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Original Research Article

Interaction of dietary replacements of fishmeal by protein blend and feeding frequency on growth performance and protein utilization of gibel carp (*Carassius gibelio* var. CAS V)

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ABSTRACT

Feeding frequency represents a potential strategy to improve the utilization of protein sources by fish. This study investigated its impact on the utilization of protein blend in gibel carp. The dietary fishmeal was totally substituted with three protein blends consisting of *Tenebrio molitor* meal, *Chlorella* meal, *Clostridium autoethanogenum* protein, cottonseed protein concentrate, at ratios of 1:1:8:2, 1:1:6:4, and 1:1:4:6, respectively. During an 8-week feeding trial, a total of 960 healthy fish (18.10 g) were randomly assigned to eight groups, each with three replicates. Then they were fed either twice daily (two meals per day) or four times daily (four meals per day) with four different diets. Higher feeding frequency increased feed intake and intestinal trypsin activity ($P < 0.05$), and up-regulated the expression levels of genes related to amino acid or peptide transporter (*pept1*, *y*$^{+}$*lat2*) and sensory receptors (*casr*, *gprc6a*, *mglur4*) in intestine ($P < 0.05$). Moreover, it accelerated muscle protein turnover by increasing free amino acid content, aspartate aminotransferase activity and *akt1* transcript levels ($P < 0.05$), ultimately promoting growth. However, higher feeding frequency reduced protein apparent digestibility and feed efficiency ($P < 0.05$). Dietary blended proteins elevated trypsin and chymotrypsin activities ($P < 0.01$). Notably, the adverse effects observed with blended proteins (ratio at 1:1:8:2) on total essential amino acid digestibility and muscle protein metabolism-related gene expression were mitigated with increased feeding frequency, thus alleviating growth inhibition. Furthermore, the blended proteins at a ratio of 1:1:6:4 increased protein apparent digestibility ($P < 0.05$), down-regulated *mstn* expression level ($P < 0.05$), and up-regulated expression levels of genes related to protein synthesis (*akt1*, *mtor*, *s6k1*, *eif4b*, *eif4e*; $P < 0.05$); thereby promoting protein utilization and muscle growth at four meals per day. Overall, feeding frequency interacted synergistically with blended proteins to influence growth and protein utilization in gibel carp, and a protein blend with a ratio of 1:1:6:4 was a superior alternative to fishmeal at both feeding frequencies. Future strategies aimed at replacing dietary fishmeal should consider the role of feeding frequency as a critical factor.

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1. Introduction

Gibel carp (*Carassius gibelio*), an omnivorous fish, contributed 2.85 million tonnes to aquaculture production in China in 2022, with production continuing to rise (Bureau of Fisheries of the Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2023). To sustain this growth, ensuring a sufficient

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supply of high-quality protein sources is crucial. Compared to the single protein source, dietary protein blends with balanced amino acids have demonstrated superior growth performance and protein utilization in aquaculture species (Li et al., 2021; Shi et al., 2017; Xue et al., 2012). In our previous study, blends of *Tenebrio molitor* meal (TMM), *Chlorella* meal (CM), *Clostridium autoethanogenum* protein (CAP), and cottonseed protein concentrate (CPC) at appropriate ratios achieved amino acid balance and improved intestine health, protein utilization, and growth in gibel carp as a substitution for fishmeal (Yu et al., 2024). In addition, these non-food protein sources offer environmentally friendly, resource-saving attributes with high supply potentials (Chen et al., 2018; Kotrbáček et al., 2015; Van Huis, 2013; Xue, 2021). In fact, the utilization of protein by fish is influenced not only by protein properties but also by feeding management, density, temperature, etc (Busti et al., 2020; Lu et al., 2020; Sun et al., 2018).

Feeding frequency is a paramount facet of feeding management that significantly impacts feed intake, growth, and feed efficiency in fish (Basto-Silva et al., 2022; Biswas et al., 2023; Wu et al., 2021). Studies have extensively examined the independent effects of feeding frequency on fish growth. Insufficient feeding frequency can lead to reduced nutrient intake and hinder fish growth (Liu et al., 2022; Wu et al., 2021). Conversely, increasing feeding frequency appropriately can promote fish growth by increasing feed intake (Basto-Silva et al., 2022; Wang et al., 2022) and improving feed efficiency (Biswas et al., 2023; Gao et al., 2022). Feeding frequency also influences the digestion rate of different protein sources, affecting the simultaneous absorption of amino acids in fish such as channel catfish (*Ictalurus punctatus*) (Ambardekar et al., 2009). Protease activity plays a crucial role in protein digestion rate determination (Estevão-Rodrigues et al., 2024; Zuo et al., 2015), and increasing feeding frequency has been shown to effectively enhance protease activity in aquatic animals (Busti et al., 2020; Xie et al., 2011; Xu et al., 2020). Furthermore, increasing feeding frequency has been demonstrated to mitigate the growth inhibition caused by replacing dietary fishmeal with soybean meal in gibel carp, attributed to increased feed intake, improved feed efficiency, and regulated amino acid absorption (Zhao, 2014). It was also reported in grass carp (*Ctenopharyngodon idellus*) that while dietary CM did not affect the protein efficiency ratio with feeding frequency increasing from two to four meals per day, dietary substitutes like soybean meal, TMM, CAP, and CPC promoted protein efficiency ratios and growth (Xia, 2022). These findings underscore the potential of increased feeding frequency to improve the utilization of substituted protein sources by fish.

Proteins digested and absorbed by fish are primarily transported to various tissues in the form of free amino acids (FAA) through blood circulation. Feeding frequency is an important factor influencing plasma levels of FAA (Cleveland and Burr, 2011; Zhao, 2014). Additionally, the increment in circulating FAA can improve growth performance by stimulating protein synthesis (Tome, 2022; Wu, 2018). The protein utilized for growth demand is mainly represented by its deposition in the fish. Protein deposition, predominantly in muscle, is regulated by synthesis and degradation pathways, where FAA content in muscle reflects protein synthesis via the mammalian target of rapamycin (mTOR) pathway (Mai et al., 2022; Tome, 2022). Muscle RING finger 1 (MuRF1) and muscle atrophy F-box (Fbxo32) are E3 ubiquitin ligases of ubiquitin-proteasome system (UPS) which regulate protein degradation (Sun et al., 2018; Yoshida and Delafontaine, 2020). However, research on the impact of feeding frequency on muscle protein metabolic processes remains limited. Therefore, the present study used blended TMM, CM, CAP, and CPC with different ratios to totally replace dietary fishmeal at two feeding frequencies, exploring the role of feeding frequency in the utilization of blended proteins and finding the optimal blend according to growth and protein utilization of gibel carp. The results will contribute theoretical bases for further improving substitution levels of dietary fishmeal by increasing feeding frequency and using blended protein sources containing balanced amino acids.

2. Materials and methods

2.1. Animal ethics statement

All experimental animal care protocols were approved by the ethic committee of the Institute of Hydrobiology, Chinese Academy of Sciences (approval: IHB20140724).

2.2. Feeding trial and sample collection

Gibel carp were obtained from a *C. gibelio* production base (Huangshi, China). The trial was conducted in pond cages (7.2 m^3; Jingzhou, China). Diet 1 was formulated with 15% fishmeal as control, while the blended proteins (TMM:CM:CAP:CPC) at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. All of the ingredients were well-ground, thoroughly mixed, and then pelleted in a diameter of 2 mm using an extruder (SLP-45; Fishery Machinery and Instrument Research Institute, Chinese Academy of Fishery Sciences, Shanghai, China). Then, pellets were oven-dried at 60 °C and stored at 4 °C. Tables 1 and 2 shows the particulars of the experimental diets. All fish were fed up to apparent satiation with control diets thrice a day (07:00, 12:00, 17:30) for two weeks. Then all the fish were fasted for 24 h. 960 healthy fish (18.10 g) were preferred and distributed into 24 cages (three cages for each diet) at random. Fish with low feeding frequency (LF) were fed at 07:00 and 17:30 for 56 days, and fish with high feeding frequency (HF) were fed at 07:00, 10:30, 14:00, and 17:30, for the same duration. Details of husbandry included; photoperiod: natural light; water temperature: 31.6 ± 2.4 °C; water ammonia nitrogen <0.2 mg/L; dissolved oxygen >5 mg/L; pH: 7.4 to 7.6.

Following completion of the 56-day trial, all fish were fasted for 12 h, anesthetized with 50 mg/L MS-222 (Sigma, USA), then bulk weighed. Two fish were taken from each cage to analyze whole-body composition. The other two fish had blood drawn from a vein with heparinized syringes and the blood was centrifuged (850 × *g*, 15 min) to obtain plasma, then the intestine and muscle tissues were gathered and quickly frozen in liquid nitrogen. The plasma samples, intestine tissues, and muscle tissues were stored at −80 °C. Another three fish were collected to calculate body index.

2.3. Digestibility determination

The unsampled fish of the feeding trial were acclimated in the recirculating aquaculture system (Wuhan, China) for two weeks. Fish were raised in cylindrical fiberglass tanks (1.06 m^3) with continuous aeration (photoperiod: 14 h light and 10 h dark; pH: 7.0–7.2; dissolved oxygen >5 mg/L; water temperature: 29.2 ± 1.3 °C; water ammonia nitrogen <0.2 mg/L). They were fed with the diets and feeding frequencies consistent with those of the feeding trial. The addition of 0.1% yttrium trioxide to feed was an indirect indicator for digestibility determination. Fecal samples were collected by siphoning at 4 h postprandial and were then freeze-dried for digestibility determination.

Table 1
Ingredients and proximate composition of the experimental diets.

Item	Blended ratios (TMM:CM:CAP:CPC)			
	0:0:0:0 (diet 1)	1:1:8:2 (diet 3)	1:1:6:4 (diet 3)	1:1:4:6 (diet 4)
Ingredients, % of dry matter				
Fishmeal[1]	15.00	0.00	0.00	0.00
TMM[2]	0.00	1.14	1.20	1.26
CM[3]	0.00	1.14	1.20	1.26
CAP[4]	0.00	9.12	7.20	5.04
CPC[5]	0.00	2.28	4.80	7.56
Rapeseed meal[6]	25.00	25.00	25.00	25.00
Soybean meal[7]	26.00	26.00	26.00	26.00
Corn starch	15.00	15.00	15.00	15.00
Microcrystalline cellulose	4.38	4.70	3.98	3.26
Fish oil	3.00	3.50	3.50	3.50
Soybean oil	3.00	3.50	3.50	3.50
Mineral premix[8]	5.00	5.00	5.00	5.00
Vitamin premix[9]	0.39	0.39	0.39	0.39
Sodium benzoate	0.02	0.02	0.02	0.02
Sodium carboxymethyl cellulose	3.00	3.00	3.00	3.00
Choline chloride	0.11	0.11	0.11	0.11
Yttrium trioxide	0.10	0.10	0.10	0.10
Proximate composition, % of dry matter				
Crude protein	34.25	34.20	34.04	33.47
Crude lipid	7.66	7.75	7.07	7.33
Ash	10.26	9.41	9.08	9.26
Gross energy, MJ/kg	18.05	18.45	18.27	17.96

TMM = *Tenebrio molitor* meal; CM = *Chlorella* meal; CAP = *Clostridium autoethanogenum* protein; CPC = cottonseed protein concentrated.

[1] Fishmeal: TASA, Fish Product Co. Ltd, Peru. Crude protein: 72.63% of dry matter. Crude lipid: 7.98% of dry matter.

[2] TMM: Guangdong Zehecheng Biotechnology Co., Ltd, Guangzhou, China. Crude protein: 76.83% of dry matter. Crude lipid: 1.11% of dry matter.

[3] CM: Demeter Bio-Tech Co., Ltd., Wuhan, China. Crude protein: 63.00% of dry matter. Crude lipid: 6.86% of dry matter.

[4] CAP: Hebei Shoulang Novel Energy Technology Co., Ltd, Tangshan, China. Crude protein: 85.82% of dry matter. Crude lipid: 0.35% of dry matter.

[5] CPC: Xinjiang Jinlan Plant Protein Co., Ltd, Xinjiang, China. Crude protein: 66.70% of dry matter. Crude lipid: 0.45% of dry matter.

[6] Rapeseed meal: Wuhan Coland Feed Co. Ltd, Wuhan, China. Crude protein: 41.81% of dry matter. Crude lipid: 3.52% of dry matter.

[7] Soybean meal: Wuhan Coland Feed Co. Ltd, Wuhan, China. Crude protein: 53.22% of dry matter. Crude lipid: 1.34% of dry matter.

[8] Mineral premix (mg/kg diet): NaCl, 500.0; $MgSO_4 \cdot 7H_2O$, 8155.6; $NaH_2PO_4 \cdot 2H_2O$, 12,500.0; KH_2PO_4, 16,000.0; $CaHPO_4 \cdot 2H_2O$, 7650.6; $FeSO_4 \cdot 7H_2O$, 2286.2; $C_6H_{10}CaO_6 \cdot 5H_2O$, 1750.0; $ZnSO_4 \cdot 7H_2O$, 178.0; $MnSO_4 \cdot H_2O$, 61.4; $CuSO_4 \cdot 5H_2O$, 15.5; $CoSO_4 \cdot 7H_2O$, 0.9; KI, 1.5; Na_2SeO_3, 0.6; corn starch, 899.7.

[9] Vitamin premix (mg/kg diet): vitamin B_1, 20; vitamin B_2, 20; vitamin B_6, 20; vitamin B_{12}, 0.02; folic acid, 5; calcium pantothenate, 50; inositol, 100; niacin, 100; biotin, 0.1; cellulose, 3522; vitamin A, 11; vitamin D, 2; vitamin E, 100; vitamin K, 10.

Table 2
Amino acid composition of the experimental diets (% of dry matter).

Item	Diet 1	Diet 2	Diet 3	Diet 4
Essential amino acids				
Lysine	2.1644	2.2620	2.2258	2.1098
Methionine	0.5599	0.5726	0.5802	0.4887
Threonine	1.3127	1.3548	1.3423	1.3317
Arginine	1.9582	2.0719	2.0547	2.0820
Leucine	2.4856	2.4548	2.5534	2.5150
Isoleucine	1.4621	1.4538	1.4643	1.4907
Valine	1.5516	1.5332	1.5496	1.6027
Phenylalanine	1.4798	1.4853	1.5196	1.5683
Histidine	0.6928	0.7272	0.6282	0.7590
Non-essential amino acids				
Asparagine	2.7664	2.7329	2.8231	2.8444
Serine	1.2910	1.3135	1.3522	1.3200
Glutamic acid	5.5653	5.6073	5.6812	5.6515
Glycine	1.4511	1.4685	1.4179	1.4491
Alanine	1.5661	1.6272	1.6123	1.5773
Tyrosine	0.9861	0.9956	1.0307	1.0406
Proline	1.5528	1.5617	1.9800	1.7391
Cysteine	2.2123	2.0924	1.7148	2.1150

Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets.

2.4. Biochemical determination

The moisture, ash, crude lipid and crude protein contents of the diets, fish, or fecal were analyzed by AOAC methods of 930.15, 942.05, 954.02 and 984.13, respectively (AOAC, 2000). The yttrium trioxide contents of diets and fecal were determined by referring to China National Standard (2014). The amino acid contents of the diets, feces, and plasma were measured by the ninhydrin post-column derivatization. The gross energy was assessed using the oxygen bomb calorimeter by the method of 9831 (ISO, 1998). More details were given in a past investigation (Yu et al., 2024).

The measurement of trypsin and chymotrypsin activities in intestines was described in a previous subject (Liu et al., 2017). The measurement of FAA, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of muscle, and protein concentration of muscle and intestine was conducted according to the instructions of the commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. qPCR analysis

The experiment analyzed the expression of key genes involved in various physiological processes: *pept1*, y^+*lat2* and *snat2* (related to amino acid or peptide transporter); *casr*, *gprc6a*, *t1r1*, *t1r3* and *mglur4* (associated with amino acid sensory receptors); *mstn*, *akt1*, *mtor*, *s6k1*, *eif4e* and *eif4b* (involved in protein synthesis); and *foxo1*, *murf1* and *fbxo32* (involved in proteolysis). RNA of muscle and intestine tissues was extracted by TRIzol reagent (Invitrogen, USA) following manufacturer's instructions and assessed RNA integrity with agarose gel electrophoresis. Then RNA was detected concentration by NanoDrop Spectrophotometer (NanoDrop Technologies, USA) and reverse transcribed by the M-MLV Synthesis Kit (Invitrogen, Shanghai, China). qPCR was carried out using LightCycle 480 II system (Roche, Basel, Switzerland). Table 3 shows the qPCR primers designed following NCBI primer BLAST service. More details were given in a previous study (Yu et al., 2024). The relative expression was calculated following Vandesompele et al. (2002). Genes *gapdh* and β-actin were serviced as the internal reference for normalizing muscle and intestine respectively.

2.6. Statistical analysis

Statistical analysis was performed using SPSS R26.0. Data were shown as mean and standard error of the mean (SEM). All data were firstly tested for normality and homogeneity of variance and then were analyzed by two-way ANOVA (significance level of $P < 0.05$) (Zare et al., 2024). The following model was used:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \alpha_i \times \beta_j + e_{ij},$$

where Y_{ij} is the observation in diet i and feeding frequency j; μ is the overall mean; α_i is the fixed effect of diet ($i = 1, 2, 3, 4$); β_j is the fixed effect of feeding frequency ($j = 1, 2$); $\alpha_i \times \beta_j$ is the interaction between diet and feeding frequency; e_{ij} is the random residual error.

When interactions between feeding frequency and diet were significant, data in different diets and two feeding frequencies were

Table 3
Primer sequences used for the analysis of mRNA expression by qRT-PCR.

Gene	GenBank accession no.	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
gapdh[1]	XM_026284269.1	AGGTCTTATGAGCACCGTTCAT	GGAGGCTGGGATAATGTTCTGA
β-actin[2]	JN006052.1	TGGGACAGAAGGACAGCTATG	AGCTCGTTGTAGAAGGTGTGA
y$^+$*lat2*	XM_026266738.1	ATCATCACTGGCCTGGTCAA	CTGTGACAATGGGCATGGAG
snat2	XM_026209285.1	TCACGATCAACACCGAGTCA	ACAGCCCAAATGTGCGAAAT
pept1	XM_026265622.1	CCGTACTCATCCTCCCCATC	TCTCGGTCTCTCCTTCCTCA
casr	AB713518.1	AACTCCTGGTCTAACGGCAA	AACACCCAACACGAAAGCTG
gprc6a	XM_026283875.1	ACGCTGTGTGTTTCATGCAT	GCAAACGATCACATACGGCT
t1r3	XM_026275959.1	TTCTGAGCAGCTGGAGAACA	CTCCACTGGACAACGCAAAA
t1r1	XM_026268818.1	TGAATGGTCTGATGAGGGCA	GTAAACACATGCTGCCACCA
mglur4	EU147495.1	CCAGTATCAGCACGACCTCT	AATCGGCGTGTCATTGTAGC
mstn	XM_026271441.1	CGCAAGACACTGTGCAATAGAA	TACATCCACGTAACGTTGGACT
akt1	XM_026286718.1	TTGTCACGAGATCGGGTTTTCT	TGATTCCCTCCTTACACAAGCC
foxo1	XM_026263271.1	AACAGCAACACCTGGGGAAA	GCCGTGAGTGGTGGTCTTTA
mtor	KF772613	TATAAGGGAAACGTGGTGGGAG	GGCTAGTGCTAGTCTTCCACTT
s6k1	EF373665.1	GCTGGAGGAGGTAAAGGACG	TCTGACACAGGTGCTGACAG
eif4b	XM_026259736.1	ACCAGGGAGATGAAGATGGAGA	GCCATCATCCCTAGAGCTACAC
eif4e	MF461722	AAAATCTGCGTCTCATCTCC	TATTCCTGTCATCCTCCCAC
murf1	XM_026290505.1	GATGCGCGTGGGTTTAGACT	TGCTGGCACGGTAGTATCAC
fbxo32	XM_026284995.1	TAATCGCTTGGACTTCTGCAGT	CTGCACAACCCTTTCCAGAATG

gapdh = glyceraldehyde-3-phosphate dehydrogenase; *y*$^+$*lat2* = Y$^+$L amino acid transporter 2-like; *snat2* = sodium-coupled neutral amino acid transporter 2; *pept1* = antigen peptide transporter 1-like; *casr* = calcium-sensing receptor; *gprc6a* = G protein-coupled receptor class C group 6 member A; *t1r3* = taste receptor type 1 member 3; *t1r1* = taste receptor type 1 member 1; *mglur4* = metabotropic glutamate receptor 4; *mstn* = myostatin; *akt1* = serine/threonine-protein kinase 1; *foxo1* = Forkhead box O1; *mtor* = mammalian target of rapamycin; *s6k1* = ribosomal protein S6 kinase 1; *eif4b* = eukaryotic translation initiation factor 4B; *eif4e* = eukaryotic translation initiation factor 4E; *murf1* = muscle RING-finger 1; *fbxo32* = muscle atrophy F-box.
[1] The reference gene for muscle tissues.
[2] The reference gene for intestine tissues.

compared with Duncan's multiple tests and independent samples *t*-test, respectively. When interactions were not significant, treatments were compared in pooled data. If the “diet effect” or “feeding frequency effect” were significant, it was reported “main effect” and the pooled data were compared with Duncan's multiple tests and independent samples *t*-test, respectively (Table 4).

3. Results

3.1. Growth performance

The different treatment manipulations did not significantly change the survival rate (Table 5). Feed intake (FI) increased

Table 4
The results of two-way ANOVA analysis with SPSS.

Item	*P*-value			Main effects (mean)					
	Feeding frequency Effect	Diet effect	Interaction	Feeding frequency of two meals per day	Feeding frequency of four meals per day	Diet 1	Diet 2	Diet 3	Diet 4
FI	<0.001	0.085	0.549	14.09	19.61				
ADC of dry matter	<0.001	<0.001	0.695	57.50	51.99	53.49^{bc}	55.19^{b}	57.87^{a}	52.44^{c}
TAA-plasma	<0.001	0.333	0.655	44.00	50.15				
TEAA-plasma	<0.001	0.395	0.631	28.47	32.44				
TNEAA-plasma	0.006	0.324	0.751	8.48	9.79				
Lysine-plasma	<0.001	0.044	0.701	3.31	3.98	3.79^{a}	3.74^{a}	3.80^{a}	3.24^{b}
Arginine-plasma	0.002	0.503	0.261	3.59	4.04				
Leucine-plasma	0.013	0.763	0.234	3.71	4.07				
Isoleucine-plasma	0.008	0.655	0.139	1.90	2.15				
Valine-plasma	0.001	0.666	0.134	4.41	4.97				
Glycine-plasma	0.027	0.425	0.897	3.31	3.76				
Alanine-plasma	0.014	0.530	0.856	5.52	6.49				
Trypsin	0.046	<0.001	0.335	694.86	862.54	227.4^{c}	1174.06^{a}	708.66^{b}	1004.68^{a}
Chymotrypsin	0.737	0.001	0.873			428.28^{b}	1149.72^{a}	814.66^{a}	969.49^{a}
ALT	0.128	0.009	0.259			4.42^{a}	3.39^{b}	2.92^{b}	3.41^{b}
AST	0.006	0.002	0.203	16.87	18.45	19.68^{a}	17.19^{b}	16.75^{b}	17.02^{b}
FAA	0.012	0.017	0.067	4.37	4.81	4.48^{b}	4.22^{b}	5.01^{a}	4.64^{ab}
pept1	<0.001	0.001	0.338	1.97	2.97	1.85^{b}	2.21^{b}	2.49^{b}	3.31^{a}
y$^+$*lat2*	0.005	0.750	0.778	1.15	1.48				
casr	0.022	0.499	0.106	1.28	1.61				
gprc6a	0.028	0.065	0.414	0.82	1.06				
akt1	0.003	0.029	0.498	1.27	1.73	1.12^{b}	1.43^{ab}	1.70^{a}	1.76^{a}
mtor	0.243	0.032	0.195			0.86^{b}	0.98^{ab}	1.23^{a}	1.14^{a}

FI = feed intake; ADC = apparent digestibility coefficient; TAA = total amino acid; TEAA = total essential amino acid; TEAA = total non-essential amino acid; ALT = alanine aminotransaminase; AST = aspartate aminotransferase; FAA = free amino acid; *y*$^+$*lat2* = Y$^+$L amino acid transporter 2-like; *pept1* = antigen peptide transporter 1-like; *casr* = calcium-sensing receptor; *gprc6a* = G protein-coupled receptor class C group 6 member A; *akt1* = serine/threonine-protein kinase 1; *mtor* = mammalian target of rapamycin.
The non-significant parameters are not shown. The interaction effect for significant parameters is unpacked and shown in Tables and Figures. The parameters which are non-significant at interaction effect and are significant at main effect are shown.
The “feeding frequency effect” and “diet effect” are compared with independent samples *t*-test and Duncan's multiple tests, respectively ($P < 0.05$). Within a row, difference superscript letters a, b, c suggest significant differences among treatments exposed to different diets.

Table 5
Growth performance and feed utilization indices of gibel carp fed the experimental diets with different feeding frequencies.

Item	Groups								SEM	*P*-value		
	LF1	LF2	LF3	LF4	HF1	HF2	HF3	HF4		Feeding frequency effect	Diet Effect	Interaction
IBW, g	18.13	18.19	18.15	18.09	18.02	18.17	18.16	18.02	0.034	0.534	0.592	0.945
SR[1], %	97.50	98.33	97.50	97.50	96.67	97.50	97.50	97.50	0.451	0.700	0.957	0.984
FI[2], g/kg MBW per day	14.66	14.18	13.68	13.84	19.80	19.70	19.58	19.37	0.584	<0.001	0.085	0.549
FBW, g	61.99^{b}	59.11^{b}	69.88^{a}	67.45^{a}	72.34^{y}*	72.71^{xy}*	75.75^{x}*	71.37^{y}	1.151	<0.001	<0.001	0.001
SGR[3], %/d	2.20^{b}	2.11^{c}	2.41^{a}	2.35^{a}	2.48*	2.48*	2.55*	2.46	0.031	<0.001	<0.001	0.001
FE[4], %	60.83^{b}*	60.04^{b}*	71.82^{a}*	69.09^{a}*	51.49	51.42	53.73	51.98	1.606	<0.001	<0.001	<0.001
PER[5]	1.77^{b}*	1.76^{b}*	2.12^{a}*	2.05^{a}*	1.50^{y}	1.50^{y}	1.59^{x}	1.54^{xy}	0.048	<0.001	<0.001	<0.001
PRE[6], %	31.82^{b}*	31.17^{b}*	37.16^{a}*	35.65^{a}*	26.26^{y}	26.70^{y}	28.18^{x}	27.23^{xy}	0.829	<0.001	<0.001	0.002

LF = low frequency; HF = high frequency; SEM = standard error of the mean; IBW = initial body weight; SR = survival rate; FI = feed intake; MBW = mean metabolic body weight; FBW = final body weight; SGR = specific growth rate; FE = feed efficiency; PER = protein efficiency ratio; PRE = protein retention efficiency.
Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively.
Values are means ($n = 3$). Within a row, difference superscript letters a, b, c and x, y indicate significant differences among treatments at feeding frequencies of two meals and four meals per day, respectively ($P < 0.05$). The asterisk (*) indicates a significant difference between diet levels and comparing feeding frequencies of two and four meals per day ($P < 0.05$).
[1] SR (%) = 100 × (final fish number/initial fish number).
[2] FI (g/kg MBW, per day) = feed consume/(MBW × days) and MBW = [(initial body weight/1000)$^{0.75}$ +(final body weight/1000)$^{0.75}$]/2.
[3] SGR (%/d) = 100 × (ln final body weight - ln initial body weight)/days.
[4] FE (%) = 100 × (weight gain + dead fish weight)/dry weight of feed.
[5] PER = (final body weight - initial body weight)/protein intake.
[6] PRE (%) = 100 × amount of body protein deposition/protein intake.

significantly at four meals per day compared to two meals per day ($P < 0.001$; Table 4). Significant interactions between feeding frequency and diet were evident in final body weight (FBW), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER) and protein retention efficiency (PRE) ($P < 0.05$; Table 5). As feeding frequency increased from two to four meals per day, FBW and SGR increased significantly in fish fed diets 1, 2 and 3 ($P < 0.05$), while FE, PER, and PRE decreased significantly in fish fed diets 1, 2, 3 and 4 ($P < 0.05$). Compared to diet 1, diet 3 significantly increased FBW, PER and PRE at both feeding frequencies ($P < 0.05$). Specifically, SGR decreased in fish fed diet 2 compared to diet 1 at two meals per day ($P < 0.05$), while differences were not observed at four meals per day. Conversely, higher FBW, SGR, FE, PER, and PRE were found in fish fed diet 4 compared to diet 1 at two meals per day ($P < 0.05$), with no significant differences observed at four meals per day.

Significant interactions between feeding frequency and diet were also observed on condition factor (CF) and hepatosomatic index (HSI) ($P < 0.05$; Table 6). At two meals per day, CF increased significantly in fish fed diet 3 ($P < 0.05$), whereas it decreased significantly in fish fed diet 2 compared to that in diet 1 ($P < 0.05$). With feeding frequency increasing from two to four meals per day, CF in fish-fed diet 2 and HSI in fish-fed diet 1 increased significantly ($P < 0.05$). The different treatment manipulations did not significantly affect whole-body proximate composition (Table 7).

3.2. Apparent digestibility coefficient (ADC) and dietary essential amino acid (EAA) composition

The digestibility trial revealed significant effects of feeding frequency and dietary blended proteins on the ADC of nutrients (Tables 4 and 8). ADC of dry matter (ADC_{DM}) at four meals per day was significantly lower than at two meals per day ($P < 0.001$). It was observed the significant interactions between feeding frequency and diet on the ADC of crude protein (ADC_{CP}), gross energy (ADC_{GE}), total essential amino acid (ADC_{TEAA}), total non-essential amino acid (ADC_{TNEAA}), and individual amino acid ($P < 0.01$). With feeding frequency increasing from two to four meals per day, ADC_{CP}, ADC_{TEAA}, and ADC_{TNEAA} decreased significantly for each diet ($P < 0.05$). Higher ADC_{CP} and ADC_{TEAA} were found in fish-fed diet 3 compared to diet 1 at both feeding frequencies ($P < 0.05$). Compared to diet 3, ADC_{TEAA} and ADC_{TNEAA} in fish-fed diet 2 decreased significantly at two meals per day ($P < 0.05$), while no significant differences were observed at four meals per day.

Table 6
Body condition indices of gibel carp fed the experimental diets with different feeding frequencies.

Item	Groups								SEM	*P*-value		
	LF1	LF2	LF3	LF4	HF1	HF2	HF3	HF4		Feeding frequency effect	Diet effect	Interaction
CF[1], g/cm^3	3.22^{b}	3.10^{c}	3.37^{a}	3.24^{b}	3.30	3.31*	3.28	3.27	0.019	0.096	0.083	0.029
VSI[2], %	11.40	12.32	12.640	11.99	12.12	11.29	12.26	11.34	0.123	0.151	0.073	0.056
HSI[3], %	2.66^{b}	3.11^{a}	3.22^{a}	3.33^{a}	3.48*	3.10	3.34	2.84	0.069	0.405	0.639	0.008

LF = low frequency; HF = high frequency; SEM = standard error of the mean; CF = condition factor; VSI = visceral somatic index; HSI = hepatosomatic index.
Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively.
Values are means ($n = 9$). Within a row, difference superscript letters a, b, c indicate a significant difference among treatments at feeding frequencies of two meals per day ($P < 0.05$). The asterisk (*) indicates a significant difference between diet levels and comparing feeding frequencies of two meals per day and four meals per day ($P < 0.05$).
[1] CF (g/cm^3) = 100 × body weight/total length^3.
[2] VSI (%) = 100 × (visceral weight/body weight).
[3] HSI (%) = 100 × (liver weight/body weight).

Table 7
Whole-body proximate composition of gibel carp fed the experimental diets with different feeding frequencies (%).

Item	Groups								SEM	*P*-value		
	LF1	LF2	LF3	LF4	HF1	HF2	HF3	HF4		Feeding frequency Effect	Diet Effect	Interaction
Moisture	72.43	73.35	71.90	70.94	70.69	69.97	71.36	72.32	0.360	0.143	1.000	0.143
Ash	4.40	4.30	4.22	4.23	4.27	4.18	4.12	4.18	0.028	0.089	0.207	0.945
Crude protein	16.93	16.76	16.71	16.65	16.75	16.94	16.96	16.90	0.070	0.453	0.987	0.741
Crude lipid	4.84	4.02	5.51	6.19	5.95	6.99	6.03	5.15	0.295	0.133	0.965	0.131

LF = low frequency; HF = high frequency; SEM = standard error of the mean.
Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively.
Values are means ($n = 3$).

Table 8
Apparent digestibility coefficients of gibel carp fed the experimental diets with different feeding frequencies (%).

Item	Groups								SEM	*P*-value		
	LF1	LF2	LF3	LF4	HF1	HF2	HF3	HF4		Feeding frequency Effect	Diet Effect	Interaction
Dry matter	56.18	57.43	61.18	55.22	50.80	52.94	54.56	49.65	0.767	<0.001	<0.001	0.695
Crude protein	84.57^{a}*	83.67^{a}*	84.42^{a}*	81.48^{b}*	72.27^{y}	79.72^{w}	79.72^{w}	74.44^{x}	0.900	<0.001	<0.001	<0.001
Gross energy	63.71^{c}	65.81^{b}*	70.03^{a}*	62.81^{c}*	61.53^{w}	62.12^{w}	62.70^{w}	58.32^{x}	0.683	<0.001	<0.001	0.001
TEAA	86.13^{b}*	85.47^{bc}*	87.54^{a}*	84.79^{c}*	77.31^{y}	83.02^{w}	83.86^{w}	81.58^{x}	0.630	<0.001	<0.001	<0.001
TNEAA	88.33^{ab}*	87.75^{bc}*	89.04^{a}*	87.21^{c}*	77.89^{y}	84.22^{w}	84.77^{w}	82.87^{x}	0.726	<0.001	<0.001	<0.001
Lysine	88.21^{a}*	87.88^{a}*	89.00^{a}*	85.58^{b}*	81.48^{y}	85.38^{w}	85.73^{w}	83.98^{x}	0.495	<0.001	<0.001	<0.001
Methionine	88.53*	88.42	89.28*	86.53*	77.44^{y}	85.34^{w}	85.93^{w}	79.67^{x}	0.879	<0.001	<0.001	0.001
Threonine	82.39^{ab}*	81.63^{bc}*	83.47^{a}*	80.36^{c}*	69.28^{y}	76.58^{w}	77.50^{w}	74.71^{x}	0.934	<0.001	<0.001	<0.001
Arginine	76.64^{b}*	77.93^{b}	83.24^{a}	76.72^{b}	69.54^{x}	78.71^{w}	78.29^{w}	75.86^{w}	0.800	0.001	<0.001	0.005
Leucine	88.65^{a}*	87.24^{b}*	89.29^{a}*	87.13^{b}*	79.70^{y}	83.93^{x}	85.96^{w}	84.52^{x}	0.608	<0.001	<0.001	<0.001
Isoleucine	88.89^{a}*	87.91^{b}*	89.27^{a}*	87.77^{b}*	81.52^{y}	84.42^{x}	86.71^{w}	84.58^{x}	0.525	<0.001	<0.001	<0.001
Valine	86.65*	84.77	86.50*	85.24*	73.35^{x}	81.75^{w}	82.32^{w}	78.69^{w}	0.926	<0.001	0.001	<0.001
Phenylalanine	88.38^{ab}*	87.52^{b}	89.28^{a}*	87.84^{ab}*	81.75^{x}	86.40^{w}	87.30^{w}	83.81^{x}	0.522	<0.001	<0.001	0.001
Histidine	90.80*	90.71*	90.76*	90.58	83.35^{y}	88.01^{x}	87.40^{x}	89.79^{w}	0.532	<0.001	<0.001	<0.001
Asparagine	88.21^{a}*	87.44^{ab}*	88.32^{a}*	86.84^{b}*	78.29^{y}	84.35^{w}	85.14^{w}	83.17^{x}	0.663	<0.001	<0.001	<0.001
Serine	88.37^{ab}*	87.05^{bc}*	89.72^{a}*	86.19^{c}*	74.18^{x}	80.95^{w}	82.33^{w}	81.97^{w}	0.996	<0.001	<0.001	<0.001
Glutamic acid	92.19^{ab}*	92.00^{b}*	92.80^{a}*	91.67^{b}*	86.30^{y}	89.59^{w}	89.90^{w}	88.26^{x}	0.443	<0.001	<0.001	<0.001
Glycine	85.80^{a}*	85.20^{a}*	85.86^{a}*	83.82^{b}*	65.12^{y}	79.71^{w}	78.70^{wx}	75.78^{x}	1.400	<0.001	<0.001	<0.001
Alanine	85.99^{ab}*	85.15^{bc}*	87.14^{a}*	83.67^{c}*	71.72^{y}	80.47^{w}	81.87^{w}	77.28^{x}	1.015	<0.001	<0.001	<0.001
Tyrosine	89.35*	88.29*	89.72*	88.30*	79.33^{y}	83.90^{w}	86.14^{w}	85.84^{x}	0.687	<0.001	<0.001	<0.001
Proline	82.57^{b}*	82.33^{b}	87.43^{a}*	83.17^{b}*	70.98^{z}	80.23^{x}	84.19^{w}	76.81^{y}	1.002	<0.001	<0.001	<0.001
Cysteine	85.65*	84.80	83.08*	84.18	75.38^{x}	80.93^{w}	76.71^{x}	81.16^{w}	0.809	<0.001	0.047	0.029

LF = low frequency; HF = high frequency; SEM = standard error of the mean; TEAA = total essential amino acids; TNEAA = total non-essential amino acids.
Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively.
Values are means ($n = 3$). Within a row, difference superscript letters a, b, c and w, x, y, z indicate significant differences among treatments at feeding frequencies of two meals and four meals per day, respectively ($P < 0.05$). The asterisk (*) indicates a significant difference between diet levels and comparing feeding frequencies of two and four meals per day ($P < 0.05$).

Table 2 showed that the composition of EAA was similar in the four experimental diets. At both two and four meals per day, the ratios of dietary digestible EAA (DEAA) to muscle EAA composition were higher in diet 3 for most EAAs compared to other diets (Table 9). In addition, the ratios of dietary DEAA to muscle EAA composition were higher at two meals per day for most EAAs compared to four meals per day.

3.3. Plasma amino acid composition

An increase in feeding frequency significantly elevated the contents of plasma total amino acids ($P < 0.001$; Table 4). Significant interactions between feeding frequency and diet were noted for plasma threonine content ($P = 0.043$; Table 10). At two meals per day, diet 3 exhibited significantly higher plasma threonine content compared to diets 1 and 2 ($P < 0.05$).

3.4. Digestion and absorption function to protein

Protease activity and amino acid or peptide transporter expression levels were used to assess intestinal digestive and absorptive capacity, revealing no significant interactions between feeding frequency and diet (Tables 4 and 11, Fig. 1A). Compared to two meals per day, significant increases were observed in trypsin activity and the mRNA expression of $y^{+}lat2$ and *pept1* at four meals per day ($P < 0.05$). The activities of trypsin and chymotrypsin were significantly decreased in fish-fed diet 1 compared to other diets ($P < 0.01$). In addition, the expression levels of *pept1* were significantly up-regulated in fish-fed diet 4 compared to other diets ($P = 0.001$).

The expression levels of *casr* and *gprc6a* in the intestine were significantly up-regulated at four meals per day compared to two meals per day ($P < 0.05$; Table 4). Feeding frequency and diet

Table 9
Ratios of dietary DEAA composition to muscle EAA composition of gibel carp fed the experimental diets with different feeding frequencies.

Item	LF1	LF2	LF3	LF4	HF1	HF2	HF3	HF4
Lysine	0.2874	0.2995	0.2994	0.2724	0.2654	0.2910	0.2884	0.2673
Methionine	0.2339	0.2377	0.2443	0.2000	0.2045	0.2294	0.2351	0.1841
Threonine	0.3405	0.3476	0.3529	0.3371	0.2863	0.3261	0.3276	0.3134
Arginine	0.3871	0.4158	0.4398	0.4113	0.3513	0.4199	0.4137	0.4067
Leucine	0.3907	0.3783	0.4030	0.3871	0.3513	0.3639	0.3880	0.3755
Isoleucine	0.4399	0.4321	0.4418	0.4433	0.4035	0.4150	0.4291	0.4272
Valine	0.4094	0.3954	0.4087	0.4158	0.3466	0.3813	0.3890	0.3838
Phenylalanine	0.4219	0.4207	0.4378	0.4448	0.3903	0.4153	0.4280	0.4245
Histidine	0.2568	0.2714	0.2492	0.2821	0.2357	0.2633	0.2400	0.2797

DEAA = digestible essential amino acid; EAA = essential amino acid; LF = low frequency; HF = high frequency.
Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively.
Values are means ($n = 3$).
Dietary each DEAA content (%) = dietary each EAA composition × apparent digestibility coefficients of each EAA.
Ratios of dietary DEAA composition to muscle EAA composition = dietary each DEAA content/muscle each EAA content.
Muscle EAA contents: lysine, 6.63%; methionine, 2.12%; threonine, 3.17%; arginine, 3.88%; leucine, 5.65%; isoleucine, 2.95%; valine, 3.28%; phenylalanine, 3.10%; histidine, 2.44%.

Table 10
Plasma free amino acid content of gibel carp fed the experimental diets with different feeding frequencies (μmol/cL).

Item	Groups								SEM	*P*-value		
	LF1	LF2	LF3	LF4	HF1	HF2	HF3	HF4		Feeding frequency effect	Diet effect	Interaction
TAA	46.33	42.93	44.96	41.79	50.40	51.12	50.37	48.70	0.865	<0.001	0.333	0.655
TEAA	29.18	27.78	29.50	27.42	32.32	33.10	32.72	31.61	0.521	<0.001	0.395	0.631
TNEAA	10.10	7.81	8.10	7.89	9.81	10.19	9.71	9.43	0.269	0.006	0.324	0.751
Lysine	3.45	3.27	3.49	3.01	4.14	4.21	4.11	3.47	0.107	<0.001	0.044	0.701
Methionine	0.80	0.68	0.75	0.77	0.72	0.71	0.77	0.63	0.019	0.298	0.394	0.307
Threonine	6.97^{b}	6.74^{b}	8.02^{a}	7.59^{ab}	9.16*	9.23*	9.31*	8.53	0.217	<0.001	0.091	0.043
Arginine	3.95	3.45	3.55	3.41	3.96	4.06	4.14	3.99	0.076	0.002	0.503	0.261
Leucine	3.87	3.83	3.77	3.36	3.94	4.14	4.01	4.20	0.075	0.013	0.763	0.234
Isoleucine	1.97	1.94	1.96	1.72	1.93	2.19	2.20	2.27	0.049	0.008	0.655	0.139
Valine	4.42	4.47	4.71	4.05	4.85	4.97	4.92	5.15	0.088	0.001	0.666	0.134
Phenylalanine	1.32	1.16	1.15	1.31	1.40	1.30	1.23	0.97	0.042	0.908	0.282	0.165
Histidine	2.43	2.24	2.11	2.19	2.21	2.29	2.04	2.40	0.041	0.941	0.141	0.277
Asparagine	0.40	0.40	0.38	0.53	0.66	0.41	0.76	0.41	0.070	0.403	0.884	0.660
Serine	2.54	2.73	2.77	2.36	2.92	2.91	2.92	2.84	0.079	0.090	0.714	0.876
Glutamic acid	0.71	0.83	0.78	0.57	0.70	0.64	0.62	0.88	0.042	0.894	0.984	0.205
Glycine	3.41	3.38	3.43	3.01	3.99	3.87	3.65	3.54	0.098	0.027	0.425	0.897
Alanine	6.10	5.51	5.47	5.00	6.59	6.75	6.39	6.24	0.190	0.014	0.530	0.856
Tyrosine	1.32	1.41	1.28	1.88	1.36	1.36	1.25	1.37	0.057	0.177	0.094	0.226
Proline	2.59	0.77	1.25	0.90	1.74	1.94	1.98	1.71	0.170	0.133	0.191	0.115
Cysteine	0.09	0.11	0.10	0.11	0.13	0.13	0.10	0.10	0.007	0.503	0.890	0.783

LF = low frequency; HF = high frequency; SEM = standard error of the mean; TAA = total amino acids; TEAA = total essential amino acids; TNEAA = total non-essential amino acids.
Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively.
Values are means ($n = 6$). Within a row, difference superscript letters a, b indicate a significant difference among treatments at feeding frequencies of two meals per day ($P < 0.05$). The asterisk (*) indicates a significant difference between diet levels and comparing feeding frequencies of two and four meals per day ($P < 0.05$).

Table 11
Protein utilization-related enzyme activities in the intestine and muscle of gibel carp fed the experimental diets with different feeding frequencies (U/g prot, unless otherwise stated).

Item	Groups								SEM	*P*-value		
	LF1	LF2	LF3	LF4	HF1	HF2	HF3	HF4		Feeding frequency Effect	Diet effect	Interaction
Intestine												
Trypsin	191.75	1178.25	512.52	896.92	263.06	1169.87	904.79	1112.43	66.587	0.046	<0.001	0.335
Chymotrypsin	490.14	1220.23	769.38	960.73	366.43	1079.21	859.95	978.26	66.501	0.737	0.001	0.873
Muscle												
ALT	4.36	3.56	2.53	2.77	4.49	3.22	3.32	4.04	0.170	0.128	0.009	0.259
AST	18.29	17.04	15.36	16.79	21.06	17.33	18.15	17.26	0.338	0.006	0.002	0.203
FAA, μmol/mg prot	4.53	3.73	4.59	4.60	4.42	4.71	5.43	4.68	0.100	0.012	0.017	0.067

LF = low frequency; HF = high frequency; SEM = standard error of the mean; ALT = alanine aminotransaminase; AST = aspartate aminotransferase; FAA = free amino acid.
Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively.
Values are means ($n = 6$).

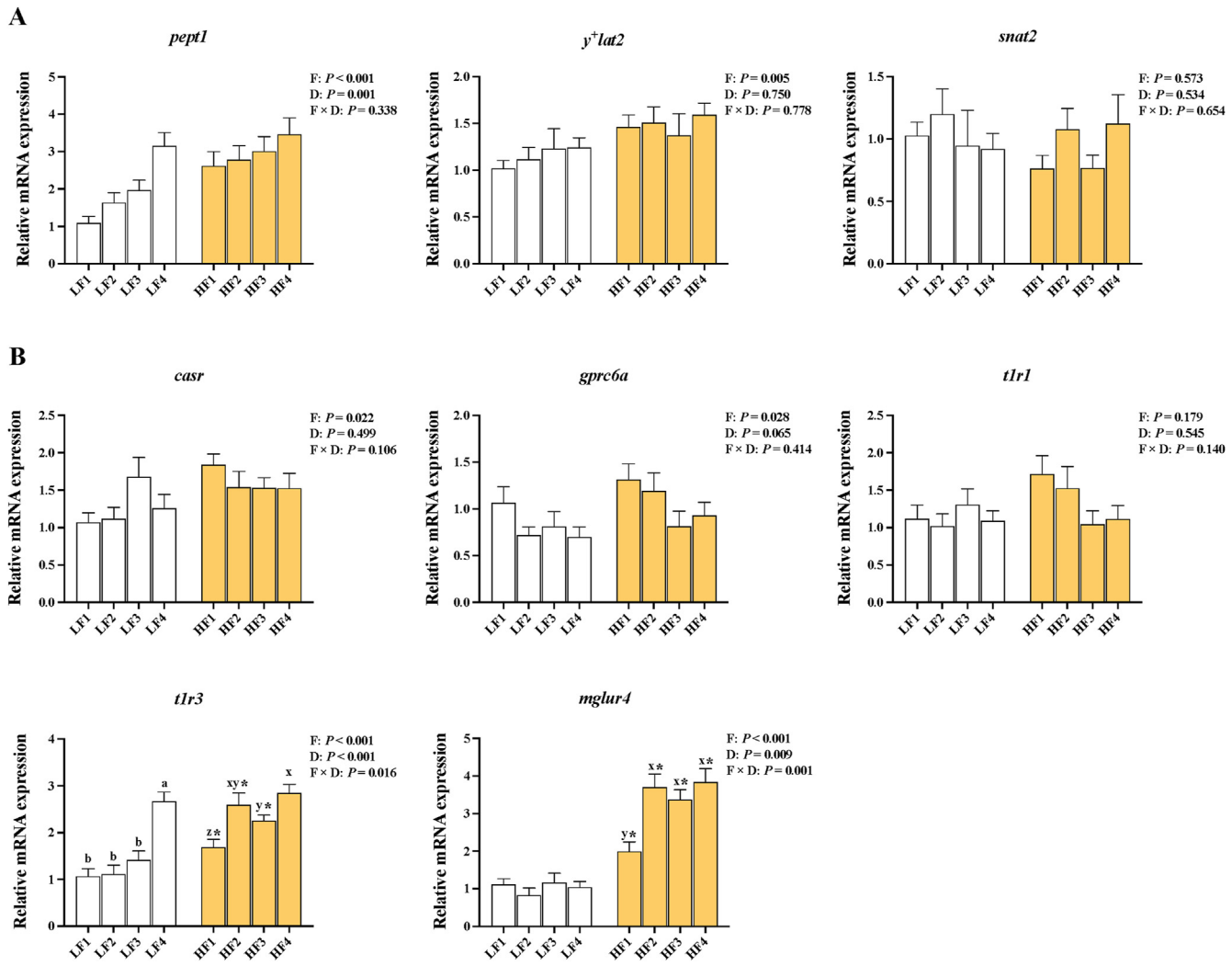

Fig. 1. The relative mRNA expression of genes related to amino acid or peptide transporter (A) and amino acid sensory receptor (B) in the intestine of gibel carp. Values are means ± SEM ($n = 6$). Difference letters a, b and x, y, z on the column indicate significant differences among treatments at feeding frequencies of two meals and four meals per day, respectively ($P < 0.05$). The asterisk (*) indicates a significant difference between diet levels and comparing feeding frequencies of two and four meals per day ($P < 0.05$). LF = low frequency; HF = high frequency. Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively. F = feeding frequency effect; D = diet effect; F × D = interaction effect; *y*$^+$*lat2* = Y$^+$L amino acid transporter 2-like; *snat2* = sodium-coupled neutral amino acid transporter 2; *pept1* = antigen peptide transporter 1-like; *casr* = calcium-sensing receptor; *gprc6a* = G protein-coupled receptor class C group 6 member A; *t1r3* = taste receptor type 1 member 3; *t1r1* = taste receptor type 1 member 1; *mglur4* = metabotropic glutamate receptor 4.

significantly interacted with the transcript levels of *t1r3* and *mglur4* ($P < 0.05$; Fig. 1B). With feeding frequency increasing, the transcript levels of *t1r3* in fish fed diets 1, 2 and 3, and the transcript levels of *mglur4* in fish fed diets 1, 2, 3 and 4 were significantly up-regulated ($P < 0.05$). In addition, significant up-regulation of *t1r3* and *mglur4* transcript levels were observed in fish-fed diets 2, 3 and 4 compared to diet 1 at four meals per day ($P < 0.05$).

3.5. Protein utilization

Consistent with plasma findings, increasing feeding frequency significantly elevated FAA contents and AST activity in the muscle ($P < 0.05$; Table 4). Fish-fed diet 3 exhibited significantly higher FAA content compared to diets 1 and 2 ($P = 0.017$), while the activities of ALT and AST were significantly decreased in fish-fed diets 2, 3 and 4 compared to diet 1 ($P < 0.01$).

The protein synthesis-related and proteolysis-related gene expression levels were used to reflect muscle protein metabolism (Fig. 2 & Table 4). The transcript levels of *akt1* in muscle were significantly up-regulated at four meals per day compared to two meals per day ($P = 0.003$). Compared to diet 1, diet 3 significantly up-regulated the transcript levels of *akt1* and *mtor* ($P < 0.05$). Feeding frequency and diet significantly interacted on the mRNA levels of *mstn*, *s6k1*, *eif4b*, *eif4e*, *foxo1* and *fbxo32* ($P < 0.05$). Specifically, in fish-fed diet 2 compared to diet 1, *s6k1* was down-regulated significantly and *foxo1* and *fbxo32* was up-regulated significantly at two meals per day ($P < 0.05$), with no significant differences observed at four meals per day. At four meals per day, diet 3 significantly down-regulated the transcript levels of *mstn* and up-regulated the transcript levels of *s6k1*, *eif4b* and *eif4e* compared to diet 1 ($P < 0.05$). As feeding frequency increased from two to four meals per day, the transcript levels of *mstn* in fish fed diet 1, the

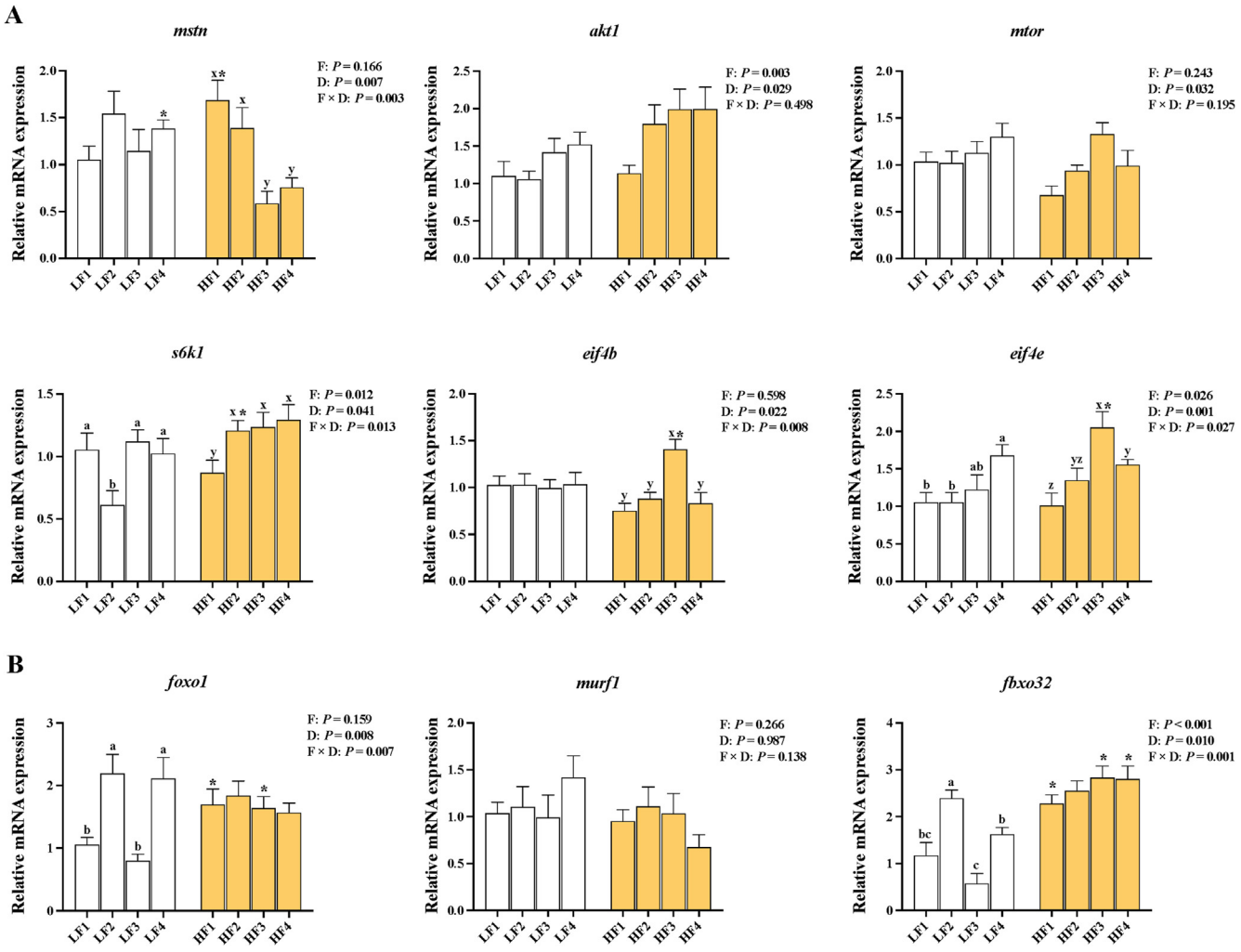

Fig. 2. The relative mRNA expression of genes related to protein synthesis (A) and proteolysis (B) in the muscle of gibel carp. Values are means ± SEM ($n = 6$). Difference letters a, b, c and x, y, z on the column indicate significant differences among treatments at feeding frequencies of two meals and four meals per day, respectively ($P < 0.05$). The asterisk (*) indicates a significant difference between diet levels and comparing feeding frequencies of two and four meals per day ($P < 0.05$). LF = low frequency; HF = high frequency. Diet 1 was formulated with 15% fishmeal as control, while the blended proteins at ratios of 1:1:8:2 (diet 2), 1:1:6:4 (diet 3) and 1:1:4:6 (diet 4) were used to fully replace fishmeal respectively, preparing four isonitrogenous and isolipidic practical diets. LF1, LF2, LF3, LF4 denote fish fed diets 1, 2, 3, 4 at two meals per day, respectively; HF1, HF2, HF3, HF4 denote fish fed diets 1, 2, 3, 4 at four meals per day, respectively. F = feeding frequency effect; D = diet effect; F × D = interaction effect; *mstn* = myostatin; *akt1* = serine/threonine-protein kinase 1; *foxo1* = Forkhead box O1; *mtor* = mammalian target of rapamycin; *s6k1* = ribosomal protein S6 kinase 1; *eif4b* = eukaryotic translation initiation factor 4B; *eif4e* = eukaryotic translation initiation factor 4E; *murf1* = muscle RING-finger 1; *fbxo32* = muscle atrophy F-box.

transcript levels of *eif4b* and *eif4e* in fish fed diet 3, the transcript levels of *foxo1* in fish fed diets 1 and 3, and the transcript levels of *fbxo32* in fish fed diets 1, 3 and 4 were significantly up-regulated ($P < 0.05$).

4. Discussion

Protein constitutes the predominant organic material in fish, and improving its utilization is pivotal for promoting growth. Additionally, both diet composition and feeding frequency exert significant influences on protein utilization (Busti et al., 2020; Zhao, 2014). In the present trial, SGR (except for diet 4) increased in fish fed to satiation at four meals per day compared to two meals per day, while FE and PRE decreased. The heightened feed intake at four meals per day primarily drives growth enhancement, yet the short intervals compromise inferior feed utilization. Similar trends were also observed in gilthead seabream (*Sparus aurata*) (Basto-Silva et al., 2022), grass carp (Wu et al., 2021) and largemouth bass (*Micropterus salmoides*) (Liu et al., 2022). Conversely, the augment of feeding frequency from one to four meals per day with satiated feeding promoted the growth of *Schizothorax wangchiachii* by increasing feed intake without affecting feed utilization (Wang et al., 2022). In addition, increased feeding frequency promoted growth through improving feed utilization under satiated feeding in tiger puffer fish (*Takifugu rubripes*) (Gao et al., 2022), and under quantitative feeding in long whiskers catfish (*Mystus gulio*) (Biswas et al., 2023) and oriental river prawn (*Macrobrachium nipponense*) (Ding et al., 2017). These indicate species-specific responses to feeding patterns. In actual aquaculture, appropriate feeding frequency and feeding pattern of each meal should be comprehensively selected according to yield requirements, economic efficiency and animal species.

Superior growth performance was observed at two meals per day in diets containing protein blends at ratios of 1:1:6:4 and 1:1:4:6, whereas inferior growth was noted in the 1:1:8:2 blend, consistent with our prior findings (Yu et al., 2024). In crucian carp, replacing fishmeal with a blend of rapeseed meal and CM improved protein utilization and growth (Shi et al., 2017). Similarly, dietary

mixture of shrimp hydrolysate and plant proteins to replace 22% fishmeal increased SGR in largemouth bass (Li et al., 2021). Thus, appropriately mixed dietary protein sources better met the growth demands of fish compared to single protein sources. However, inappropriate protein source blends inhibited protein utilization and growth of aquaculture animals (Burr et al., 2012; Villanueva-Gutiérrez et al., 2020). CF serves as an important index to evaluate the economic value, which is higher in fish exhibiting superior growth (Zheng et al., 2023a,b). At two meals per day, higher CF was found in fish fed diet 3. The negative effect of diet 2 and the positive effect of diet 4 on growth were offset by enlarging feeding frequency from two to four meals per day. Similarly, it was reported that increasing the feeding frequency from two to four meals per day ameliorated poor feed utilization and asynchronous amino acid absorption issues when soybean meal replaced dietary fishmeal in gibel carp (Zhao, 2014). Furthermore, the increase in the feeding frequency from two to four meals per day had no effect on the protein utilization of CM but enhanced the utilization of soybean meal, TMM, CAP, and CPC, promoting growth in grass carp (Xia, 2022). Thus, interactions between feeding frequency and protein source influenced fish growth and feed utilization. Subsequently, the substitution levels of dietary fishmeal in aquaculture can be further improved, not only by using complementary blended protein sources, but also by increasing feeding frequency. Additionally, two feeding patterns: satiation feeding and quantitative feeding, are typically employed when discussing the effect of feeding frequency on fish. Increasing feeding frequency with quantitative feeding, which is not adopted in the present trial, holds considerable potential in enhancing growth performance by promoting feed utilization (Biswas et al., 2023; Ding et al., 2017). Future research may take into account the contributions of increasing feeding frequency with quantitative feeding in improving the utilization of protein sources.

As known, the demand of fish for feed protein is essentially a demand for amino acids (Halver and Hardy, 2002). Despite similar AA profiles among the four diets in our study, the chemical composition of dietary AAs did not majorly impact growth. The apparent digestibility of protein is an important index to evaluate animal digestion and absorption function (Liu et al., 2024). In our study, nutrient ADC decreased in fish fed at four meals per day compared to two meals per day. Poor nutrient digestibility in aquaculture animals was not conducive to feed utilization (Hernández et al., 2021; Lin et al., 2023; Poolsawat et al., 2021). In gilthead seabream, ADC_{CP} increased with feeding frequency from one to three meals per day, which subsequently declined with further increase to six meals per day (Gilannejad et al., 2019). EAAs are the most important part of the demand for dietary protein (Halver and Hardy, 2002). ADC_{CP} and ADC_{TEAA} increased in fish fed with diet 3, indicating better digestion and absorption of protein. In addition, ADC_{TEAA} in fish-fed diet 2 decreased at two meals per day but not at four meals per day, in line with results on growth performance. The ideal dietary protein model for fish correlates with the composition of muscle or whole-body EAA (Peres and Oliva-Teles, 2009; Yu et al., 2024; Zhu et al., 2011). The dietary DEAA of fish fed diet 3 or fed at two meals per day was closer to the muscle EAA profile, corroborating the results on feed utilization.

Protein digestion and absorption primarily occur in gibel carp intestine, where proteases break down proteins into amino acids or peptides for absorption via transporters on the intestinal brush membrane (Debnath and Saikia, 2021). Sodium-coupled neutral amino acid transporter 2 (*snat2*) transports alanine and other small, polar neutral amino acids, while y^+L-type amino acid transporter 2 (*y^+lat2*) favors cationic amino acids (Bröer, 2008). Additionally, peptide transporter 1 (*pept1*) facilitates the absorption of dipeptides and tripeptides (Spanier and Rohm, 2018). In the present trial, trypsin activity and expression levels of *pept1* and *y^+lat2* were up-regulated in fish fed four meals per day compared to two meals per day. Similarly in gilthead seabream, trypsin and chymotrypsin activities in the proximal intestine increased at higher feeding frequency (Busti et al., 2020), and expression levels of *pept1* were up-regulated in the gastrointestinal tract with five meals per day or continuous feeding compared to one or three meals per day (Gilannejad et al., 2021). In addition, pancreatic and intestinal trypsin activities of large yellow croaker (*Pseudosciaena crocea*, Richardson) increased at eight meals per day compared to two and four meals per day (Xie et al., 2011). Thus, the appropriate increase in feeding frequency enhanced protein digestion and absorption in fish. Moreover, the intestinal trypsin and chymotrypsin activities increased with fish-fed blended proteins. In pearl gentian grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂), dietary CPC instead of 36% fishmeal in diets enhanced intestinal trypsin and chymotrypsin activities (Chen et al., 2020). Moreover, TMM instead of 30% fishmeal in diets increased alkaline protease activities of rainbow trout (*Oncorhynchus mykiss*) (Melenchón et al., 2020), and CAP instead of 25% fishmeal in diets increased trypsin activity of abalone (*Haliotis discus hannai*) (Wu et al., 2022). However, trypsin activity in Pacific white shrimp (*Litopenaeus vannamei*) varied with CM replacing fishmeal content (Pakravan et al., 2018). Thus, the specificity of protein blends enhanced protein digestion in fish. Amino acid sensory receptors, such as calcium-sensing receptors (CaSR), G protein-coupled receptor class C group 6 member A (GPRC6A), taste receptor type 1 member 1 (T1R1), taste receptor type 1 member 3 (T1R3), and metabotropic glutamate receptor 4 (mGluR4), can sense amino acids present in the intestinal cavity to transmit nutritional signals, thereby mobilizing amino acid metabolism quickly (Calo et al., 2021; Efeyan et al., 2015; Wauson et al., 2012). The expression levels of *casr*, *gprc6a*, and *mglur4* were up-regulated at higher feeding frequency. At four meals per day, the up-regulated transcript levels of *t1r3* and *mglur4* were found in fish-fed blended proteins. Thus, both high feeding frequency and protein blends enhanced amino acid sensing in gibel carp intestine.

Protein is predominantly present as FAA circulating in the bloodstream to various tissues. Plasma FAA content was higher at four meals per day compared to two meals per day in the present trial. Similarly, higher plasma EAA levels were reported 5 h postprandially of gibel carp fed at six meals per day compared to two meals per day (Zhao, 2014). In addition, rainbow trout exhibited increased plasma FAA levels with higher feeding levels (Cleveland and Burr, 2011). An augment in circulating FAA stimulated protein synthesis and ultimately improved growth performance (Tome, 2022; Wu, 2018). At two meals per day, it was observed that plasma threonine content was higher in fish-fed diet 3. Threonine plays an important role in growth, immunity, and protein synthesis in fish (Habte-Tsion et al., 2016; Zhao et al., 2020).

Protein deposition primarily occurs in muscle and is regulated by processes of anabolism and catabolism. ALT and AST are critical aminotransferases involved in amino acid metabolism (Chandel, 2021; Kumar et al., 2017). In the present trial, the expression levels of protein synthesis-related gene *akt1* and proteolysis-related gene *fbxo32* (except for diet 2), AST activity, and FAA content of muscle increased with increasing feeding frequency. Sufficient FAA supported vigorous protein synthesis, alongside the enhancement of proteolysis and amino acid metabolism, was conducive to the renewal of FAAs, ultimately facilitating protein turnover. Similar mechanisms were observed in grass carp, where protein turnover was accelerated by motivating UPS and mTOR pathways, promoting muscle growth (Zhang et al., 2021). Myostatin (MSTN) is a key regulator that inhibits muscle growth (Sharma et al., 2015). Reduced *mstn* expression levels, coupled with increased expression levels of muscle protein synthesis genes (*akt1*,

mtor, *s6k1*, *eif4b*, *eif4e*), and heightened FAA content were evident in fish-fed diet 3 at four meals per day. Similarly, replacing fishmeal with appropriately mixed proteins contained balanced amino acids improved growth in largemouth bass by up-regulating mTOR pathway expression (Li et al., 2021). These suggested that diet 3 increased muscle FAA pools and stimulated protein synthesis, thereby enhancing protein deposition and muscle growth. At two meals per day, up-regulation of proteolysis genes (*foxo1*, *fbxo32*) and down-regulation of *s6k1* were observed in fish fed diet 2, consistent with findings in turbot (*Scophthalmus maximus* L.) that the substitution of fishmeal with inappropriate blended proteins inhibited mTOR phosphorylation levels (Wang et al., 2016). Diet 2 decreased protein synthesis and increased protein degradation at two meals per day, which was not conducive to protein deposition. However, dietary diet 2 had no adverse effects on protein deposition at four meals per day, aligning with the results for ADC_{TEAA} and SGR. These imply that altering the feeding frequency influences the protein utilization process of fish to different protein sources, thus mitigating the adverse effects on growth.

5. Conclusion

At four meals per day compared to two meals per day, feed intake increased, intestinal digestion and absorption enhanced, FAA pools increased, and muscle protein turnover accelerated, thus promoting growth. However, ADC_{TEAA} decreased and feed intake exceeded growth requirements, leading to reduce feed utilization. Moreover, an increase in feeding frequency mitigated the negative effects of dietary unsuitable blended proteins on growth by altering the protein utilization process. Dietary blended proteins (TMM:CM:CAP:CPC) in the ratio of 1:1:6:4 completely replacing fishmeal promoted growth performance at both feeding frequencies. Therefore, the strategies of a reasonable increase in feeding frequency and appropriate mixing of different protein sources can be used to cope with the insufficient supply of fishmeal in aquaculture.

CRediT authorship contribution statement

Yongning Yu: Writing – original draft, Investigation, Formal analysis, Data curation. **Yu Wang:** Investigation, Data curation. **Junyan Jin:** Writing – review & editing, Supervision, Data curation. **Dong Han:** Methodology, Conceptualization. **Xiaoming Zhu:** Methodology. **Haokun Liu:** Methodology, Conceptualization. **Zhimin Zhang:** Methodology. **Yunxia Yang:** Methodology. **Shouqi Xie:** Supervision, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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References

Ambardekar AA, Reigh RC, Williams MB. Absorption of amino acids from intact dietary proteins and purified amino acid supplements follows different time-courses in channel catfish (*Ictalurus punctatus*). Aquaculture 2009;291(3–4):179–87.
AOAC (Association of Official Analytical Chemists). Official methods of analysis. 16th ed. Gaithersburg. MD: Association of Official Analytical Chemists; 2000.
Basto-Silva C, Couto A, Rodrigues J, Oliva-Teles A, Navarro I, Kaiya H, Capilla E, Guerreiro I. Feeding frequency and dietary protein/carbohydrate ratio affect feed intake and appetite regulation-related genes expression in gilthead seabream (*Sparus aurata*). Comp Biochem Physiol Mol Integr Physiol 2022;267:111168.
Biswas G, Kumar P, Mandal B, Ghoshal TK, De D, Bera A, Kailasam M. Effects of feeding frequency on growth, survival, body composition and size variation in long whiskers catfish, *Mystus gulio* (Hamilton, 1822) fry reared in net cage system. Region Stud Mar Sci 2023;61:102851.
Bröer S. Amino acid transport across mammalian intestinal and renal epithelia. Physiol Rev 2008;88(1):249–86.
Bureau of Fisheries of the Ministry of Agriculture and Rural Affairs of the People's Republic of China. China Fishery statistical yearbook. Beijing, China: China Agriculture Press; 2023.
Burr GS, Wolters WR, Barrows FT, Hardy RW. Replacing fishmeal with blends of alternative proteins on growth performance of rainbow trout (*Oncorhynchus mykiss*), and early or late stage juvenile Atlantic salmon (*Salmo salar*). Aquaculture 2012;334–337:110–6.
Busti S, Bonaldo A, Dondi F, Cavallini D, Yúfera M, Gilannejad N, Moyano FJ, Gatta PP, Parma L. Effects of different feeding frequencies on growth, feed utilisation, digestive enzyme activities and plasma biochemistry of gilthead sea bream (*Sparus aurata*) fed with different fishmeal and fish oil dietary levels. Aquaculture 2020;529:735616.
Calo J, Blanco AM, Comesana S, Conde-Sieira M, Morais S, Soengas JL. First evidence for the presence of amino acid sensing mechanisms in the fish gastrointestinal tract. Sci Rep 2021;11(1):4933.
Chandel NS. Amino acid metabolism. Cold Spring Harbor Perspect Biol 2021;13:a040584.
Chen G, Yin B, Liu H, Tan B, Dong X, Yang Q, Chi S, Zhang S. Effects of fishmeal replacement with cottonseed protein concentrate on growth, digestive proteinase, intestinal morphology and microflora in pearl gentian grouper (♀*Epinephelus fuscoguttatus* × ♂*Epinephelus lanceolatu*). Aquacult Res 2020;51(7):2870–84.
Chen J, Daniell J, Griffin D, Li XL, Henson MA. Experimental testing of a spatio-temporal metabolic model for carbon monoxide fermentation with *Clostridium autoethanogenum*. Biochem Eng J 2018;129:64–73.
China National Standard. Inorganic chemicals for industrial use—determination of impurity element—inductively coupled plasma-mass spectrometry method (ICP-MS). GB/T 30903-2014. Beijing: Standards Press of China; 2014.
Cleveland BM, Burr GS. Proteolytic response to feeding level in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 2011;319(1–2):194–204.
Debnath S, Saikia SK. Absorption of protein in teleosts: a review. Fish Physiol Biochem 2021;47(2):313–26.
Ding Z, Kong Y, Zhang Y, Li J, Cao F, Zhou J, Ye J. Effect of feeding frequency on growth, body composition, antioxidant status and mRNA expression of immunodependent genes before or after ammonia-N stress in juvenile oriental river prawn, *Macrobrachium nipponense*. Fish Shellfish Immunol 2017;68:428–34.
Efeyan A, Comb WC, Sabatini DM. Nutrient-sensing mechanisms and pathways. Nature 2015;517(7534):302–10.
Estevão-Rodrigues T, Fernandes H, Moutinho S, Filipe D, Fontinha F, Magalhães R, Couto A, Ferreira M, Gamboa M, Castro C, Belo I, Salgado J, Oliva-Teles A, Peres H. Effect of solid-state fermentation of Brewer's spent grain on digestibility and digestive function of european seabass (*Dicentrarchus labrax*) juveniles. Anim Feed Sci Technol 2024;315:116018.
Gao XQ, Wang X, Wang XY, Li HX, Xu L, Huang B, Meng XS, Zhang T, Chen HB, Xing R, Liu BL. Effects of different feeding frequencies on the growth, plasma biochemical parameters, stress status, and gastric evacuation of juvenile tiger puffer fish (*Takifugu rubripes*). Aquaculture 2022;548:737718.
Gilannejad N, Moyano FJ, Martínez-Rodríguez G, Yúfera M. The digestive function of gilthead seabream juveniles in relation to feeding frequency. Aquaculture 2021;531:735867.
Habte-Tsion HM, Ren M, Liu B, Ge X, Xie J, Chen R. Threonine modulates immune response, antioxidant status and gene expressions of antioxidant enzymes and antioxidant-immune-cytokine-related signaling molecules in juvenile blunt snout bream (*Megalobrama amblycephala*). Fish Shellfish Immunol 2016;51:189–99.
Halver JE, Hardy RW. Fish nutrition. 3rd ed. Elsevier Science; 2002.
Hernández C, Lizárraga-Velázquez CE, Contreras-Rojas D, Sánchez-Gutiérrez EY, Martínez-Montaño E, Ibarra-Castro L, Peña-Marín ES. Fish meal replacement by

corn gluten in feeds for juvenile spotted rose snapper (*Lutjanus guttatus*): effect on growth performance, feed efficiency, hematological parameters, protease activity, body composition, and nutrient digestibility. Aquaculture 2021;531:735896.
ISO (International Organization for Standardization). Animal feeding stuffs, animal products, and faeces or urine-Determination of gross calorific value- Bomb calorimeter method. Standard 9831. Geneva, Switzerland: International Organization for Standardization; 1998.
Kumar S, Sándor Zs J, Nagy Z, Fazekas G, Havasi M, Sinha AK, De Boeck G, Gál D. Potential of processed animal protein versus soybean meal to replace fish meal in practical diets for European catfish (*Silurus glanis*): growth response and liver gene expression. Aquacult Nutr 2017;23(5):1179–89.
Li SL, Dai M, Qiu HJ, Chen NS. Effects of fishmeal replacement with composite mixture of shrimp hydrolysate and plant proteins on growth performance, feed utilization, and target of rapamycin pathway in largemouth bass, *Micropterus salmoides*. Aquaculture 2021;533:736185.
Lin H, Deng Y, Zhu D, Yang Q, Zhou X, Tan B, Feng L, Chi S. Effects of partially replacing fishmeal with corn gluten meal on growth, feed utilization, digestive enzyme activity, and apparent nutrient digestibility for juvenile white shrimp, *Litopenaeus vannamei*. Front Vet Sci 2023;10:1162599.
Liu H, Jin J, Zhu X, Han D, Yang Y, Xie S. Effect of substitution of dietary fish meal by soybean meal on different sizes of gibel carp (*Carassius auratus gibelio*): digestive enzyme gene expressions and activities, and intestinal and hepatic histology. Aquacult Nutr 2017;23(1):129–47.
Liu X, Zou D, Wang Y, Zhuang Y, Liu Y, Li Y, Sun Z, Ye C. Replacement of fish meal with cottonseed protein concentrate in Chinese mitten crab (*Eriocheir sinensis*): nutrient digestibility, growth performance, free amino acid profile, and expression of genes related to nutrient metabolism. Anim Nutr 2024;17:447–62.
Liu Y, Lei M, Victor H, Wang Z, Yu C, Zhang G, Wang Y. The optimal feeding frequency for largemouth bass (*Micropterus salmoides*) reared in pond and in-pond-raceway. Aquaculture 2022;548:737464.
Lu KL, Cai LS, Wang L, Song K, Zhang CX, Rahimnejad S. Effects of dietary protein/energy ratio and water temperature on growth performance, digestive enzymes activity and non-specific immune response of spotted seabass (*Lateolabrax maculatus*). Aquacult Nutr 2020;26(6):2023–31.
Mai KS, Xue M, He G, Xie SQ, Kaushik SJ. Protein and amino acids. In: Fish nutrition. 4rd ed. 2022. p. 181–302.
Melenchón F, Larrán AM, de Mercado E, Hidalgo MC, Cardenete G, Barroso FG, Fabrikov D, Lourenço HM, Pessoa MF, Tomás-Almenar C. Potential use of black soldier fly (*Hermetia illucens*) and mealworm (*Tenebrio molitor*) insectmeals in diets for rainbow trout (*Oncorhynchus mykiss*). Aquacult Nutr 2020;27(2):491–505.
Pakravan S, Akbarzadeh A, Sajjadi MM, Hajimoradloo A, Noori F. *Chlorella vulgaris meal* improved growth performance, digestive enzyme activities, fatty acid composition and tolerance of hypoxia and ammonia stress in juvenile Pacific white shrimp *Litopenaeus vannamei*. Aquacult Nutr 2018;24(1):594–604.
Peres H, Oliva-Teles A. The optimum dietary essential amino acid profile for gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 2009;296(1–2):81–6.
Poolsawat L, Yang H, Sun YF, Li XQ, Liang GY, Leng XJ. Effect of replacing fish meal with enzymatic feather meal on growth and feed utilization of tilapia (*Oreochromis niloticus* × *O. aureus*). Anim Feed Sci Technol 2021;274:114895.
Sharma M, McFarlane C, Kambadur R, Kukreti H, Bonala S, Srinivasan S. Myostatin: expanding horizons. IUBMB Life 2015;67(8):589–600.
Shi X, Chen F, Chen GH, Pan YX, Zhu XM, Liu X, Luo Z. Fishmeal can be totally replaced by a mixture of rapeseed meal and *Chlorella* meal in diets for crucian carp (*Carassius auratus gibelio*). Aquacult Res 2017;48(11):5481–9.
Spanier B, Rohm F. Proton coupled oligopeptide transporter 1 (PepT1) function, regulation, and influence on the intestinal homeostasis. Compr Physiol 2018;8(2):843–69.
Sun Y, Liang X, Chen J, Tang R, Li L, Li D. Change in ubiquitin proteasome system of grass carp *Ctenopharyngodon idellus* reared in the different stocking densities. Front Physiol 2018;9:837.
Tome D. Efficiency of free amino acids in supporting muscle protein synthesis. J Nutr 2022;152(1):3–4.
Van Huis A. Potential of insects as food and feed in assuring food security. Annu Rev Entomol 2013;58(1):563–83.
Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:research0034.1.
Villanueva-Gutiérrez E, González-Félix ML, Gatlin DM, Perez-Velazquez M. Use of alternative plant and animal protein blends, in place of fishmeal, in diets for juvenile totoaba, *Totoaba macdonaldi*. Aquaculture 2020;529:735698.
Wang C, Xie S, Zheng H, Chen F, Fang Y. Effects of feeding frequency on the growth, body composition and SOD, GPX and HSP70 gene expression in *Schizothorax wangchiachii*. Aquacult Rep 2022;22:100942.
Wang Q, He G, Mai K, Xu W, Zhou H. Fishmeal replacement by mixed plant proteins and maggot meal on growth performance, target of rapamycin signalling and metabolism in juvenile turbot (*Scophthalmus maximus L.*). Aquacult Nutr 2016;22(4):752–8.
Wauson EM, Zaganjor E, Lee AY, Guerra ML, Ghosh AB, Bookout AL, Chambers CP, Jivan A, McGlynn K, Hutchison MR, Deberardinis RJ, Cobb MH. The G protein-coupled taste receptor T1R1/T1R3 regulates mTORC1 and autophagy. Mol Cell 2012;47(6):851–62.
Wu B, Huang L, Chen J, Zhang Y, Chen X, Wu C, Deng X, Gao J, He J. Effects of feeding frequency on growth performance, feed intake, metabolism and expression of *fgf21* in grass carp (*Ctenopharyngodon idellus*). Aquaculture 2021;545:737196.
Wu G. Principles of animal nutrition. 1st ed. Boca Raton: CRC Press; 2018.
Wu Z, Yu X, Guo J, Fu Y, Guo Y, Pan M, Zhang W, Mai K. Replacement of dietary fish meal with *Clostridium autoethanogenum* protein on growth performance, digestion, mTOR pathways and muscle quality of abalone *Haliotis discus hannai*. Aquaculture 2022;553:738070.
Xia S. Effects of feeding frequency and feed protein source on growth performance of grass carp [Master's Thesis]. China: Huazhong Agricultural University; 2022.
Xie F, Ai Q, Mai K, Xu W, Ma H. The optimal feeding frequency of large yellow croaker (*Pseudosciaena crocea*, Richardson) larvae. Aquaculture 2011;311(1–4):162–7.
Xu W, Xu Y, Su H, Hu X, Xu Y, Li Z, Wen G, Cao Y. Effects of feeding frequency on growth, feed utilization, digestive enzyme activity and body composition of *Litopenaeus vannamei* in biofloc-based zero-exchange intensive systems. Aquaculture 2020;522:735079.
Xue M, Yun B, Wang J, Sheng H, Zheng Y, Wu X, Qin Y, Li P. Performance, body compositions, input and output of nitrogen and phosphorus in Siberian sturgeon, *Acipenser baerii Brandt*, as affected by dietary animal protein blend replacing fishmeal and protein levels. Aquacult Nutr 2012;18(5):493–501.
Xue M. Processing of cottonseed protein concentrated and its nutrient values in aquatic feed. Feed Ind 2021;42(12):1–5.
Yoshida T, Delafontaine P. Mechanisms of IGF-1-mediated regulation of skeletal muscle hypertrophy and atrophy. Cells 2020;9:1970.
Yu Y, Wang X, Jin J, Han D, Zhu X, Liu H, Zhang Z, Yang Y, Xie S. Effects of the replacement of dietary fishmeal by the blend of *Tenebrio molitor* meal, *Chlorella* meal, *Clostridium autoethanogenum* protein, and cottonseed protein concentrate on growth, protein utilization, and intestinal health of gibel carp (*Carassius gibelio*, CAS V). Aquacult Nutr 2024;2024:1–18.
Zare M, Esmaeili N, Hosseini H, Hosseini Choupani SM, Akhavan S, Salini M, Rombenso A, Stejskal V. Do optimum dietary protein and early mild stress events prepare oscar (*Astronotus ocellatus*) for a stressful future? Aquacult Rep 2024;34:101854.
Zhang L, Xu N, Liu X, Onxayvieng K, Liu L, Tang R, Li D. Exercise training accelerates UPS- and mTOR-mediated protein turnover of grass carp *Ctenopharyngodon idella*. Aquaculture 2021;545:737252.
Zhao S. Effects of feeding frequency and dietary protein formulation on gibel carp (*Carassius auratus gibelio*): growth, feed utilization and blood free essential amino acids dynamics [Doctoral degree thesis Dissertation]. China: Institute of Hydrobiology, Chinese Academy of Sciences; 2014.
Zhao Y, Jiang Q, Zhou XQ, Xu SX, Feng L, Liu Y, Jiang WD, Wu P, Zhao J, Jiang J. Effect of dietary threonine on growth performance and muscle growth, protein synthesis and antioxidant-related signalling pathways of hybrid catfish *Pelteobagrus vachellifemale* symbol × *Leiocassis longirostrismale* symbol. Br J Nutr 2020;123(2):121–34.
Zheng JL, Zhang HT, Gao L, Chen X, Zhu QL, Han T. Combined effects of crowding stress and low salinity on GH/IGF axis, antioxidant response, and HPI axis in largemouth bass (*Micropterus salmoides*) larvae. Aquaculture 2023a;578:740036.
Zheng X, Liao X, Zhang M, Mao J, Chen Y, Lan S, Yin Z, Yu F, Vasquez HE, Gu Z. The effect of aquarium color background on the survival, growth performance, body coloration, and enzymatic activity of laboratory cultured *Cherax quadricarinatus* juveniles. Aquacult Rep 2023b;32:101699.
Zhu H, Gong G, Wang J, Wu X, Xue M, Niu C, Guo L, Yu Y. Replacement of fish meal with blend of rendered animal protein in diets for Siberian sturgeon (*Acipenser baerii Brandt*), results in performance equal to fish meal fed fish. Aquacult Nutr 2011;17(2):E389–95.
Zuo J, Ling B, Long L, Li T, Lahaye L, Yang C, Feng D. Effect of dietary supplementation with protease on growth performance, nutrient digestibility, intestinal morphology, digestive enzymes and gene expression of weaned piglets. Anim Nutr 2015;1(4):276–82.