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Original Research Article

An emerging role of vitamin D_3 in amino acid absorption in different intestinal segments of on-growing grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Vitamin D_3 (VD_3), an essential nutrient for animals, has been demonstrated to stimulate the uptake of certain amino acids. However, the role of VD_3 in the intestine, the primary site for digestion and absorption of nutrients, remains poorly characterized. Here, the grass carp (*Ctenopharyngodon idella*) was studied to assess the influence of different doses of VD_3 (15.2, 364.3, 782.5, 1,167.9, 1,573.8, and 1,980.1 IU/kg) on growth performance, intestinal morphology, digestive absorption, amino acid transport, and potential signaling molecule levels in a feeding experiment. As a result, dietary VD_3 improved growth performance, intestinal structure, and digestive and brush border enzyme activities. Additionally, most intestinal free amino acids and their transporters were upregulated after VD_3 intake, except for Ala, Lys, Asp, Leu, solute carrier (*SLC*) *7A7*, *SLC1A5*, and *SLC1A3* mRNA in different segments, Leu and *SLC6A14* mRNA in the proximal intestine, and *SLC7A5* mRNA in the mid and distal intestine. In the crucial target of rapamycin (TOR) signal pathway of amino acid transport, the gene and protein expression of TOR, S6 kinase 1, and activating transcription factor 4 were elevated, whereas 4E-binding protein 1 was decreased, further suggesting an advanced amino acid absorption capacity in the fish due to VD_3 supplementation. Based on percentage weight gain, feed efficiency, and trypsin activity, the VD_3 requirements of on-growing grass carp were estimated to be 968.33, 1,005.00, and 1,166.67 IU/kg, respectively. Our findings provide novel recommendations for VD_3 supplementation to promote digestion and absorption capacities of fish, contributing to the overall productivity of aquaculture.

1. Introduction

Vitamin D_3 (VD_3) is of importance for animals (Mora et al., 2008). It is obtained directly from the diet by all vertebrates, including fish (Dusso et al., 2005), and is transported and absorbed by the intestines (Haddad et al., 1993). Fish accumulate VD_3 throughout their lifetime, and appropriate levels are maintained through diet (Graff et al., 2002). VD_3 deficiency reduces specific growth rate (SGR) and feed conversion ratio in juvenile Siberian sturgeon, *Acipenser baerii* (Wang et al., 2017), juvenile black carp, *Mylopharyngodon piceus* (Wu et al., 2020), and orange-spotted

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grouper, *Epinephelus coioides* (He et al., 2021). Growth performance and feed utilization in fish are associated with intestinal digestion and absorption functions (Zhang et al., 2019), especially in the case of stomachless fish (Le et al., 2019). However, the influence of VD_3 on the intestine, the primary site of digestion and absorption, and the related underlying mechanism remain poorly understood. Chen et al. (2017) reported that, in the primary human trophoblast cells, VD_3 facilitated the absorption of certain amino acids (AAs) by accelerating the activity of associated amino acid transporters (AATs), indicating that VD_3 may influence the absorption capacity on the intestine. Moreover, different intestinal sections of fish exhibit varied digestive abilities (Yuan et al., 2020). Therefore, the specific effects of VD_3 are likely to vary throughout the intestine and need to be further studied.

The digestion and absorption capacities of fish are reflected by the activities of digestive enzymes (e.g., trypsin and chymotrypsin) and intestinal brush border enzymes (e.g., alkaline phosphatase [AKP] and Na^+-K^+-ATPase) (García-Gasca et al., 2006). To date, just one study has explored the relationship between VD_3 and digestive enzymes in the animal gut; the study showed that VD_3 improved the activity of chymotrypsin in laying hens (Korének et al., 2000). However, no report has focused on the influence of VD_3 on brush border enzymes in animal intestines. Butyrate and manganese have been proved to elevate the brush border enzyme activities (e.g. trypsin, chymotrypsin, lipase, and amylase) and the levels of creatine kinas (CK) and γ-glutamyl transpeptidase (γ-GT), respectively, in the intestines of grass carp (Tang et al., 2016; Tian et al., 2017). VD_3 can increase butyrate and manganese levels in cells (Claro da Silva et al., 2016; Tanaka et al., 1990). Furthermore, these findings suggest that VD_3 affects the digestive and brush border enzymes activities in the animal intestine, and this requires further explanation.

Digestive enzymes are close-connected with nutrient digestion (Mourad and Saadé, 2011). Dietary protein, a fundamental nutrient, is decomposed into AAs and small peptides in the intestines (Coomer et al., 1993). The absorption and transportation of AAs and small peptides occur via AATs and small peptide transporters, respectively (Daniel, 2004; Ogihara et al., 1999). The AATs can be classified into four types based on their transport mechanisms and substrate specificity: neutral and cationic AATs (such as solute carrier [*SLC*]*7A7*), cationic AATs (such as *SLC7A1*), neutral AATs (such as *SLC7A5*), and anionic AATs (such as *SLC1A2*) (Perland and Fredriksson, 2017). *SLC15A1* is a peptide transporter (*PepT1*) that is highly expressed in animal intestines (Christensen, 1990). Chen et al. (2017) demonstrated that VD_3 increases the activity of *SLC38A2* in the primary human trophoblast cells. However, to our knowledge, no studies have explored the influence of VD_3 on the absorption capacity of AAs and small peptides in animal intestines. In rat skeletal muscle cells, *GSK3* enhanced *SLC38A2* mRNA expression (Stretton et al., 2019), and VD_3 increased GSK3 contents in a C2C12 cell line (Salles et al., 2013). Moreover, low levels of insulin decreased *SLC7A1* expression in umbilical cord veins endothelial cells (González et al., 2011). VD_3 stimulated pancreatic insulin secretion (Zeitz et al., 2003). Therefore, VD_3 potentially influences the absorptive capacity of AAs and relevant transporters in the intestines, which warrants a detailed study.

AAs and peptides are regulated by the target of rapamycin (TOR) signaling pathway (Benner et al., 2011). The mammalian TOR (mTOR) signaling regulates AATs by controlling activating transcription factor 4 (*ATF4*) mRNA, which is negatively regulated by 4E-binding protein 1 (4E-BP1) (Park et al., 2017). Studies investigating the relationship between VD_3 and the TOR signaling pathway during intestinal assimilation of animals are limited. In cardiac muscle cells, the expression of *mTOR* and ribosomal protein S6 kinase 1 (*S6K1*) can be activated by protein kinase C (*PKC*) (Moschella et al., 2007), the expression of which is mediated by VD_3 in chondrocytes (Sylvia et al., 1996). Additionally, in the C2C12 muscle cell line, VD_3 can activate c-Jun N-terminal kinase (*JNK*) expression (Buitrago et al., 2006), which inhibits *ATF4* mRNA expression in MC3T3-E1 cells (Matsuguchi et al., 2009). These studies indicate that VD_3 presumably accommodates AAs and peptide transporters in the animal intestine through the TOR signaling pathway, which requires further exploration.

Grass carp (*Ctenopharyngodon idella*), originally distributed in China, has now been introduced in over 100 countries and is considered one of the most important freshwater aquaculture species (Wu et al., 2012). Currently, no research has focused on the VD_3 requirements of grass carp. Furthermore, the reported nutritional requirements of fish are based on different indicators (Fang et al., 2020). Hence, it is of worthiness to estimate the VD_3 requirements of on-growing grass carp based on the various indicators.

In this study, we hypothesize that VD_3 supplementation increases the growth performance and intestinal digestion and absorption capacities of grass carp (*C. idella*) through a mechanism related to the TOR signaling pathway. To test this hypothesis, we aimed to investigate the effects of VD_3 on intestinal structural integrity, digestive enzyme (trypsin, chymotrypsin, and amylase), and brush border enzyme (AKP, Na^+/K^+-ATPase, γ-GT, and CK) activities in different segments of the grass carp intestine. To the best of our knowledge, this is the first investigation on VD_3 as it pertains to AATs, PepT1 transport, and associated signaling molecules (TOR/S6K1, 4E-BP1, and ATF4) in each intestinal segment of on-growing grass carp. This study offers insight into the VD_3 requirements of on-growing grass carp according to various indicators and can be used to guide the production of ideal commercial feeds, improving animal health and yield in grass carp aquaculture operations.

2. Materials and methods

The current study was approved by the procedures and guidelines of the University of Sichuan Agricultural Animal Care Advisory Committee (No. PXR-2019114009).

2.1. Experimental diets

The formula and approximate components of the experimental diets are displayed in Table 1. Protein sources were mainly composed with soybean protein concentrate, gelatin, and casein, with linseed and soy oil chosen as lipid sources. Dietary protein content reached 300 g/kg feed, which has been demonstrated to be adequate by the National Research Council (NRC, 2011). Different levels of VD_3 (500,000 IU/g) was provided: 0 (un-supplemented control), 400, 800, 1,200, 1,600, and 2,000 IU/kg feed, and corn starch was used to adjust dietary weight. Following analysis approaches of Takeuchi et al. (1984), the final amounts of VD_3 in each treatment were determined as 15.2 (un-supplemented control), 364.3, 782.5, 1,167.9, 1,573.8, and 1,980.1 IU/kg feed. The ingredients were mixed, pressed into pellets, and stored at −20 °C.

2.2. Experimental design, procedure and sample collection

This feeding trial was conducted in an authorized experimental base (Dayi County, Sichuan province, China). Experimental fish were acquired from a local farm (Chengdu, China). The fish selected for experiments were visibly healthy, with no parasites found under microscopic examination. Fish were acclimatized for 4 weeks before the experiment. Before the experiment, fish were provided with a basal diet (un-supplemented) for 2 weeks to reduce pre-accumulated levels of VD_3. At the beginning of the feeding

Table 1
Composition and nutrient levels of basal diet (dry matter basis, g/kg).

Item	Content
Ingredients	
Casein	135.0
Gelatin	44.7
Soybean protein concentrate	220.0
α-starch	240.0
Corn starch	209.1
linseed oil	17.7
Soy oil	14.5
Cellulose	50.0
$Ca(H_2PO_4)_2$	14.8
Vitamin premix [1]	10.0
Mineral premix [2]	20.0
Vitamin D_3 (VD_3) premix [3]	10.0
Choline chloride (50%)	10.0
Ethoxyquin (30%)	0.5
DL-Met	3.7
Total	1,000.0
Nutrients content[4]	
Crude protein	300.0
Crude fat	37.8
n-3 PUFAs	10.4
n-6 PUFAs	9.6
n-3: n-6 PUFAs	10.8
Available phosphorus	4.0
VD_3, μg/kg	3.8

PUFAs = polyunsaturated fatty acids.
[1] One kilogram of vitamin premix contained the following (g/kg): DL-α-tocopherol acetate (50%), 12.58; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.09; ascorbyl acetate (95%), 4.31; niacin (99%), 4.04; meso-inositol (98%), 19.39; calcium-D-pantothenate (98%), 3.85; riboflavin (80%), 0.73; pyridoxine hydrochloride (98%), 0.62; retinyl acetate (500,000 IU/g), 2.10. All ingredients were diluted with maize starch to 1 kg.
[2] One kilogram of mineral premix contained the following (g/kg): $FeSO_4.H_2O$ (30.0% Fe), 12.2500; $MgSO_4.H_2O$ (15.0% Mg), 200.0000; $ZnSO_4.H_2O$ (34.5% Zn), 8.2460; $MnSO_4.H_2O$ (31.8% Mn), 2.6590; $CuSO_4.5H_2O$ (25.0% Cu), 0.9560; Na_2SeO_3 (44.7% Se), 0.0168; KI (76.9% I), 0.0650g. All ingredients were diluted with maize starch to 1 kg.
[3] VD_3 premix: premix was added to obtain graded level of VD_3 and the amount of maize starch was reduced to compensate.
[4] The contents of crude protein and crude lipid were measured values on the air-dried matter basis. Supplement of available P, n-3 PUFAs, and n-6 PUFAs were calculated according to NRC (2011).

experiment, 540 fish were weighed (257.24 ± 0.63 g, mean ± SD) and randomly divided into 18 culture cages (140 cm × 140 cm × 140 cm), with 30 fish in each cage. Six different doses of VD_3 treatments were performed with 3 replicates. All cages were located in outdoor freshwater ponds, and microporous aeration was used throughout the trial. The dissolved oxygen concentration in each cage was >6 mg/L. Water temperature and pH were 28.0 ± 2.1 °C and 7.0 ± 0.2, respectively. All fish were subject to natural light conditions, with approximately 11 h light and 13 h darkness. Fish were fed to satiation four times daily for 10 weeks. A 100 cm^2 disk was equipped at the bottom of each cage to collected the feed residue. This was then dried and weighed to calculate feed intake (FI).

Toward the end of the 70-day feeding experiment, the fish were weighed and counted to determine growth performance. Twelve fish were randomly selected from each treatment and euthanized in a benzocaine bath (50.0 mg/L). The intestines were immediately removed, their length and weight were recorded, and tissues were preserved at −80 °C (Shao et al., 2021). On the basis of intestinal anatomical structure of grass carp (Ni and Wang, 1999), intestines were divided into proximal intestine (PI), mid intestine (MI), and distal intestine (DI) segments. The PI segment was determined as the length from the sphincter to the first turn, and the DI was defined as the last turn to the anus.

2.3. Histological analysis

Four percent paraformaldehyde was used to fix the different intestinal segments for subsequent histologic assessment (Song et al., 2021). After serial dehydration in gradually increasing concentrations of ethanol, the tissues were then xylene equilibration and paraffin embedded (Tsertou et al., 2020). The samples were dissected into 4-μm slides, dyed with hematoxylin and eosin (H&E), and observed for morphological structures using a Nikon TS100 light microscope (Tokyo, Japan).

2.4. Biochemistry assay

The PI, MI, and DI segments were homogenized in ice-cold 0.9% sterile physiological saline (1:10, wt/vol), incubated on ice for 30 min, and centrifuged at 6,000 × *g* at 4 °C for 20 min to collect the supernatant (Heidarieh et al., 2013). A bicinchoninic acid (BCA) assay kit was used to determine the protein concentration of the supernatant (Beyotime, Nanjing, China) according to the manufacturer's instructions. Activities of digestive enzymes trypsin, chymotrypsin, and amylase were determined following the process described by Ma et al. (2019), Korének et al. (2000), and Eshaghzadeh et al. (2015), respectively. AKP, Na^+/K^+-ATPase, γ-GT, and CK activities were measured according to Zhou et al. (2012), Bessey et al. (1946), Bauermeister et al. (1983), and Tanzer and Gilvarg (1959), respectively.

2.5. Free amino acid (FAA) analysis

For each sample, 400 mg tissue was homogenized in a 10% sulfosalicylic acid solution and centrifuged at 6,000 × *g* at 4 °C for 10 min. The collected supernatants were percolated through 0.22-μm filters for FAA measurements. The FAA content was analyzed using an automated AA analyzer (L-8080; Hitachi, Tokyo, Japan).

2.6. Real-time quantitative polymerase chain reaction (PCR)

Total intestinal RNA extraction, reverse transcription, and quantitative real-time PCR were performed following standard procedure by Zhao et al. (2020). Briefly, intestinal RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China), and treated with DNase I. The purity and quantity of RNA were identified via agarose gel (1.5%) electrophoresis and spectrophotometry (A260/280 nm ratio) analysis, respectively. Successively, RNA was reverse transcribed into complementary DNA using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). Oligonucleotide primers were adopted for quantitative real-time PCR (Table 2). The amplification efficiency of all genes was calculated based on the standard curve of a 10-step dilution sequence, which confirmed that the primer amplification efficiency was about 100%. β-actin was used as the reference gene for normalization.

2.7. Western blot analysis

The separation of tissue protein homogenate, antibodies, and the western blotting procedure were performed as previously reported (Huang et al., 2021). In brief, protein samples of equal mass (40 μg per row) were extracted, separated on sodium dodecyl sulfate (SDS)-glycine polyacrylamide gel, and then

Table 2
Real-time PCR primer sequences.

Gene	Forward sequences of primers (5′ to 3′)	Reverse sequences of primers (5′ to 3′)	Temperature, °C	GenBank number
SLC7A7	GATAGCCATCACTTTCTCCAAC	GTCCCATACTTCACATAGGCA	58.0	JX013494
SLC7A6	GCAGAGTTGGGCACGACAATCAC	GGAAGAGCGGCTGAACCAAGTAG	58.0	KC206055
SLC1A2a	ATCCATGGTGCCATATTCCTG	CAACGGAAAGTTACAGGCAGAG	59.6	KY200977
SLC1A3	GGAATGCTTTCGTCATCCTCAC	CAGAGCGGCCATACCTGTAATT	61.0	KY200978
SLC1A5	ACACTTTCTTGCTGGGATTGGT	GGATGGTGATGACCTGGACAAA	62.0	KU559898
SLC6A14	TCATTGCGTACCCTGATGCTCT	ACTTCAGAACTTTGGGATAGGCA	62.5	KX823959
SLC6A19b	ACCACTGGAAAGGCTGTTTATGT	ACCGCATCTTGCTCACAGTTATT	62.0	KX823960
SLC7A1	CGACATCACTGAACCCTCCAAC	TGCACAGGCTGAACAGGACACT	62.0	KY200979
SLC7A5	CAACATGAGCCGACCAGGAGAC	CCAGCGACAACCCGACTGAACC	62.0	KY200980
SLC7A8	TGGTGAGAAGCTGTTGGGAGTGA	GCAAGTGAAGAGTAGGGCTGGAA	59.6	GU474428
SLC7A9	TTCTACAGTCTTCTGCCGTTGC	AGAGCTGGAGAAGGCGTGTAAC	61.5	KX823958
SLC38A2	AGAAGAGTCCTGCAAACCCAA	CACAAACATTCCCAGAAACGA	61.7	KY200981
SLC6A6	CTCCGCAAGAACAAGACACTC	CCCACACAGCGAGCAGAC	62.5	KX682391
SLC15A1	TGCTCTTGTTGTGTTCATCG	CTCTCTCTTGGGGTATTGCTT	62.0	KC782748
atp1a1a.1	TGCCATTGTAGCCGTAAC	GGTGCCCAAAGGTAGAGG	60.3	JX854442
atp1a1a.4	GAGGTCGTTGCTGGTGAT	CAGTGAGGGAAGAGTTGTC	55.9	KM112094
CK	CTCCTCGTTCACCCAGAC	CAGCATCAAGGGATACGC	61.4	JX854444
TOR	TCCCACTTTCCACCAACT	ACACCTCCACCTTCTCCA	61.4	JX854449
S6K1	TGGAGGAGGTAATGGACG	ACATAAAGCAGCCTGACG	59.4	EF373673.1
4E-BP1	GCTGGCTGAGTTTGTGGTTG	CGAGTCGTGCTAAAAAGGGTC	60.3	KT757305.1
ATF4	CAACATTAGCAGCGGCAA	GTGAGCGGGTGAACGGT	56.3	AY437846.1
β-actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	61.4	M25013

SLC = solute carrier; *atp1a1a.1* = Na^+/K^+-ATPase alpha-subunit isoform 1; *atp1a1a.4* = Na^+/K^+-ATPase alpha-subunit isoform 4; *CK* = creatine kinase; *TOR* = target of rapamycin; *S6K1* = ribosomal protein S6 kinase 1; *4E-BP1* = eIF4E-binding protein 1; *ATF4* = activating transcription factor.

transferred to the polyvinylidene-fluoride membrane. The membrane was blocked with blocking solution (0.5%) for 2 h at room temperature, and then incubated with primary antibody overnight at 4 °C. Primary antibodies against β-actin (1:3,000), anti-total TOR (1:1,000), p-$TOR^{Ser2448}$ (1:1,000), and p-ATF^{Ser219}(1:1,000) were purchased from Affinity BioReagents (Golden, Colorado, USA); antibodies against p-$S6K1^{Thr389}$ (1:1,000) and p-4E-$BP1^{Thr\ 37/46}$ (1:1,000) were obtained from Affinity Technology (Shanghai, China). We washed the membranes, incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h, and visualized the immune complexes under ChemiDoc imaging system (Bio-Rad, USA).

2.8. Statistical analyses

Calculations for growth performance parameters are listed in Table 3. Data were presented as means ± SD, as indicated in the table legends. Statistical tests were performed using SPSS Statistics v.25 (IBM Corp., Armon, NY, USA). Samples were analyzed using one-way analysis of variance and Duncan's multiple range test to evaluate statistical differences among treatments at the level of $P < 0.05$. We used a nonparametric Kruskal–Wallis test for normalization if the data did not display normal distribution. Regression and correlation analyses were performed using the broken-line model and Pearson's correlation, respectively.

Table 3
Index formula for growth performance of on-growing grass carp (*Ctenopharyngodon idella*).

Item	Formulas
PWG	PWG (%) = 100 × [FBW (g/fish) − IBW (g/fish)]/IBW (g)
SGR	SGR (%/d) = 100 × [ln FBW (g/fish) − ln IBW (g/fish)]/d
FE	FE (%) = 100 × [FBW (g/fish) − IBW (g/fish)]/FI (g/fish)
ISI	ISI (%) = 100 × Wet intestine weight (g)/Wet body weight (g)
ILI	ILI (%) = 100 × Wet intestine length (cm)/Wet body length (cm)

PWG = percentage of weight gain; FBW = final body weight; IBW = initial body weight; SGR = specific growth rate; FE = feed efficiency; FI = feed intake; ISI = intestinal somatic index; ILI = intestinal length index.

3. Results

3.1. Growth performance and histopathology of on-growing grass carp

During and at end of the 70 days feeding trial, the survival rates of all experimental grass carp were 100%. As displayed in Table 4, final body weight (FBW), percentage weight gain (PWG), SGR, FI, feed efficiency (FE), intestinal length (IL), and intestinal weight (IW) increased significantly ($P < 0.05$) compared with those of the control for VD_3 supplementation up to 1,167.9 IU/kg, and decreased significantly ($P < 0.05$) at higher concentrations. Intestinal somatic index (ISI) and intestinal length index (ILI) increased minimally for dietary VD_3 levels up to 1,167.9 IU/kg but decreased slightly at higher levels. The folds height in the three intestinal segments increased with VD_3 levels up to 1,167.9 IU/kg ($P < 0.05$), above which they decreased in all segments. The histopathological analysis results (Fig. 1) revealed that VD_3 deficiency was associated with inflammatory cell infiltration in the PI, blood capillary hyperemia in the MI, and blood capillary hyperemia and goblet cell hyperplasia in the DI.

3.2. Digestive and absorption enzyme activities, and Na^+/K^+-ATPase alpha-subunit isoform 1 (atp1a1a1), atp1a1a4, and creatine kinase (CK) mRNA levels

All intestinal digestion enzyme activities under different VD_3 levels are displayed in Table 5. Peak levels of trypsin and amylase activities were observed with dietary VD_3 supplementation levels of 782.5 and 1,167.9 IU/kg, respectively, and activities plateaued at higher values ($P > 0.05$). Chymotrypsin activity increased slightly along with an enhancement in dietary VD_3, up to 1,167.9 IU/kg, and declined slightly at higher levels. The levels of brush border enzymes are presented in Table 6. The AKP activities in the PI, MI, and DI slightly increased with the supplementation of dietary VD_3 levels up to 1,167.9, 1,573.8 and 1,167.9 IU/kg, respectively, and they were lower in all intestinal segments at higher VD_3 levels. Na^+/K^+-ATPase activities were increased by the supplementation of dietary VD_3, with the highest levels at VD_3 supplementation level of 1,167.9

Table 4
Growth performance and intestinal growth of on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of VD_3 for 10 weeks.

Item	VD_3 level, IU/kg diet						*P*-value
	15.2 (control)	364.3	782.5	1,167.9	1,573.8	1,980.1	
IBW[1], g/fish	257.00 ± 0.67	257.11 ± 0.38	258.00 ± 0.67	257.11 ± 0.77	257.33 ± 0.67	256.89 ± 0.38	0.328
FBW[1], g/fish	854.99 ± 14.42^{a}	965.39 ± 15.01^{c}	1,051.49 ± 13.21^{d}	1,129.67 ± 14.73^{e}	973.48 ± 10.55^{c}	883.08 ± 10.87^{b}	<0.001
PWG[1], %	232.68 ± 5.76^{a}	275.48 ± 5.86^{c}	306.85 ± 5.28^{d}	339.37 ± 7.03^{e}	276.02 ± 3.33^{c}	242.57 ± 4.45^{b}	<0.001
SGR[1], %/d	1.72 ± 0.02^{a}	1.89 ± 0.02^{c}	2.01 ± 0.01^{d}	2.11 ± 0.02^{e}	1.90 ± 0.02^{c}	1.76 ± 0.02^{b}	<0.001
FI[1], g/fish	927.47 ± 1.11^{a}	1,051.64 ± 1.66^{c}	1,084.60 ± 1.93^{d}	1,143.64 ± 0.91^{e}	1,051.06 ± 2.69^{c}	954.36 ± 1.36^{b}	<0.001
FE[1], %	0.64 ± 0.02^{a}	0.67 ± 0.01^{bc}	0.73 ± 0.01^{d}	0.76 ± 0.01^{e}	0.68 ± 0.01^{c}	0.66 ± 0.01^{ab}	<0.001
IL[2], cm	61.36 ± 2.48^{a}	64.92 ± 2.52^{b}	69.85 ± 4.95^{c}	68.83 ± 4.89^{c}	65.00 ± 3.07^{b}	62.92 ± 2.85^{ab}	<0.001
ISI[2], %	1.68 ± 0.07^{a}	1.76 ± 0.10a^{bc}	1.79 ± 0.05^{bc}	1.82 ± 0.07^{c}	1.76 ± 0.17^{abc}	1.73 ± 0.11^{ab}	0.02
IW[2], g	14.28 ± 0.91^{a}	16.83 ± 0.81^{b}	18.52 ± 1.16^{c}	20.12 ± 1.91^{d}	17.13 ± 1.35^{b}	15.1 ± 1.27^{a}	<0.001
ILI[2], %	175.78 ± 6.87^{a}	179.74 ± 7.64^{ab}	186.43 ± 13.46^{bc}	187.99 ± 10.54^{c}	181.23 ± 7.50^{abc}	179.02 ± 7.21^{ab}	0.015
Folds height							
PI[3], μm	1,274.17 ± 191.08^{a}	1,520.17 ± 192.56^{b}	1,734.50 ± 190.88^{bc}	1,914.50 ± 254.33^{c}	1,641.50 ± 195.55^{b}	1,573.83 ± 192.98^{b}	<0.001
MI[3], μm	1,001.17 ± 115.53^{a}	1,220.67 ± 177.77^{b}	1,280.83 ± 176.36^{bc}	1,445.33 ± 200.79^{c}	1,320.17 ± 153.27^{bc}	1,201.67 ± 202.87^{ab}	0.004
DI[3], μm	976.83 ± 140.05^{a}	1,017.67 ± 130.43^{a}	1,141.00 ± 133.27^{ab}	1,214.67 ± 161.81^{b}	1,049.67 ± 140.06^{ab}	1,004.67 ± 128.87^{a}	0.046

IBW = initial body weight; FBW = final body weight; PWG = percentage of weight gain; SGR = specific growth rate; FI = feed intake; FE = feed efficiency; IL = intestinal length; ISI = intestinal somatic index; IW = intestinal weight; ILI = intestinal length index; PI = proximal intestine; MI = mid intestine; DI = distal intestine.
$^{a-e}$ Mean values within a row with different superscript letters indicate significant difference (one-way ANDOVA and Duncan's multiple-range tests, $P < 0.05$).
[1] Values are means ± SD for 3 replicate groups, with 30 fish in each group.
[2] Values are means ± SD ($n = 3$).
[3] Values are means ± SD ($n = 6$).

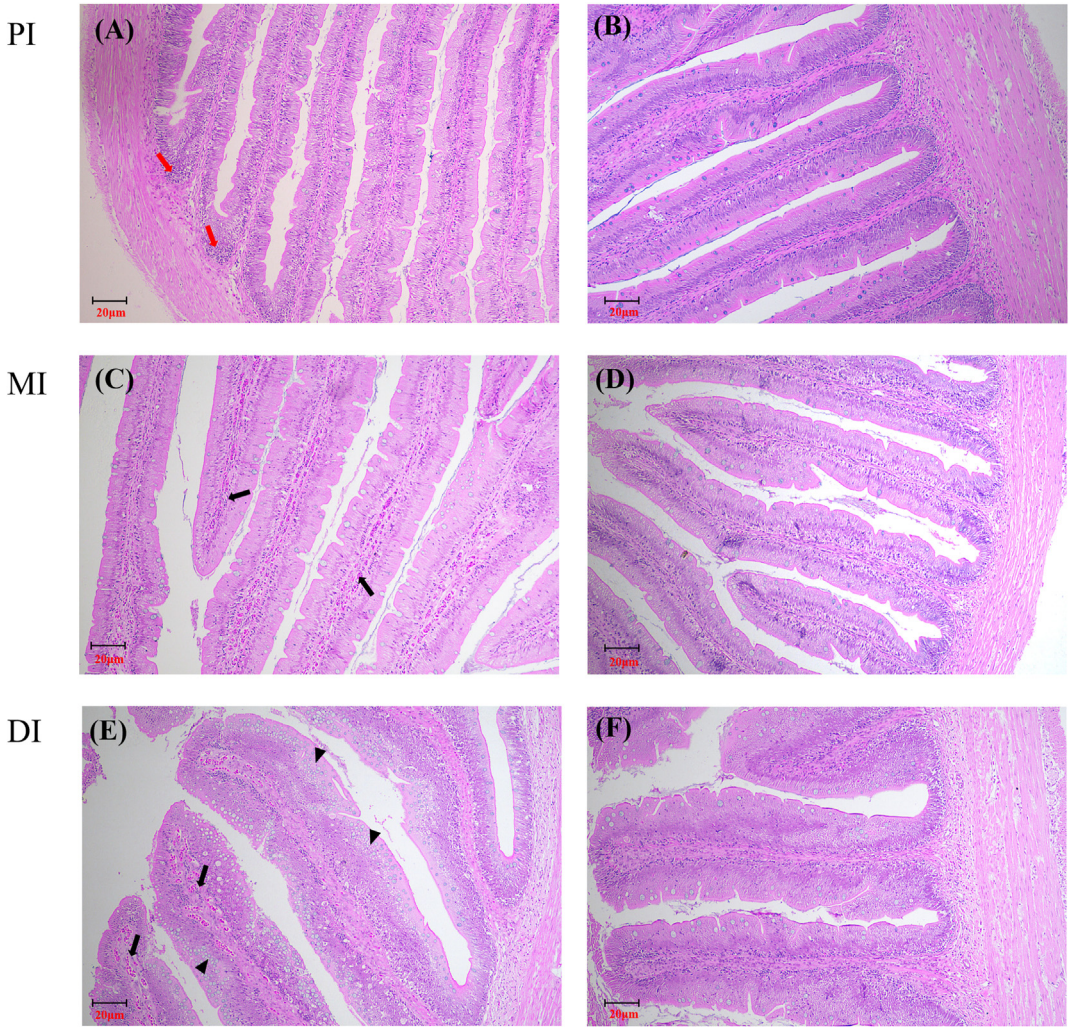

Fig. 1. The hematoxylin and eosin (H&E) histology of PI, MI, and DI (100×) in on-growing grass carp fed diets containing different levels of VD_3 (IU/kg). (A), (C), and (E), 15.2 IU/kg diet (Un-supplement control); (B), (D), and (F), 1,167.9 IU/kg diet. Red arrowhead, black arrowhead, and triangle showed, respectively, the inflammatory cell infiltration, the blood capillary hyperemia, and the goblet cell hyperplasia. VD_3 = vitamin D_3; PI = proximal intestine; MI = mid intestine; DI = distal intestine.

Table 5
Digestive enzymes activities in the intestine of the on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of vitamin D_3 for 10 weeks (U/g tissue)[1].

Item	VD_3 level, IU/kg diet						*P*-value
	15.2 (control)	364.3	782.5	1,167.9	1,573.8	1,980.1	
Trypsin	0.82 ± 0.08[a]	0.98 ± 0.09[b]	1.25 ± 0.11[c]	1.22 ± 0.13[c]	1.20 ± 0.09[c]	1.02 ± 0.13[c]	<0.001
Chymotrypsin	9.07 ± 0.77[a]	9.13 ± 0.74[a]	10.61 ± 0.82[b]	12.02 ± 1.32[c]	11.99 ± 1.09[b]	11.39 ± 0.83[b]	<0.001
Amylase	1,009.12 ± 86.67[a]	1,180.49 ± 66.10[bc]	1,245.55 ± 75.92[c]	1,293.35 ± 139.44[c]	1,164.97 ± 127.77[bc]	1,091.18 ± 102.44[ab]	0.001

[a-c] Mean values within a row with different superscript letters indicate significant difference (one-way ANOVA and Duncan's multiple-range tests, $P < 0.05$).
[1] Values are means ± SD ($n = 6$).

Table 6
Brush border enzymes of the PI, MI, and DI in on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of VD_3 for 10 weeks[1].

Item	VD_3 level, IU/kg diet						*P*-value
	15.2 (control)	364.3	782.5	1,167.9	1,573.8	1,980.1	
PI							
AKP	69.11 ± 7.09[a]	75.45 ± 4.26[abc]	78.7796 ± 7.23[bc]	80.96 ± 7.47[c]	74.21 ± 1.99[abc]	72.61 ± 4.19[ab]	0.017
Na^+/K^+-ATPase	190.96 ± 11.63[a]	207.50 ± 8.49[b]	220.49 ± 11.41[bc]	231.28 ± 11.95[c]	209.23 ± 18.51[b]	207.97 ± 10.02[b]	<0.001
γ-GT	23.58 ± 2.46[a]	26.91 ± 2.52[bc]	27.00 ± 2.55[bc]	29.05 ± 2.71[c]	26.04 ± 2.70[abc]	23.79 ± 2.19[ab]	0.006
CK	91.77 ± 5.73[a]	107.47 ± 11.34[a]	110.69 ± 10.55[ab]	128.18 ± 13.52[b]	115.62 ± 12.56[ab]	106.60 ± 10.60[a]	0.037
MI							
AKP	63.53 ± 5.38[a]	66.14 ± 6.32[ab]	72.97 ± 6.91[bc]	73.19 ± 6.30[bc]	74.77 ± 7.53[c]	69.52 ± 4.36[abc]	0.024
Na^+/K^+-ATPase	123.13 ± 12.41[a]	131.46 ± 12.10[ab]	139.54 ± 15.55[bc]	149.07 ± 11.76[c]	129.74 ± 11.60[ab]	126.31 ± 8.73[ab]	0.01
γ-GT	20.98 ± 2.24[a]	24.16 ± 1.76[b]	26.93 ± 1.68[c]	27.85 ± 2.34[c]	23.72 ± 2.13[b]	21.79 ± 1.68[ab]	0.002
CK	100.34 ± 7.24[a]	105.69 ± 11.04[ab]	120.42 ± 9.86[c]	122.83 ± 6.83[c]	113.91 ± 10.33[bc]	112.67 ± 9.34[bc]	<0.001
DI							
AKP	58.41 ± 6.70[a]	60.01 ± 6.88[a]	64.80 ± 5.16[bc]	70.65 ± 6.54[c]	63.28 ± 6.69[bc]	58.95 ± 6.00[a]	0.02
Na^+/K^+-ATPase	106.30 ± 7.60[a]	111.11 ± 9.26[a]	119.60 ± 5.66[ab]	127.78 ± 18.02[b]	116.06 ± 9.52[ab]	107.00 ± 11.36[a]	0.015
γ-GT	18.21 ± 0.77[a]	19.47 ± 0.79[ab]	21.38 ± 2.78[c]	21.26 ± 1.40[c]	20.35 ± 2.22[ab]	18.94 ± 1.56[a]	0.019
CK	50.29 ± 5.66[a]	56.38 ± 6.59[abc]	58.53 ± 4.64[bc]	60.21 ± 4.98[c]	55.21 ± 3.87[abc]	53.08 ± 5.55[ab]	0.034

PI = proximal intestine; MI = mid intestine; DI = distal intestine; AKP = alkaline phosphatase, mmol of nitrophenol released/g tissue; Na^+/K^+-ATPase, μmol of phosphorus released/g tissue; γ-GT = γ-glutamyl transpeptidase, mmol of 5-amino-2-nitrobenzoate released/g tissue; CK = creatine kinase, μmol of phosphorus released/g tissue.
[a-c] Mean values within a row with different superscript letters indicate significant difference (one-way ANOVA and Duncan's multiple-range tests, $P < 0.05$).
[1] Values are means ± SD ($n = 6$).

IU/kg diet, and at higher levels maintained constant level in the PI and MI ($P > 0.05$), while decreasing slightly in the DI. The activities of γ-GT reached the highest values at dietary VD_3 levels of 1,167.9, 1,167.9, and 782.5 IU/kg in PI, MI, and DI segments, respectively. At higher levels, the activities decreased slightly in the PI and significantly in the MI and DI ($P < 0.05$). Dietary VD_3 supplementation elevated CK activities within all intestinal segments, and peak values were obtained in the 1,167.9 IU/kg treatment, decreasing minimally at higher levels.

Intestinal *CK*, *atp1a1a1*, and *atp1a1a4* mRNA levels under different VD_3 treatments are presented in Fig. 2. Following dietary VD_3 supplementation of up to 1,167.9 IU/kg, the *CK* mRNA was upregulated minimally in the PI and DI but significantly in the MI ($P < 0.05$) and then was downregulated slightly in all segments. *atp1a1a1* levels increased significantly in the PI and MI under VD_3 supplementation up to 1,167.9 IU/kg ($P < 0.05$) but increased only minimally in the DI; it level was decreased at higher supplement levels. The mRNA levels of *atp1a1a4* increased slightly with VD_3 supplementation up to 1,167.9 IU/kg in the three intestinal segments and decreased slightly in the PI; however, *atp1a1a4* mRNA levels exhibited a stabilizing trend in the MI and DI above 1,167.9 IU/kg treatment.

3.3. FAA profile of intestinal segments

As displayed in Table 7, with dietary VD_3 supplementation of 1,167.9 IU/kg, the Met, Gly, Trp, Thr, Ile, Phe, Gly, Pro, Ser, Tyr, Tau, His, Arg, His, and Glu contents increased in the PI, but were lower at higher treatment levels. Levels of Val and Cys elevated with an increase in VD_3 levels up to 782.5 IU/kg and then decreased at higher levels. However, VD_3 had no effect on Leu in the PI ($P > 0.05$). In the MI, the contents of Met, Trp, Thr, Ile, Leu, Val, Phe, Ser, Tyr, Tau, Arg, His, and Glu increased with dietary VD_3 levels up to 1,167.9 IU/kg and decreased slightly at higher levels ($P > 0.05$). The contents of Gly and Cys minimally increased with increasing VD_3 levels up to 1,573.8 IU/kg and then decreased minimally at higher levels ($P > 0.05$). The content of Pro was higher in VD_3 supplement groups than that in control groups and stayed relatively constant. In the DI, levels of Met, Thr, Ile, Leu, Val, Phe, Pro, Ser, Tyr, Cys, Tau, Arg, His, and Glu increased with the supplementation of VD_3 up to 1,167.9 IU/kg, and decreased slightly above this level ($P > 0.05$). Tyr and Gly contents enhanced with the VD_3 levels increased up to 1,573.8 IU/kg, decreasing slightly at higher levels. Notably, VD_3 supplementation did not affect Ala, Lys, or Asp contents in any intestinal segment ($P > 0.05$).

3.4. Intestinal AATs and PepT1 mRNA levels

As illustrated in Fig. 3, VD_3 supplementation up to 1,167.9 IU/kg corresponded to increased *SLC7A6*, *SLC7A1*, *SLC7A5*, *SLC7A8*, *SLC38A2*, *SLC6A19b*, and *SLC15A1* mRNA levels in the PI; at higher treatment levels, all were downregulated. *SLC7A9*, *SLC6A6*, and *SLC1A2* mRNA was upregulated with VD_3 supplementation of up to 1,573.8, 782.5, and 782.5 IU/kg diet, respectively, and under higher VD_3 concentrations, they declined minimally. No significant differences were observed in *SLC6A14* levels in the PI ($P > 0.05$). In the MI, as dietary VD_3 levels increased from 15.2 to 1,167.9 IU/kg, *SLC7A6*, *SLC7A1*, *SLC38A2*, *SLC6A19b*, *SLC1A2*, and *SLC15A1* mRNA expression increased, and then their expression decreased at higher VD_3 concentrations. The mRNA levels of *SLC7A9*, *SLC7A8*, and *SLC6A6* all increased with VD_3 supplementation up to 782.5 IU/kg, and were downregulated at higher levels. The peak level of *SLC6A14*

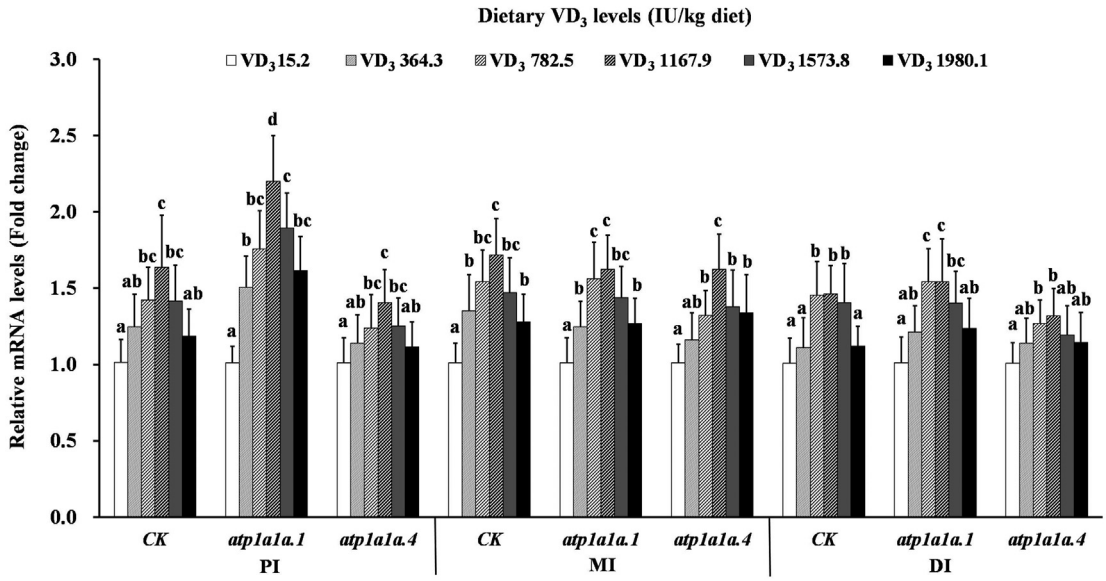

Fig. 2. Relative expression of *CK*, *atp1a1a.1*, and *atp1a1a.4* in the PI, MI, and DI of on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of VD_3 for 10 weeks. Mean values ± SD. $n = 6$ for each bar. Different letters above the bars indicate significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$). VD_3 = vitamin D_3; PI = proximal intestine; MI = mid intestine; DI = distal intestine; CK = creatine kinase.

was found at VD_3 supplementation of 1,573.8 IU/kg. Notably, VD_3 had no marked effect on *SLC7A5* in the MI ($P > 0.05$). In the DI, with the supplementation of VD_3 up to 1,167.9 IU/kg, *SLC7A6*, *SLC7A9*, *SLC7A1, SLC7A8*, *SLC38A2*, *SLC6A6*, *SLC1A2*, and *SLC15A1* mRNA levels were upregulated and then downregulated at higher levels. Following VD_3 supplementation up to 782.5 IU/kg, *SLC6A14*, and *SLC6A19b* mRNA levels in the DI increased and then decreased slightly at higher levels of dietary VD_3. The mRNA levels of *SLC7A5* were not affected by VD_3 in the DI ($P > 0.05$). Notably, *SLC7A7*, *SLC1A5*, and *SLC1A3* mRNA levels exhibited no marked changes in on-growing grass carp ($P > 0.05$).

3.5. Intestinal mRNA and protein phosphorylation levels of TOR, 4E-BP1, S6K1, and ATF4 of on-growing grass carp

As shown in Fig. 4, *TOR* mRNA was upregulated at VD_3 supplementation levels of 782.5, 1,167.9, and 1,167.9 IU/kg diet in the PI, MI, and DI, respectively, and downregulated at higher levels. In addition, *S6K1* mRNA was upregulated slightly with VD_3 supplementation of 1,167.9, 1,167.9, and 782.5 IU/kg within the PI, MI, and DI, respectively, and downregulated at higher levels. Furthermore, the mRNA levels of *4E-BP1* decreased significantly with dietary VD_3 supplementation of 1,167.9 IU/kg ($P < 0.05$) and increased slightly in the MI and DI ($P < 0.05$); however, levels remained steady in the PI ($P > 0.05$). *ATF4* mRNA was upregulated with VD_3 supplementation up to 1,167.9 IU/kg diet and slightly downregulated in the MI and DI at higher levels; however, a stabilizing trend was observed in the PI ($P > 0.05$).

Protein levels of signaling molecules in the three intestinal segments of on-growing grass carp with varying VD_3 supplementation are illustrated in Fig. 5. No significant differences were detected in protein levels of T-TOR within the PI, MI, or DI ($P > 0.05$) under different VD_3 levels. The p-TOR protein levels increased with VD_3 supplementation up to 1,167.9, 1,167.9, and 782.5IU/kg diets in the PI, MI, and DI, respectively, and were lower under higher levels in the PI and MI, while remaining stable in the DI ($P > 0.05$). Protein levels of p-4E-BP1 decreased with VD_3 supplementation up to 1,167.9 IU/kg and plateaued in the PI and DI at higher levels ($P > 0.05$), while increasing minimally in the MI. Increasing levels of p-S6K1 were observed in all segments, with increasing VD_3 supplementation up to 1,167.9 IU/kg diet. Levels decreased slightly in the PI and significantly in the MI at higher levels ($P < 0.05$), while plateauing in the DI. The p-ATF4 protein levels reached the highest level when VD_3 levels were 1,167.9 IU/kg, 1,573.8, and 1,167.9 IU/kg in the PI, MI, and DI, respectively, and then stabilized in the DI ($P > 0.05$); however, the levels decreased significantly in the PI and MI segments ($P < 0.05$).

4. Discussion

It has been demonstrated that VD_3 increases the uptake of certain AAs by facilitating the activities of AATs, such as *SLC38A2* in cultured cells (Chen et al., 2017). However, the function of VD_3 in the intestine, the primary position for nutrients digestion and absorption, is not fully understood. In the current study, for the first time, to our knowledge, we explored the role and mechanisms of VD_3 in the intestinal absorption of nutrients by analyzing its effects on digestion and brush border enzyme activities, AAs and AATs levels in different intestinal segments in fish, as well as the key TOR signaling pathway.

4.1. VD_3 deficiency decreased growth performance and disrupted intestinal morphology of fish

Reductions in FBW, PWG, and SGR were observed in fish under VD_3 deficiency (control group, 15.2 IU/kg), which was consistent with observations of Wuchang bream, *Megalobrama amblycephala* (Hong et al., 2015) and Atlantic salmon, *Salmo salar* L (Graff et al., 2002). Fish growth performance is associated with FI and FE (Zhang et al., 2013); thus, our findings revealed that VD_3 deficiency was associated with poor growth. Additionally, fish growth, reflected by the IL, ISI, IW, and ILI indices, is closely related to intestinal development (Wu et al., 2011). Our results demonstrated that dietary VD_3 deficiency decreased IL, ISI, IW, and ILI, suggesting VD_3 deficiency impairs growth and development of fish intestines.

In fish, intestinal growth is correlated with structural integrity (Zhang et al., 2013). Pathological changes in intestinal structure reflected in the form of inflammatory cell infiltration, goblet cell

Table 7
Free amino acid contents of the PI, MI, and DI in on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of VD_3 for 10 weeks (mg/100g tissue) [1].

Item	VD_3 level, IU/kg diet						*P*-value
	15.2 (control)	364.3	782.5	1,167.9	1,573.8	1,980.1	
PI							
Neutral amino acids							
Met	0.83 ± 0.06^{a}	0.89 ± 0.09^{a}	1.11 ± 0.13^{b}	1.12 ± 0.13^{b}	1.03 ± 0.15^{ab}	0.84 ± 0.10^{a}	0.023
Trp	0.69 ± 0.06^{a}	0.75 ± 0.10^{ab}	0.89 ± 0.04^{bc}	0.99 ± 0.10^{c}	0.78 ± 0.10^{ab}	0.75 ± 0.05^{ab}	0.006
Thr	1.21 ± 0.08^{a}	1.40 ± 0.15^{ab}	1.52 ± 0.15^{b}	1.57 ± 0.20^{ab}	1.30 ± 0.10^{a}	1.22 ± 0.05^{a}	0.050
Ile	1.10 ± 0.10^{a}	1.30 ± 0.08^{abc}	1.37 ± 0.13^{bc}	1.47 ± 0.21^{c}	1.32 ± 0.13^{abc}	1.15 ± 0.09^{ab}	0.039
Ala	1.72 ± 0.11	1.73 ± 0.11	1.80 ± 0.11	1.84 ± 0.08	1.79 ± 0.15	1.72 ± 0.09	0.766
Leu	2.11 ± 0.16	2.29 ± 0.16	2.32 ± 0.15	2.62 ± 0.17	2.44 ± 0.35	2.36 ± 0.17	0.980
Val	1.20 ± 0.10^{a}	1.41 ± 0.12^{ab}	1.60 ± 0.08^{b}	1.58 ± 0.15^{b}	1.36 ± 0.12^{ab}	1.29 ± 0.20^{a}	0.018
Phe	1.35 ± 0.10^{a}	1.42 ± 0.08^{ab}	1.56 ± 0.03^{b}	1.81 ± 0.15^{c}	1.58 ± 0.17^{b}	1.41 ± 0.13^{ab}	0.002
Gly	1.10 ± 0.12^{a}	1.24 ± 0.08^{a}	1.32 ± 0.17^{ab}	1.52 ± 0.16^{b}	1.21 ± 0.16^{a}	1.12 ± 0.11^{a}	0.028
Pro	1.13 ± 0.09^{a}	1.38 ± 0.10^{bc}	1.44 ± 0.11^{c}	1.46 ± 0.08^{c}	1.30 ± 0.05^{bc}	1.28 ± 0.07^{b}	0.004
Ser	1.27 ± 0.09^{a}	1.29 ± 0.11^{ab}	1.55 ± 0.09^{bc}	1.68 ± 0.26^{c}	1.54 ± 0.08^{c}	1.33 ± 0.10^{ab}	0.016
Tyr	1.52 ± 0.16^{a}	1.73 ± 0.23^{ab}	1.87 ± 0.19^{bc}	2.16 ± 0.18^{c}	1.83 ± 0.19^{ab}	1.65 ± 0.10^{ab}	0.014
Cys	0.21 ± 0.02^{a}	0.26 ± 0.03^{ab}	0.36 ± 0.04^{c}	0.32 ± 0.04^{bc}	0.31 ± 0.06^{bc}	0.29 ± 0.02^{bc}	0.010
Tau	1.33 ± 0.14^{a}	1.50 ± 0.20^{ab}	1.67 ± 0.16^{bc}	1.84 ± 0.09^{c}	1.54 ± 0.16^{ab}	1.39 ± 0.12^{ab}	0.016
Basic amino acids							
Lys	1.25 ± 0.09	1.30 ± 0.09	1.41 ± 0.07	1.35 ± 0.11	1.30 ± 0.13	1.21 ± 0.12	0.336
Arg	1.49 ± 0.08^{a}	1.62 ± 0.10^{a}	1.81 ± 0.17^{ab}	2.16 ± 0.25^{c}	1.87 ± 0.18^{bc}	1.52 ± 0.12^{a}	0.002
His	0.45 ± 0.07^{a}	0.62 ± 0.11^{b}	0.66 ± 0.05^{bc}	0.77 ± 0.06^{c}	0.76 ± 0.05^{c}	0.63 ± 0.02^{b}	0.001
Acidic amino acids							
Asp	1.99 ± 0.14	2.10 ± 0.15	2.10 ± 0.13	2.00 ± 0.19	1.96 ± 0.22	1.95 ± 0.07	0.776
Glu	2.12 ± 0.24^{a}	2.32 ± 0.14^{abc}	2.56 ± 0.17^{cd}	2.82 ± 0.22^{d}	2.50 ± 0.25^{bcd}	2.15 ± 0.09^{ab}	0.007
MI							
Neutral amino acids							
Met	0.75 ± 0.09^{a}	0.97 ± 0.15^{ab}	1.22 ± 0.14^{bc}	1.37 ± 0.18^{c}	1.23 ± 0.10^{bc}	0.98 ± 0.18^{ab}	0.003
Trp	0.56 ± 0.07^{a}	0.66 ± 0.08^{ab}	0.79 ± 0.05^{bc}	0.89 ± 0.09^{c}	0.85 ± 0.11^{c}	0.67 ± 0.05^{ab}	0.001
Thr	1.31 ± 0.21^{a}	1.46 ± 0.17^{a}	1.65 ± 0.12^{ab}	1.84 ± 0.27^{b}	1.41 ± 0.10^{a}	1.32 ± 0.10^{a}	0.018
Ile	1.08 ± 0.14^{a}	1.18 ± 0.15^{abc}	1.40 ± 0.09^{cd}	1.50 ± 0.10^{d}	1.33 ± 0.12^{bcd}	1.13 ± 0.13^{ab}	0.009
Ala	1.84 ± 0.13	1.91 ± 0.24	1.94 ± 0.28	1.84 ± 0.16	1.77 ± 0.11	1.65 ± 0.04	0.455
Leu	2.03 ± 0.16^{a}	2.16 ± 0.10^{a}	2.33 ± 0.29^{ab}	2.65 ± 0.26^{b}	2.40 ± 0.20^{ab}	2.18 ± 0.17^{a}	0.042
Val	1.33 ± 0.16^{a}	1.49 ± 0.10^{ab}	1.64 ± 0.10^{b}	1.68 ± 0.15^{b}	1.47 ± 0.15^{ab}	1.36 ± 0.09^{a}	0.029
Phe	1.39 ± 0.11^{a}	1.60 ± 0.20^{abc}	1.68 ± 0.13^{bc}	1.75 ± 0.12^{c}	1.44 ± 0.15^{ab}	1.44 ± 0.10^{ab}	0.037
Gly	1.043 ± 0.15^{a}	1.11 ± 0.10^{a}	1.27 ± 0.14^{ab}	1.48 ± 0.20^{b}	1.50 ± 0.11^{b}	1.13 ± 0.11^{a}	0.005
Pro	1.15 ± 0.10^{a}	1.39 ± 0.10^{b}	1.47 ± 0.13^{b}	1.59 ± 0.09^{b}	1.41 ± 0.17^{b}	1.36 ± 0.13^{b}	0.019
Ser	1.17 ± 0.12^{a}	1.40 ± 0.17^{ab}	1.49 ± 0.13^{b}	1.61 ± 0.16^{bc}	1.57 ± 0.11^{c}	1.36 ± 0.08^{ab}	0.003
Tyr	1.51 ± 0.20^{a}	1.86 ± 0.19^{b}	1.99 ± 0.13^{b}	2.07 ± 0.14^{b}	2.01 ± 0.20^{b}	1.80 ± 0.13^{ab}	0.016
Cys	0.22 ± 0.02^{a}	0.27 ± 0.02^{a}	0.34 ± 0.04^{b}	0.37 ± 0.05^{bc}	0.42 ± 0.04^{c}	0.36 ± 0.04^{bc}	<0.001
Tau	1.40 ± 0.15^{a}	1.48 ± 0.08^{ab}	1.64 ± 0.11^{bc}	1.81 ± 0.11^{c}	1.77 ± 0.11^{c}	1.42 ± 0.11^{a}	0.002
Basic amino acids							
Lys	1.47 ± 0.15	1.50 ± 0.13	1.57 ± 0.08	1.45 ± 0.10	1.41 ± 0.11	1.41 ± 0.10	0.573
Arg	1.483 ± 0.12^{a}	1.62 ± 0.15^{ab}	1.75 ± 0.14^{b}	2.16 ± 0.29^{b}	1.85 ± 0.24^{b}	1.68 ± 0.10^{ab}	0.016
His	0.45 ± 0.06^{a}	0.54 ± 0.09^{ab}	0.67 ± 0.07^{bc}	0.74 ± 0.06^{d}	0.73 ± 0.06^{cd}	0.68 ± 0.04^{b}	<0.001
Acidic amino acids							
Asp	1.88 ± 0.17	2.05 ± 0.10	2.20 ± 0.10	2.05 ± 0.29	1.93 ± 0.22	1.83 ± 0.18	0.261
Glu	2.07 ± 0.25^{a}	2.22 ± 0.21^{ab}	2.58 ± 0.13^{bc}	2.63 ± 0.26^{c}	2.29 ± 0.23^{abc}	2.20 ± 0.13^{ab}	0.036
DI							
Neutral amino acids							
Met	0.74 ± 0.11^{a}	0.83 ± 0.09^{a}	1.02 ± 0.14^{b}	1.24 ± 0.08^{c}	1.15 ± 0.10^{bc}	1.04 ± 0.10^{bc}	0.001
Trp	0.57 ± 0.05^{a}	0.63 ± 0.09^{a}	0.82 ± 0.04^{bc}	0.91 ± 0.07^{c}	1.02 ± 0.10^{bc}	0.87 ± 0.05^{ab}	0.001
Thr	1.32 ± 0.19^{a}	1.40 ± 0.12^{ab}	1.63 ± 0.11^{b}	1.65 ± 0.14^{b}	1.52 ± 0.09^{ab}	1.35 ± 0.11^{a}	0.033
Ile	1.20 ± 0.19^{a}	1.21 ± 0.14^{a}	1.32 ± 0.10^{a}	1.58 ± 0.10^{b}	1.39 ± 0.12^{ab}	1.37 ± 0.09^{ab}	0.029
Ala	1.70 ± 0.18	1.85 ± 0.22	1.82 ± 0.18	1.87 ± 0.23	1.75 ± 0.10	1.67 ± 0.05	0.662
Leu	2.10 ± 0.23^{a}	2.42 ± 0.25^{abc}	2.64 ± 0.27^{bc}	2.81 ± 0.22^{c}	2.54 ± 0.19^{abc}	2.33 ± 0.27^{ab}	0.043
Val	1.49 ± 0.17^{a}	1.58 ± 0.17^{a}	2.05 ± 0.29^{b}	2.14 ± 0.27^{b}	1.77 ± 0.29^{ab}	1.57 ± 0.23^{a}	0.026
Phe	1.42 ± 0.16^{a}	1.47 ± 0.08^{a}	1.80 ± 0.19^{bc}	1.96 ± 0.27^{c}	1.51 ± 0.13^{ab}	1.45 ± 0.10^{a}	0.008
Gly	1.12 ± 0.14^{a}	1.14 ± 0.08^{a}	1.33 ± 0.13^{ab}	1.54 ± 0.19^{b}	1.57 ± 0.11^{b}	1.47 ± 0.11^{b}	0.003
Pro	1.15 ± 0.09^{a}	1.37 ± 0.15^{ab}	1.51 ± 0.19^{b}	1.63 ± 0.12^{b}	1.45 ± 0.17^{b}	1.39 ± 0.13^{ab}	0.031
Ser	1.37 ± 0.10^{a}	1.64 ± 0.18^{b}	1.68 ± 0.16^{b}	1.94 ± 0.16^{c}	1.65 ± 0.15^{b}	1.56 ± 0.07^{ab}	0.009
Tyr	1.57 ± 0.25^{a}	1.76 ± 0.20^{ab}	2.02 ± 0.15^{bc}	2.16 ± 0.26^{c}	1.70 ± 0.23^{ab}	1.61 ± 0.11^{a}	0.024
Cys	0.24 ± 0.03^{a}	0.30 ± 0.04^{ab}	0.37 ± 0.05^{bc}	0.44 ± 0.06^{c}	0.43 ± 0.05^{c}	0.34 ± 0.03^{b}	0.001
Tau	1.40 ± 0.18^{a}	1.50 ± 0.14^{abc}	1.66 ± 0.20^{abc}	1.78 ± 0.12^{c}	1.74 ± 0.15^{bc}	1.46 ± 0.11^{ab}	0.045
Basic amino acids							
Lys	1.44 ± 0.17	1.49 ± 0.13	1.55 ± 0.12	1.56 ± 0.11	1.56 ± 0.17	1.49 ± 0.08	0.842
Arg	1.45 ± 0.15^{a}	1.62 ± 0.12^{ab}	1.82 ± 0.21^{bc}	2.16 ± 0.30^{b}	1.87 ± 0.14^{bc}	1.68 ± 0.16^{ab}	0.011
His	0.41 ± 0.04^{a}	0.53 ± 0.07^{b}	0.61 ± 0.04^{bc}	0.75 ± 0.06^{d}	0.67 ± 0.05^{cd}	0.60 ± 0.09^{bc}	<0.001
Acidic amino acids							
Asp	1.89 ± 0.14	2.08 ± 0.13	2.29 ± 0.21	2.25 ± 0.19	2.10 ± 0.33	1.97 ± 0.20	0.223
Glu	2.15 ± 0.22^{a}	2.56 ± 0.36^{ab}	2.76 ± 0.31^{ab}	3.11 ± 0.45^{b}	2.72 ± 0.39^{ab}	2.34 ± 0.16^{a}	0.048

PI = proximal intestine; MI = mid intestine; DI = distal intestine.
[a-d] Mean values within a row with different superscript letters indicate significant difference (one-way ANOVA and Duncan's multiple-range tests, $P < 0.05$).
[1] Values are means ± SD ($n = 6$).

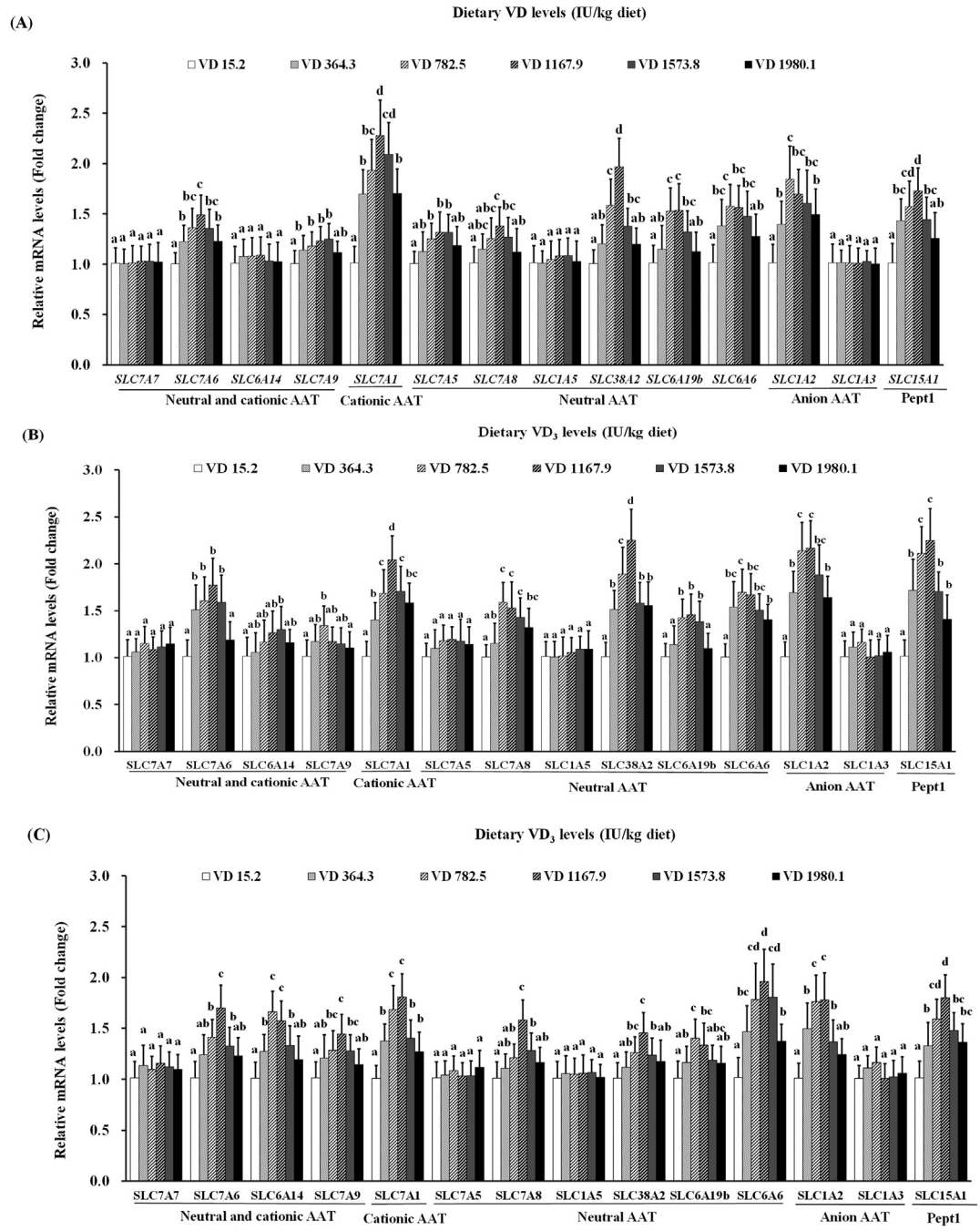

Fig. 3. Relative expression of amino acid transporters in the PI (A), MI (B), and DI (C) of the on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of VD_3 for 10 weeks. Mean values ± SD. $n = 6$ for each bar. Different letters above the bars indicate significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$). PI = proximal intestine; MI = mid intestine; DI = distal intestine.

hyperplasia (Erben et al., 2014), and blood capillary hyperemia (Wu et al., 2016). In the current study, histopathological examinations revealed that dietary VD_3 deficiency caused pathological changes in the three intestinal segments (Fig. 1), indicating damage to fish intestinal structure integrity. Intestinal health affects the intestinal function, including digestion and absorption capacity (Liu, 2015). In addition, an increase in intestinal villus height promotes the contact area between the tract and chyme and, in turn, enhances the digestion and absorption of nutrients (Wang et al., 2020). Accordingly, we assessed the influence of VD_3 on digestion and absorption capacities in fish intestines.

4.2. VD_3 deficiency decreased fish digestion and absorption capacities

The presence of digestive enzymes reflects nutrient digestion capacity in fish (Johnston et al., 2004). Our results showed that dietary VD_3 deficiency decreased trypsin, chymotrypsin, and

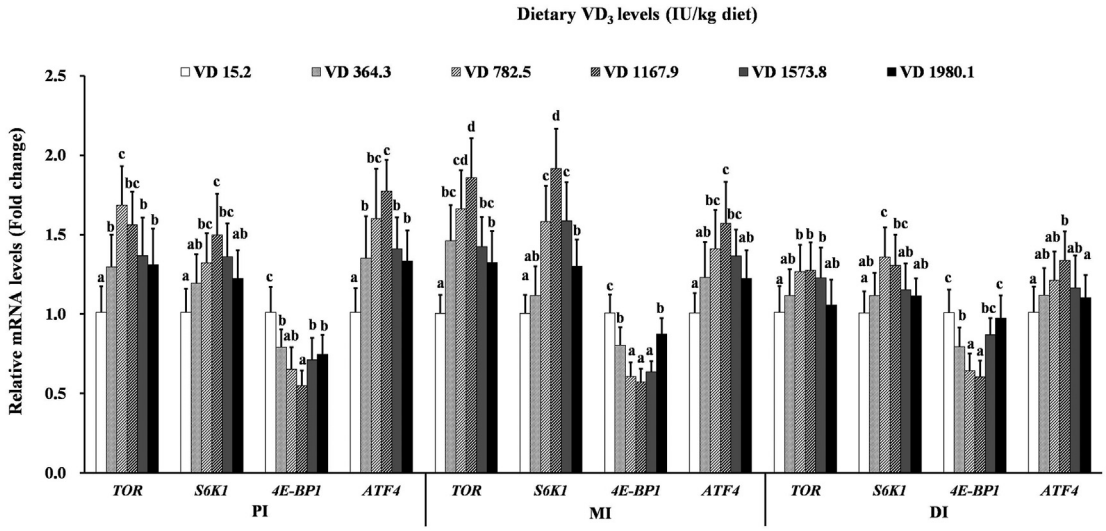

Fig. 4. Relative expression of *TOR*, S6K1, *4E-BP1*, and *ATF4* in the PI, MI, and DI of the on-growing grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of VD_3 for 10 weeks. Mean values ± SD. $n = 6$ for each bar. Different letters above the bars indicate significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$). *TOR* = total target of rapamycin; *S6K1* = S6 kinase 1; *4E-BP1* = eukaryotic translation initiation factor 4E-binding protein 1; *ATF4* = activating transcription factor 4; PI = proximal intestine; MI = mid intestine; DI = distal intestine.

amylase activities in the intestines of on-growing grass carp. Nutrient absorption is mainly influenced by brush border activities (Silva et al., 2010), particularly AKP, Na^+/K^+-ATPase, γ- GT, and CK, which are responsible for the final stage of nutrient degradation and assimilation (Jiang et al., 2014). To the best of our knowledge, this is the first study to examine the effect of VD_3 on absorption enzymes of different intestinal segments in animals. Our results revealed that VD_3 deficiency decreased brush border enzyme activities and downregulated *CK*, *atp1a1a1*, and *atp1a1a4* mRNA levels in different intestinal segments of fish. In rats, brush border enzyme activities indicate the function of the growth status of epithelial cells (Hodin et al., 1995), which is reflected by IL, ILI, and fold height. In the present study, dietary VD_3 supplementation increased these indexes. It is known that VD_3 mediates the differentiation of epithelial cells to establish small intestinal villi and, in turn, promotes nutrient absorption (Christakos et al., 2020). The enterocyte absorption of dipeptides and tripeptides relies on microvillous peptidases without hydrolysis (Jose et al., 1997). γ-GT is involved in peptide transport by catalyzing the hydrolysis of γ-glutamyl peptide bonds and simultaneously provides membrane transport of amino acids (Rust 2002). CK is vital for large or demanding energy transfers with large energy demands, as it catalyzes the transfer of phosphate to creatine in an ATP-dependent manner. In our study, VD_3 deficiency depressed γ-GT and CK levels, suggesting that VD_3 reduces amino acid and energy transport. To support this assumption, we investigated the influence of VD_3 on the absorption of AAs and small peptides in the three intestinal segments.

4.3. VD_3 deficiency decreased AA and peptide absorption in fish intestines

AAs are absorbed efficiently within epithelial cells in the animal digestive tract (Sans et al., 2021). FAA contents in tissue can reflect the absorption capacity of intestinal AAs (Berge et al., 2004). AA and small peptide transport in fish intestines require AATs and SLC15A1 (Rosario et al., 2016). Enteral AAs are mainly transported by their corresponding AATs in animals (Appendix Table 1) (Bröer, 2008; Hyde et al., 2003). In this study, we investigated, for the first time, the effect of VD_3 on AAs absorption in the gut.

4.3.1. VD_3 deficiency decreased neutral and basic AA absorption capacity partly related to neutral AATs and neutral cationic AATs in the intestine of fish

The absorption of neutral and basic AAs in intestine depends on neutral AATs and neutral and cationic AATs (Wu, 2013). The present results demonstrated that VD_3 deficiency decreased the contents of most neutral AAs (except Leu in the PI, and Ala in all three segments) and basic AAs (except Lys), as well as the regulation of most neutral and cationic AATs (except *SLC6A14* in the PI, and *SLC7A7* in all three segments) and of cationic AAT mRNA in different segments. Correlation analysis (Appendix Table 2) revealed that the abundance of AAT mRNA positively correlated with coincident FAA concentration. These results indicate that decreased AA levels in fish intestines may be related to a decrease in relative AAT mRNA.

Some of the notable phenomena observed with respect to the FAA and AATs merit an further investigation. First, the lack of influence of VD_3 over Leu content in the PI may be related to *SLC6A14*, which is highly responsible for Leu transport (Anderson et al., 2008). The results also indicated that VD_3 had no influence on *SLC6A14* expression in the PI. Second, VD_3 had no impact on the *SLC7A7* levels, which could be attributed to the affected Lys content. *SLC7A7* was high responsible for Lys transport in the intestine (Sperandeo et al., 2007). A study also demonstrated that knock out of *SLC7A7* disrupts intestinal reabsorption with Lys (Bodoy et al., 2019). Our results showed that VD_3 supplementation did not influence Lys contents, which was consistent with the observation regarding the impact of VD_3 on *SLC7A7* mRNA levels. Third, VD_3 deficiency downregulated *SLC6A14* mRNA expression in the MI and DI (rather than PI), which could be correlated with butyrate, *IL-1β*, and iNOS. Butyrate increased the mRNA of *IL-1β* in the MI and DI of young grass carp (Tian et al., 2017). iNOS can be activated by *IL-1β* in myeloid suppressor cells (Popovic et al., 2007), and iNOS also upregulates *SLC6A14* in the carcinoma cells of the cervix (Gupta et al., 2006). Tanaka et al. (1990) demonstrated that VD_3 enhanced butyrate in HT-29 cell lines. Therefore, VD_3 deficiency could decrease butyrate production, and, in turn, reduce *IL-1β* in the MI and DI; *IL-1β* would also reduce the amount of iNOS that could decrease *SLC6A14* expression. Fourth, VD_3 reduced *SLC7A5* abundance only in the PI, which may be due to zinc and *TGF-β1*. In the PI (not in the MI and DI) of young grass carp, zinc increased *TGF-*

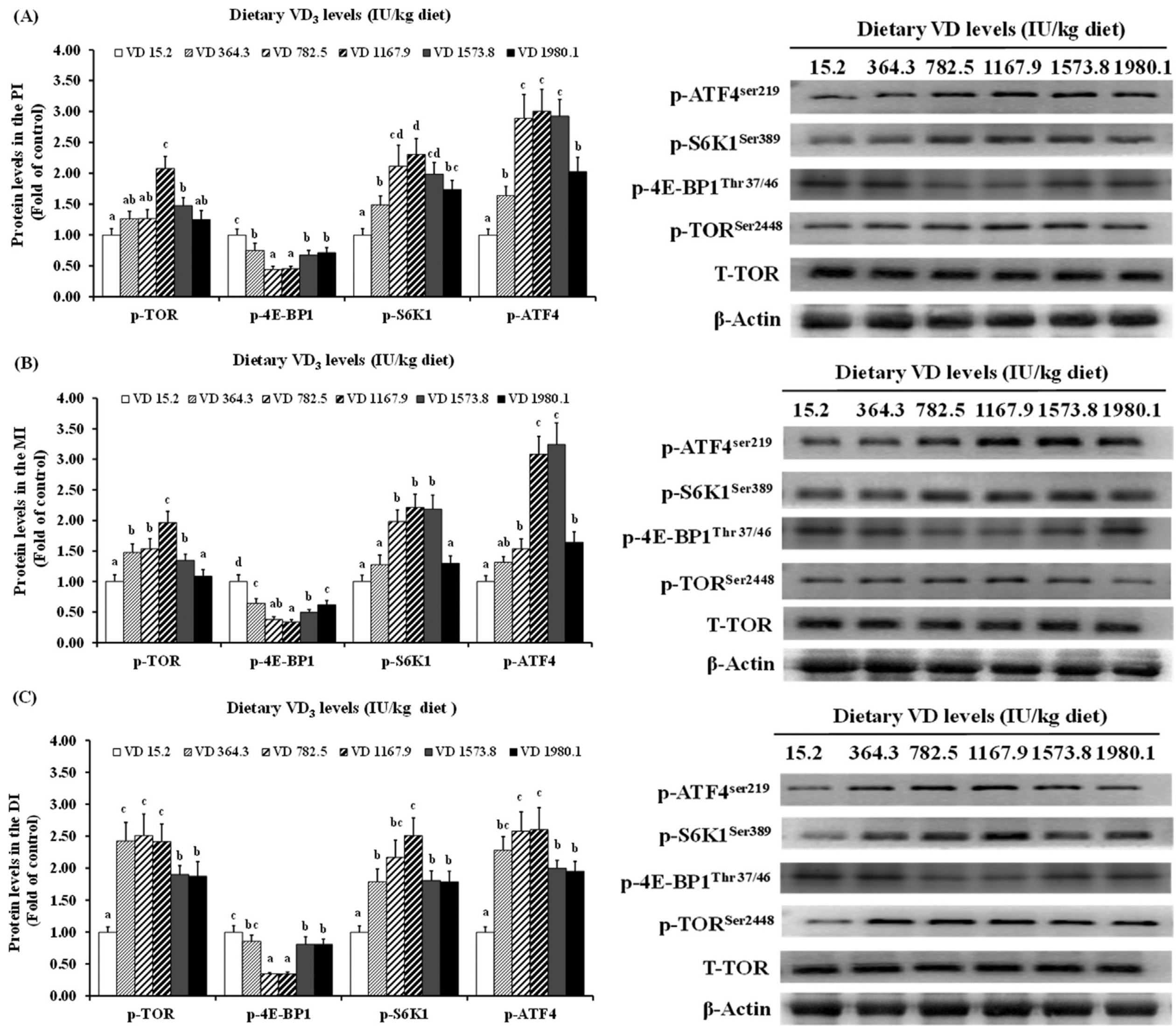

Fig. 5. Western blot analysis of protein expression of genes in the PI (A), MI (B), and DI (C) of the on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of VD_3 (IU/kg) for 10 weeks. Mean values ± SD. $n = 6$ for each bar. Different letters above the bars indicate significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$). PI = proximal intestine; MI = mid intestine; DI = distal intestine.

β1 expression (Song et al., 2017), and *TGF-β1* promoted the expression of *SLC7A5* (Zhao et al., 2017). VD_3 significantly increases the zinc levels in bone (Worker and Migicovsky, 1961). Therefore, we speculated that VD_3 could increase zinc contents and *TGF-β1* expression, resulting in upregulated *SLC7A5* expression in the PI. Fifth, VD_3 deficiency had no effect on *SLC1A5* expression, which could be associated with the unchanged content of Ala. Ala was identified as substrate specificity of *SLC1A5*. *SLC1A5* displayed the high affinity with Ala and is involved in Ala transport (Scalise et al., 2015). Our result revealed that VD_3 treatment did not affect the content of Ala, which supported our hypothesis.

4.3.2. VD_3 deficiency decreased the acidic AA absorption capacity partly related to anionic AAT in the intestine of fish

Acidic AAs (Asp and Glu) can be adjusted by anionic AATs (*SLC1a2a* and *SLC1A3*) in the intestine (Bröer, 2008). The present study showed that VD_3 deficiency caused a decline in acidic AAs (except Asp). The correlation analysis (Appendix Table 3) indicated that the expression of these AATs correlated with coincident FAA concentrations. The lack of change to Asp may be due to Ala. Ala and α-ketoglutarate can be catalyzed by aspartate aminotransferase and thus form Asp (Schindhelm et al., 2006). In the present study, VD_3 did not affect Asp content, which may support our assumption.

SLC1A3 mRNA levels were not affected by dietary VD_3 across the intestinal segments. This observation could potentially be related to insulin. The activity of *SLC1A3* is unaffected by insulin in adipocytes (Krycer et al., 2017). VD_3 stimulates pancreatic insulin secretion (Zeitz et al., 2003). Therefore, VD_3 may not affect *SLC1A3* mRNA abundance because of insulin, which requires further research.

4.3.3. VD_3 deficiency decreased AAs absorption capacity partly related to SLC15A1 in the intestine of fish

The physiological function of intestinal peptide transport (*SLC15A1*) is to absorb dipeptides and tripeptides resulting from the digestion of dietary proteins (Frazier et al., 2008; Yang et al., 1999). In our study, we found that *SLC15A1* mRNA abundance, as well as most FAA levels, decreased in the three intestinal segments under VD_3 deficiency. The probable cause for the decreased *SLC15A1* levels may be associated with intestine weight. The small intestine weights in chickens are strongly positive correlated with *SLC15A1*

gene expression (Li et al., 2013). In this study, we observed that VD_3 increased intestine weight, although further validation of this hypothesis is required.

4.4. VD_3 deficiency suppressed AA and peptide transporters partly associated with TOR, ATF4, 4E-BP1, and S6K1 signaling in on-growing grass carp intestine

mTORC1 regulates AATs by suppressing *4E-BP1*, which inhibits *ATF4* in HEK293T cells (Park et al., 2017), and phosphorylates *S6K1*, which controls the expression of AATs and PepT1 in humans (Benner et al., 2011; Hay and Sonenberg, 2004; Rosario et al., 2016). The present study stands as a first step to explore the mechanistic effects of VD_3 on absorption in animal intestinal segments. The results demonstrated that VD_3 deficiency downregulated *S6K1*, *ATF4*, and p-*TOR* mRNA levels and upregulated *4E-BP1* mRNA in all intestinal segments. Furthermore, a correlation analysis (Appendix Table 3) revealed that most AATs and *SLC15A1* were correlated positively with *TOR*, *S6K1*, and *ATF4* and negatively related to *4E-BP1* in different intestinal segments. Therefore, we inferred that VD_3 mediates AA transport and is potentially related to TOR signaling molecules in fish intestines.

4.5. Excess levels of VD_3 led to adverse effects on fish growth

Compared with the results observed at optimal VD_3 supplement levels (1,167.9 IU/kg diet), excess VD_3 (1,980.1IU/kg diet) led to significantly decreased growth performance and feed utilization. This agrees with results from studies on juvenile Siberian sturgeon (Wang et al., 2017), juvenile black carp (Wu et al., 2020), and orange-spotted grouper (He et al., 2021). This phenomenon could be associated with 1) reduced ability for digestion and absorption; 2) increased oxidative stress and decreased antioxidant capacity (He et al., 2021); 3) depressed immune response (Jiang et al., 2015); or 4) increased risk of skeletal deformities (Darias et al., 2010).

4.6. Comparison of VD_3 requirements for on-growing grass carp based on different indices

Based on PWG (Fig. 6A) and FE (Fig. 6B), the estimated optimal VD_3 supplement level was 968.33 and 1,005.00 IU/kg, respectively. Alternatively, on basis of trypsin activity (Fig. 6C), VD_3 requirements are estimated to be 1,166.67 IU/kg diet. A higher activity of trypsin was required compared to growth performance, indicating that greater amounts of VD_3 were required for optimal digestion.

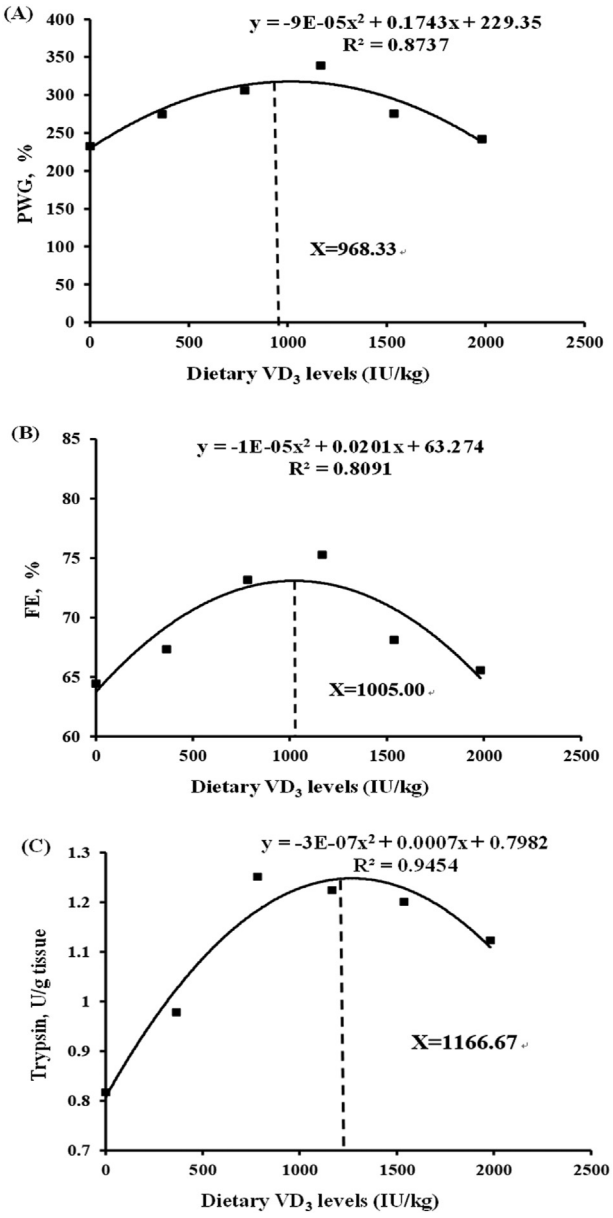

Fig. 6. Quadratic regression analysis of PWG (%, A), FE (%, B) and trypsin activity (U/g tissue, C) for the on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of VD_3 (IU/kg) for 10 weeks.

5. Conclusions

To the best of our knowledge, the present study is the first to identify the effects of VD_3 on growth performance, absorption capacity, AATs, and *PepT1* in different intestinal segments through a mechanism associated to the TOR signaling pathway. TOR signaling, supported by dietary VD_3, caused the increase of AAT, *PepT1*, and brush border enzyme mRNA levels. These conclusions were supported by the findings that VD_3 deficiency compromised the integrity of intestinal structure; decreased trypsin, chymotrypsin, amylase, CK, γ-GT, Na^+/K^+-ATPase, and AKP activities; downregulated mRNA expression of *atp1a1a1*, *atp1a1a4*, and *CK*; depressed the levels of most neutral, basic, and acidic AAs, in partial correlation with the downregulation of most corresponding AATs, and in relation to the TOR signaling pathway (*TOR*/[*4E-BP1*/*S6K1* and *ATF4*]). According to the results of quadratic regression analysis of PWG (Fig. 6A), FE (Fig. 6B), and the activity of trypsin (Fig. 6C), the dietary VD_3 requirement of on-growing grass carp (257.24 ± 0.40 g) was estimated to be 968.33, 1,005.00, and 1,166.67 IU/kg, respectively. These findings provide new insights into the development of VD_3 supplements to improve productivity in the aquaculture of grass carp.

Author contributions

Yao Zhang: Manuscript writing, Formal analysis; **Chao-Nan Li**: Investigation; **Wei-Dan Jiang**: Data curation; **Pei Wu**, **Yang Liu**: Methodology; **Sheng-Yao Kuang**, **Ling Tang**, **Hua-Wei Li**: Resources; **Xiao-Wan Jin**, **Hong-Mei Ren**: Management; **Xiao-Qiu Zhou**, **Lin Feng**: Conceptualization, Supervision. **Lin Feng** had primary responsibility for the final content of the manuscript. All authors carefully read and approved the final revision of the manuscript.

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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Appendix Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.05.004.

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