1. Weight gain in common carp during 90 days of culture

(Table 4). PCA biplot of fish blood parameters indicated that the first principal component explained (Dim 1) 63.5% and the second principal component explained (Dim 2) 27.2% variability of the data. RBC, PCV, MCV, Hb and WBC showed maximum variability and highly correlated with the first principal component while MCH and MCHC had less variability and correlated with the second principal component, RBC showed a positive correlation with Hb, while negative correlation with WBC (Fig. 3a). The stress that fish experience when handled for sampling could be the reason for this fluctuation in haematological parameters. A significant increase in the number of WBC was associated with stressful situation in fish (Abhijith *et al*., 2012). However, according to Ahmed and Ahmad (2020), dietary nutrients in fish influence haematological parameters.

Proximate composition

PCA biplot for feed showed that the first principal component explained 59.4% and the second principal component explained 30.9% data variability. Fat, ash and protein in feed showed maximum variability and highly correlated with the first principal component and moisture had less variability and correlated with the second principal component (Fig. 3b). PCA biplot of fish carcass showed that the first principal component explained 38.2% and

Table 3. Growth, survival rate, and feed conversion ratio of common carp in different treatments

Parameters	Treatments				
	T0	T1	T2	T3	T4
Initial weight (g)	2.40 ± 0.10^{a}	2.33 ± 0.15^{a}	2.40 ± 0.10^{a}	2.33 ± 0.15^{a}	2.36 ± 0.15^{a}
Final weight (g)	7.12 ± 0.11^{b}	7.00 ± 0.44^{b}	8.05 ± 0.30^{ab}	8.72 ± 1.49^{a}	8.20 ± 1.28^{ab}
Weight gain (%)	197.19 ± 15.98^{b}	200.48 ± 11.83^{b}	235.49 ± 2.45^{ab}	273.30 ± 53.46^{a}	247.99 ± 58.66^{ab}
SGR (% day^{-1})	1.20 ± 0.06^{a}	1.22 ± 0.04^{a}	1.34 ± 0.01^{a}	1.45 ± 0.17^{a}	1.37 ± 0.19^{a}
Survival rate (%)	93.33 ± 3.33^{a}	90.00 ± 10.00^{ab}	86.00 ± 3.33^{ab}	80.00 ± 5.77^{b}	83.33 ± 3.33^{ab}
FCR (%)	1.57 ± 0.16^{a}	1.37 ± 0.09^{ab}	1.35 ± 0.06^{ab}	1.25 ± 0.23^{b}	1.29 ± 0.17^{ab}

Data are expressed as mean±SE. Different superscripts in the same row indicate significant difference at 5% level.

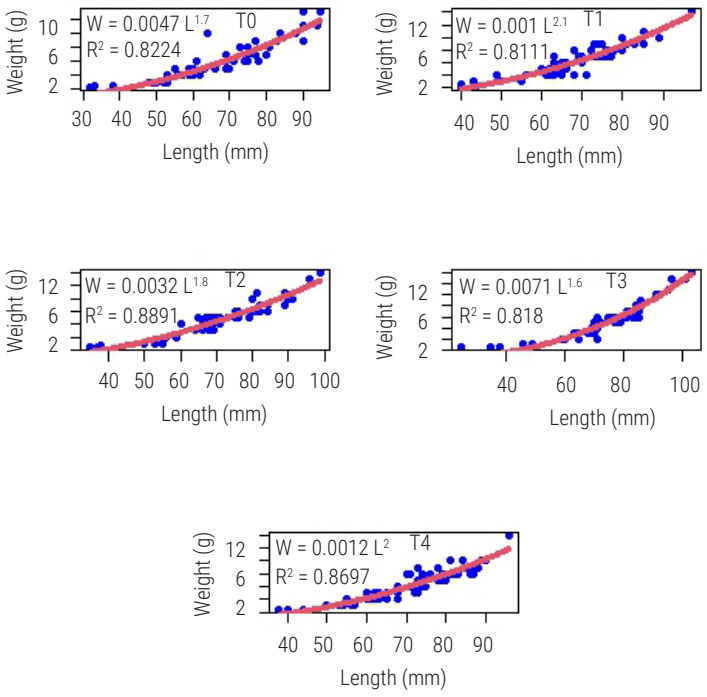

Fig. 2. Length-weight relationship in experimental fish

Table 4. Haematological parameters of experimental fish in different treatments

Parameters	Treatments				
	T0	T1	T2	T3	T4
Hb (g dl^{-1})	7.17±0.20$_{a}$	5.90±0.45$_{ab}$	4.80±0.56$_{b}$	4.67±0.24$_{b}$	6.07±0.94$_{ab}$
MCH (g dl^{-1})	28.27±0.32	27.60±0.38	28.97±2.98	25.90±0.61	26.90±1.11
MCHC (pg)	33.67±0.33$_{a}$	34.00±0.00$_{ab}$	38.33±4.33$_{b}$	33.00±0.58$_{ab}$	32.00±0.58$_{a}$
MCV (fl)	84.17±0.43$_{a}$	81.10±1.10$_{abc}$	75.53±2.23$_{c}$	77.73±0.69$_{bc}$	84.27±4.39$_{ab}$
PCV (%)	21.33±0.67$_{a}$	17.33±1.33$_{abc}$	12.67±1.67$_{c}$	14.00±0.58$_{bc}$	19.00±3.00$_{ab}$
RBC (million mm^{-3})	2.53±0.07$_{b}$	2.13±0.13$_{b}$	1.67±0.17$_{a}$	1.80±0.06$_{a}$	2.23±0.27$_{b}$
WBC (Thousand mm^{-3})	31.47± 34.29	49.53±31.4	84.67± 32.60	86.00±34.16	42.67±35.20

Data are expressed as mean±SE. Different superscripts in the same row indicate significant difference at 5% level.

second principal component explained 27.2% variability of the data. Fat, ash and protein in fish showed maximum variability and highly correlated with the first principal component, and moisture had less variability and correlated with the second principal component (Fig. 3c). According to PCA, the formulated fish feed and fish carcass showed similar patterns in terms of fat, ash, protein and moisture level variability among treatments. Nekoubin and Sudagar (2013) stated that feeding grass carp with Azolla meal significantly affected the lipid content in its carcass. In the present study also, the best protein and fat in fish carcass were obtained at 40% Azolla meal and the highest protein content in the formulated diet was obtained at 30% incorporation of Azolla meal (Table 5). In general, the body fat content gradually increases with increase in dietary protein level because the excess dietary protein content in these diets gets deaminated and deposited as body fat (Ahmed and Ahmad, 2020). However, El-Sayed (2008) reported that protein and lipid contents in fish carcasses are negatively correlated with Azolla meal levels in the diets, while body ash content is positively correlated in Nile tilapia. According to Goswami *et al.* (2022), fish-fed a duckweed based diet exhibited higher lipid content compared to those fed a diet without duckweed. Similar to previous studies, these findings suggests that the protein level in the diet plays a key role in both growth and fat deposition in fish, with high protein diets leading increased body fat (Ahmed and Ahmad, 2020). However, Datta (2011) reported that protein content in *L. rohita* remained unaffected by the incorporation of Azolla meal in the diet, even when incorporated at levels ranging from 15 to 35%.

The results of the study clearly indicate that optimal growth performance, protein content in fish carcasses and haematological parameters can be achieved with a 30% Azolla meal diet for common carp fry. As Azolla meal diet has been shown to enhance immune functions of common carp (Magouz *et al.*, 2020), it can effectively replace 30% of MOC in common carp diet resulting in superior health and meat quality. However, this has to be further

Table 5. Proximate composition of fish carcass and fish feed (on dry weight basis)

Components	Parameters (%)	Treatments				
		T0	T1	T2	T3	T4
Fish carcass	Moisture	71.18±1.08^{a}	71.42±1.24^{a}	73.30±2.02^{a}	72.03±0.90^{a}	73.34±0.85^{a}
	Protein	46.45±2.77^{ab}	50.81±0.76^{a}	40.71±1.28^{c}	44.68±2.44^{bc}	51.88±0.57^{a}
	Fat	22.98±2.14^{b}	39.43±1.43^{a}	31.84±1.48^{ab}	34.24±1.81^{a}	38.74±5.51^{a}
	Ash	88.77±0.95^{b}	91.66±0.39^{a}	89.42±0.25^{ab}	88.62±0.09^{b}	89.04±0.81^{ab}
Fish Feed	Moisture	3.44±0.29	4.85±1.20	5.34±0.60	5.54±1.15	3.91±0.29
	Protein	22.33±0.30^{d}	23.76±0.21^{b}	23.49±0.18^{bc}	24.58±0.26^{a}	23.23±0.22^{c}
	Fat	13.79±0.18^{a}	14.01±0.15^{a}	12.14±0.25^{b}	8.67±0.13^{c}	6.33±0.17^{d}
	Ash	92.07±0.08^{a}	90.92±0.05^{b}	89.02±0.12^{c}	87.64±0.19^{d}	86.96±0.01^{e}

Data are expressed as mean±SE. Different superscripts in the same row indicate significant difference at 5% level.

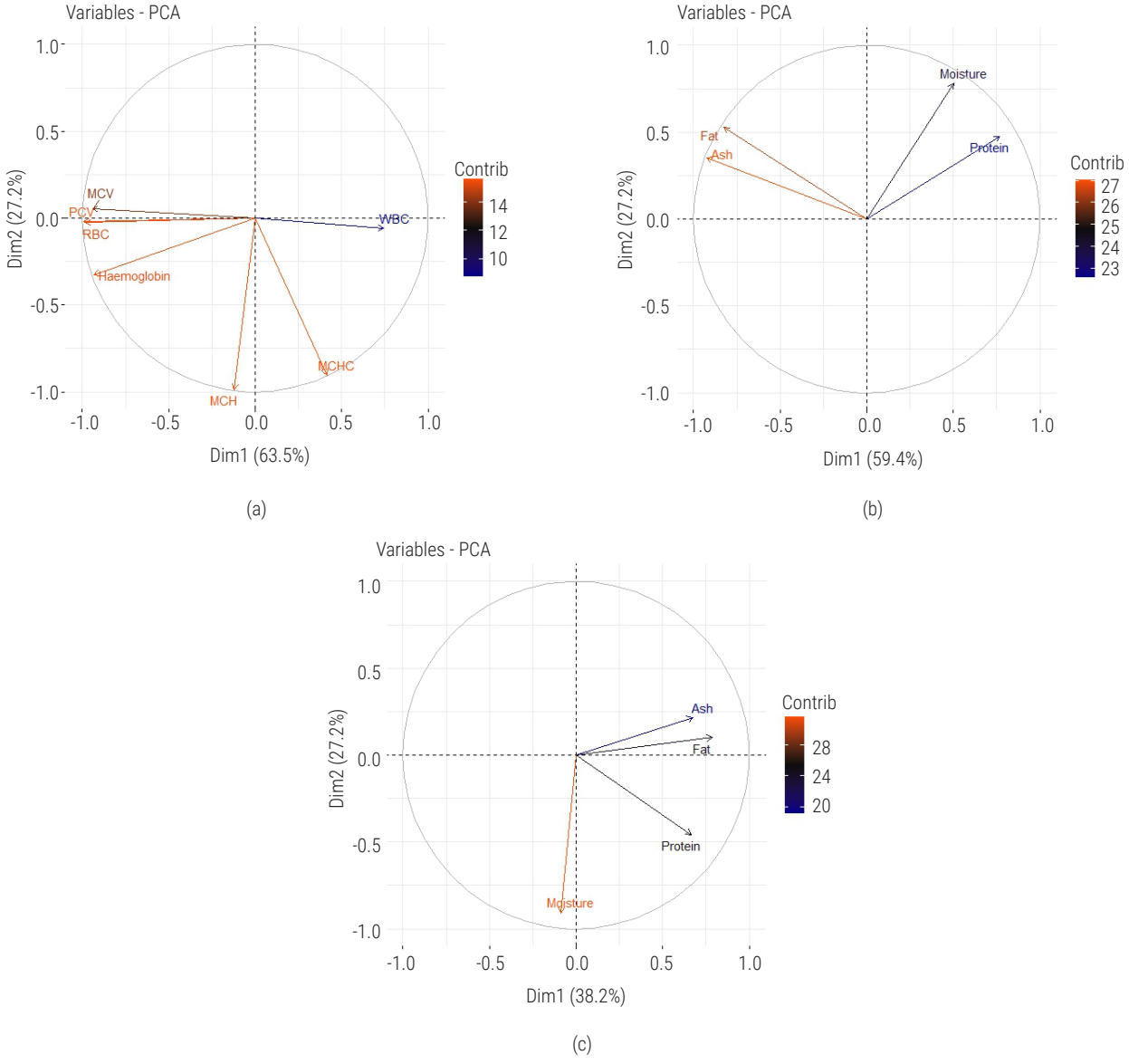

Fig. 3. PCA Biplot of (a) blood parameters, (b) proximate composition in feed and (c) fish carcass

confirmed by studying the digestibility of Azolla meal and effect of anti-nutritional factors at different stages of growth of common carp.

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