

Vitamin A deficiency suppresses fish immune function with differences in different intestinal segments: the role of transcriptional factor *NF-κB* and *p38* mitogen-activated protein kinase signalling pathways

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Abstract

The present study investigated the effects of dietary vitamin A on immune function in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of young grass carp (*Ctenopharyngodon idella*). Fish were fed graded levels of dietary vitamin A for 10 weeks, and then a challenge test using an injection of *Aeromonas hydrophila* was conducted for 14 d. The results showed that, compared with the optimum vitamin A level, vitamin A deficiency significantly decreased fish growth performance, increased enteritis morbidity, decreased intestinal innate humoral immune response and aggravated intestinal inflammation. However, liver-expressed antimicrobial peptide 2A/B mRNA in the DI and *IL-6*, *IL-17D*, *IL-10*, transforming growth factor (*TGF*)- β 1 and *TGF*- β 2 mRNA in the PI were not affected by vitamin A levels. Meanwhile, vitamin A deficiency disturbed inflammatory cytokines in the PI, MI and DI, which might be partly linked to p38 mitogen-activated protein kinase (*p38MAPK*) signalling and *NF-κB* canonical signalling pathway (*IκB* kinase β (*IKKβ*), *IKKγ*, inhibitor of κ B α , *NF-κB p65* and *c-Rel*) rather than *NF-κB* non-canonical signalling pathway (*NF-κB p52* and *IKKα*). However, the signalling molecules *NF-κB p65* and *p38MAPK* did not participate in regulating cytokines in the PI. These results suggested that vitamin A deficiency decreased fish growth and impaired intestinal immune function, and that different immune responses in the PI, MI and DI were mediated partly by *NF-κB* canonical signalling and *p38MAPK* signalling pathways. On the basis of percentage of weight gain, to protect fish against enteritis morbidity and acid phosphatase activity, the optimum dietary vitamin A levels were estimated to be 0.664, 0.707 and 0.722 mg/kg, respectively.

Key words: Vitamin A deficiency; Intestinal segments; Immune responses; *NF-κB* canonical signalling; *p38* Mitogen-activated protein kinase; Grass carp

Vitamin A is an essential nutrient for the normal growth of fish⁽¹⁾. Previous studies from our laboratory have indicated that vitamin A deficiency could decrease weight gain (WG), specific growth rate (SGR) and feed utilisation of Jian carp (*Cyprinus carpio* var. Jian)⁽²⁾. To our knowledge, fish growth is closely related to intestinal health, which is strongly associated with intestinal immune function⁽³⁾. A previous study has shown that damages to the intestinal immune system could lead to bacterial translocation and enteritis and even result in high mortality in

fish⁽⁴⁾. However, no reports have addressed the effects of vitamin A on immune function in the intestine of fish so far.

In fish, the intestinal immune function is strongly linked to a variety of immune compounds such as lysozyme (LA), acid phosphatase (ACP), antibacterial peptides, complement and cytokines⁽⁵⁾. It has been reported that the expression of cytokines could be regulated by the signalling molecule intracellular *NF-κB p65* and the inhibitor of κ B α (*IκBα*) in rats⁽⁶⁾ as well as by the signalling molecule p38 mitogen-activated protein kinase

Abbreviations: ACP, acid phosphatase; C3, complement 3; C4, complement 4; cDNA, complementary DNA; DI, distal intestine; FE, feed efficiency; FI, feed intake; IFN- γ 2, interferon γ 2; *IκB*, inhibitor of κ B; *IKK*, *IκB* kinase; LA, lysozyme; LEAP, liver-expressed antimicrobial peptide; MI, mid intestine; PI, proximal intestine; *p38MAPK*, p38 mitogen-activated protein kinase; PWG, percentage of weight gain; SGR, specific growth rate; TGF, transforming growth factor.

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(*p38MAPK*) in humans⁽⁷⁾. To date, a limited number of studies have explored the effects of vitamin A on animal intestinal immune function, which focus on only cytokines^(8–10), secretory IgA and Toll-like receptors (*TLR2*, *TLR4* and myeloid differentiation primary response gene (88) (*MyD88*) mRNA levels⁽⁹⁾. Nevertheless, no studies have explored the mechanism of the effects of vitamin A on inflammatory cytokines production through *NF-κB* (including *p65/p52* and *c-Rel*), *IκBα*, *IKK* (including *IKKα*, *IKKβ* and *IKKγ*) and *p38MAPK* signalling in animals. It has been reported that vitamin A can inhibit polysaccharide synthesis in chick embryo cells⁽¹¹⁾. Liu *et al.*⁽¹²⁾ showed that polysaccharides can suppress the expression of the antibacterial peptide *hepcidin* in rat liver. Meanwhile, an available study showed that vitamin A could strengthen the phagocytic ability of neutrophils in rats⁽¹³⁾. In humans, neutrophils were able to generate LA for defence against pathogenic micro-organisms⁽¹⁴⁾. Moreover, in the rat, vitamin A supplementation could decrease glutamate uptake in hippocampus slices⁽¹⁵⁾. One study showed that high glutamate concentrations could up-regulate cytokine *TNF-α* mRNA levels in rats⁽¹⁶⁾. In addition, Zorn & Sauro demonstrated that vitamin A could activate protein kinase C (*PKC*) in rat splenic cells⁽¹⁷⁾. Some studies have shown that *PKC* is an important intermediary in the *NF-κB* pathway in drosophila cells⁽¹⁸⁾ and in the *p38MAPK* pathway in chicken chondrocytes⁽¹⁹⁾. These observations implied a connection between vitamin A and innate humoral immune components, inflammatory cytokines as well as the series of *NF-κB* and *p38MAPK* signalling molecules in the animal intestine. Thus, further studies to systematically evaluate the effects of vitamin A on animal intestinal immune function will be of great value.

The fish intestine shows regional immune specialisation in different segments. In Atlantic salmon, higher levels of major histocompatibility complex class II^(20,21) and T-cell markers⁽²¹⁾ in the hindgut *v.* the foregut have been reported. Moreover, Rombout & Berg⁽²²⁾ reported that in the second gut segment of carp more resident intraepithelial macrophages were found compared with the first segment. To our knowledge, vitamin A (retinol) can be metabolised to retinoic acid (RA) to exert most of its functions including its immunological function in specific subsets of intestinal dendritic cells (DC) and macrophages of animals^(23–25). Thus, it is valuable to investigate the effects of vitamin A on fish immune function in different intestinal segments.

Grass carp (*Ctenopharyngodon idellus*) is one of the most important freshwater aquaculture species worldwide⁽²⁶⁾. At present, the vitamin A requirement of grass carp has been determined in only juveniles, based on the percentage of WG (PWG)⁽²⁷⁾. However, the nutrient requirements of fish may vary with different growth stages^(28,29) and different physiological indicators⁽³⁰⁾. Therefore, it is worthy to investigate the dietary vitamin A requirements of grass carp in different growth stages and based on different indicators.

In the present study, we proposed a hypothesis that dietary vitamin A deficiency could impair fish immune function in different intestinal segments. To test this hypothesis, we first systemically investigated the effects of vitamin A on fish enteritis morbidity, innate humoral immune-related components, inflammatory cytokines and the related signalling molecules in the proximal intestine (PI), mid intestine (MI) and distal

intestine (DI). Meanwhile, we determined the vitamin A requirements of young grass carp based on different indicators, which may provide a partial basis for formulating the most appropriate feed for grass carp.

Methods

Experimental diet preparation

The experimental diet formulation is given in Table 1. Fishmeal, soyabean protein concentrate and gelatin were offered as dietary protein sources, and soyabean oil was provided as the dietary lipid source. The dietary protein level was fixed at 30%, which has been shown to be adequate for grass carp as described by Khan *et al.*⁽³¹⁾. Different concentrations of retinyl acetate (172 mg/g) were added to a basal diet constituting six different diets at 0 (un-supplemented control), 0.206, 0.413, 0.619, 0.963 and 1.307 mg/kg diet, and the amount of maize starch was reduced to compensate. The final vitamin A concentrations in each experimental diet were analysed by HPLC assay according to Moren *et al.*⁽³²⁾ to be 0.006 (un-supplemented control), 0.209, 0.416, 0.618, 0.965 and 1.306 mg/kg diet, respectively. All ingredients were mixed, pelleted and stored at -20°C until use, which was similar to the method described by Guimarães *et al.*⁽²⁹⁾.

Feeding trial and experimental management

After an acclimation period of 4 weeks as described by Shaik Mohamed *et al.*⁽³³⁾, fish were fed a vitamin A-deficient diet for 2 weeks to diminish the body storage of vitamin A according to

Table 1. Formulation and nutrient content of the basal diet

Ingredients (g/kg)	Nutrients content (g/kg)		
Fishmeal	155.50	Crude protein*	297.13
Soyabean protein concentrate	262.50	Crude lipid*	35.78
Gelatin	31.30	<i>n</i> -3†	5.00
α -Starch	240.00	<i>n</i> -6†	10.00
Maize starch	163.23	Available P†	8.40
Soyabean oil	19.31		
Cellulose	50.00		
L-Met (98 %)	4.01		
Ca(H ₂ PO ₄) ₂	28.65		
Vitamin premix‡	10.00		
Mineral premix§	20.00		
Vitamin A premix	10.00		
Choline chloride (60 %)	5.00		
Ethoxyquin (30 %)	0.50		

* Crude protein and crude lipid contents were measured values.

† Available P, *n*-3 and *n*-6 contents were calculated according to NRC (2011).

‡ Per kg of vitamin premix (g/kg): cholecalciferol (172 mg/g), 0.40; 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%), 0.11; ascorbyl acetate (95%), 4.31; niacin (99%), 2.58; meso-inositol (98%), 19.39; calcium-D-pantothenate (98%), 2.56; riboflavin (80%), 0.63; pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with maize starch to 1 kg.

§ Per kg of mineral premix (g/kg): MnSO₄·H₂O (31.8% Mn), 1.8900; MgSO₄·H₂O (15.0% Mg), 200.0000; FeSO₄·H₂O (30.0% Fe), 24.5700; ZnSO₄·H₂O (34.5% Zn), 8.2500; CuSO₄·5H₂O (25.0% Cu), 0.9600; KI (76.9% I), 0.0668 g; Na₂SeO₃ (44.7% Se), 0.0168. All ingredients were diluted with maize starch to 1 kg.

|| Vitamin A premix: premix was added to obtain graded level of vitamin A and the amount of maize starch was reduced to compensate.

Campeche *et al.*⁽²⁸⁾. Thereafter, a total of 540 fish with an average initial weight of 262.02 (SD 0.45) g were randomly assigned to eighteen experimental cages (1.4 length×1.4 width×1.4 height in metres) in three tanks – that is, six cages with thirty fish per cage in each tank. Each tank was randomly assigned to one of three replicates of the six experimental dietary treatments. Each cage was equipped with a 100-cm-diameter disc in the bottom to collect uneaten food according to the method described by Luo *et al.*⁽³⁴⁾. The experimental design included six treatments with three replicates; the respective diet was fed four times per day for 70 d. The fish were fed to apparent satiation in the feeding trial, just as several previous studies have reported^(35,36). After feeding for 30 min, the uneaten food was collected, dried and weighed to calculate the feed intake (FI) according to the method of Mundheim *et al.*⁽³⁷⁾. Water temperature was set at 28 (SD 2)°C throughout the experiment. The pH and dissolved oxygen levels were maintained at 7.0 (SD 0.2) and not <6.0 mg/l, respectively, and the experimental units were under a natural light and dark cycle, similar to that described by Luo *et al.*⁽³⁴⁾. Fish from each cage were weighed at the beginning and at the end of the 70-d feeding trial to determine the PWG, SGR and feed efficiency (FE). The formulae used for calculation are described below.

Challenge test and sample collection

The challenge trial was performed in a similar manner to the trial described by Kuang *et al.*⁽³⁸⁾. After analysing fish growth performance at the end of the feeding trial, fifteen fish from each treatment group were randomly collected with similar body weights and moved into labelled cages according to Liu *et al.*⁽³⁹⁾. As described by Wu *et al.*⁽⁴⁰⁾, the fish were acclimated in the cages for 5 d. Next, all fish were intraperitoneally injected with *Aeromonas hydrophila* (College of Veterinary Medicine, Sichuan Agricultural University) at a dose rate of 2.5×10^8 colony-forming unit/ml per g fish body weight to begin the challenge test. The challenge dose was selected as an appropriate dose that could effectively induce inflammation and consequently enable the investigation on fish reactivity against a threatening disease without causing death according to our preliminary test (data not shown). The challenge test was conducted for 14 d according to Nya & Austin⁽⁴¹⁾. During the infectious trial, the experimental conditions were the same as the feeding trial. After completion of the challenge experiment, all fish from each treatment were anaesthetised in a benzocaine bath as described by Chen *et al.*⁽⁴²⁾. A scoring system was designed to evaluate the severity of fish intestinal inflammation, which was similar to the results of Song *et al.*⁽⁴³⁾ and Knudsen *et al.*⁽⁴⁴⁾. Subsequently, the intestines of the fish were quickly removed and classified according to the position of the turns in the intestine: the PI was anterior to the first turn, the MI was the region located between the first turn and the last turn and the DI was posterior to the last turn^(45,46). Thereafter, the intestinal samples were frozen in N₂ and stored at -80°C until subsequent analysis of immune parameters.

Intestinal immune parameters assay

The intestinal samples of six fish in each treatment were homogenised on ice in 10 volumes (w/v) of ice-cold physiological saline and centrifuged at 6000 g at 4°C for 20 min; next, the collected supernatant was stored at -20°C as described by Kuang *et al.*⁽³⁸⁾ for subsequent analyses. ACP activity was determined according to the method of Zhao *et al.*⁽⁴⁷⁾. LA activity was analysed according to Chi *et al.*⁽⁴⁸⁾. Complement 3 (C3) and complement 4 (C4) components were determined according to the method of Liu *et al.*⁽⁴⁹⁾.

Real-time quantitative PCR

The procedures of RNA isolation, RT and quantitative real-time PCR were similar to the description from a previous study in our laboratory⁽⁵⁰⁾. In brief, total RNA samples of six fish per treatment were isolated from the intestines using RNAiso Plus (Takara) according to the manufacturer's instructions, followed by DNase I treatment. RNA quality and quantity were assessed by agarose gel (1%) electrophoresis and spectrophotometric (A260:280 nm ratio) analysis, respectively. Next, RNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScript™ RT Reagent Kit (Takara) according to the manufacturer's instructions. For quantitative real-time PCR, specific primers were designed according to the sequences cloned in our laboratory or the published sequences for grass carp (Table 2). All real-time PCR analyses were performed in six biological replicates, and two technical replicates were analysed for each biological replicate. A total of 15 µl of the PCR reaction system consisted of 7.5 µl of SYBR Premix Ex Taq II (2×), 0.6 µl of forward primers (10 µM), 0.6 µl of reverse primers (10 µM), 2 µl of cDNA and 4.3 µl of DNase-free dH₂O. To detect any possible DNA contamination, real-time quantitative PCR was also performed in RNA control samples in the absence of RT during cDNA synthesis. A no-template control of nuclease-free water was also included in each run. All the real-time PCR reactions were performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc.) using a SYBR® PrimeScript RT-PCR Kit II. According to the results of our preliminary experiment concerning the evaluation of internal control genes, *β-actin* was used as a reference gene to normalise cDNA loading, which was stably expressed in the present experiment (data not shown). After the final PCR cycle, melting curves were systematically monitored to ensure that only one fragment was amplified. The amplification efficiency of the housekeeping and target genes was calculated using the standard curves of the specific gene generated from 10-fold serial dilutions, as described by Jiang *et al.*⁽⁵¹⁾. Pooled cDNA samples of the intestine were used to generate the calibration curves in this study. After verification that the primers amplified with an efficiency of approximately 100%, the $2^{-\Delta\Delta C_T}$ method was used to calculate the relative mRNA levels of all the genes according to Livak & Schmittgen⁽⁵²⁾.

Calculations and statistical analysis

Growth performance parameters were calculated on the basis of the following formulae: growth performance was assessed in



Table 2. Real-time PCR primer sequences

Target gene	Primer sequence		Temperature (°C)	Accession number
	Forward (5' → 3')	Reverse (5' → 3')		
<i>β-Defensin</i>	TTGCTTGTCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	58.4	KT445868
<i>Hepcidin</i>	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	59.3	JQ246442.1
<i>LEAP-2A</i>	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	59.3	FJ390414
<i>LEAP-2B</i>	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	59.3	KT625603
<i>TNF-α</i>	CGCTGCTGTCTGCTTCAC	CCTGGTCTGGTTCACTC	58.4	HQ696609
<i>IFN-γ2</i>	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	60.4	JX657682
<i>IL-1β</i>	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	57.1	JQ692172
<i>IL-6</i>	CAGCAGAATGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	62.3	KC535507.1
<i>IL-8</i>	ATGAGTCTTAGAGGCTCTGGGT	ACAGTGAGGGCTAGGAGGG	60.3	JN663841
<i>IL-12p35</i>	TGGAAGAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	55.4	KF944667.1
<i>IL-12p40</i>	ACAAAGATGAAAACTGGAGGC	GTGTGTGGTTTAGTAGGAGCC	59.0	KF944668.1
<i>IL-15</i>	CCTTCCAACAATCTCGCTTC	AACACATCTCCAGTTCTCCTT	61.4	KT445872
<i>IL-17D</i>	GTGTCCAGGAGAGCACCAAG	GCGAGAGGCTGAGGAAGTTT	62.3	KF245426.1
<i>IL-4/13A</i>	CTACTGCTCGCTTTGCTGT	CCCAGTTTTTCAGTTCTCTCAGG	55.9	KT445871
<i>IL-4/13B</i>	TGGGAGCAGACCTCAACAAC	TTCAGGACCTTTGCTGCTTG	55.9	KT625600
<i>IL-10</i>	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	61.4	HQ388294
<i>IL-11</i>	GGTTCAAGTCTCTCCAGCGAT	TGCGTGTATTGTTTCAGCCA	57.0	KT445870
<i>TGF-β1</i>	TTGGGACTTGTGCTCTAT	AGTCTGCTGGGATGTTT	55.9	EU099588
<i>TGF-β2</i>	TACATTGACAGCAAGGTGGTG	TCTTGTGGGATGATGAGTT	55.9	KM279716
<i>NF-κB p52</i>	TCAGTGTAACGACAACGGGAT	ATACTTCAGCCACACCTCTCTTAG	58.4	KM279720
<i>NF-κB p65</i>	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	62.3	KJ526214
<i>c-Rel</i>	CGCTCTATGCTTCCAGATTTACC	ACTGCCACTGTTCTTGTTCACC	59.3	KT445865
<i>IκBa</i>	TCTTGCCATTATTCACGAGG	TGTTACCACAGTCATCCACCA	62.3	KJ125069
<i>IKKα</i>	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCCATA	60.3	KM279718
<i>IKKβ</i>	GTGGCGGTGGATTATTGG	GCACGGGTTGCCAGTTTG	60.3	KP125491
<i>IKKγ</i>	AGAGGCTCGTCATAGTGG	CTGTGATTGGCTGCTTT	58.4	KM079079
<i>p38MAPK</i>	TGGGAGCAGACCTCAACAAT	TACCATCGGGTGGCAACATA	60.4	KM112098
<i>β-Actin</i>	GGCTGTGCTGTCCCTGTA	GGGCATAACCTCGTAGAT	61.4	M25013

LEAP-2, liver-expressed antimicrobial peptide 2; *TNF-α*, tumour necrosis factor α; *IFN-γ2*, interferon γ2; *TGF-β*, transforming growth factor β; *IκBa*, inhibitor of κB; *IKK*, IκB kinase; *p38MAPK*, p38 mitogen-activated protein kinase.

terms of PWG, SGR, FI and FE.

$$\text{PWG (\%)} = 100 \times (\text{final weight} - \text{initial weight}) / \text{initial weight}$$

$$\text{SGR (\%)} = 100 \times (\ln(\text{final weight}) - \ln(\text{initial weight})) / \text{d}$$

$$\text{FE (\%)} = 100 \times (\text{final weight} - \text{initial weight}) / \text{FI}$$

All data were subjected to a one-way ANOVA followed by Duncan's multiple-range test to evaluate significant differences among treatments at $P < 0.05$ with SPSS 20.0 (SPSS Inc.), similar to the method of Li *et al.*⁽⁴⁶⁾. The requirements of dietary vitamin A based on PWG and intestinal health indicators were estimated by a broken-line or quadratic regression model according to the method of Chen *et al.*⁽⁴²⁾.

Results

Growth performance parameters of the fish

The effects of graded levels of dietary vitamin A on fish growth parameters are shown in Table 3. The initial body weight of fish was not significantly different among the groups. The final body weight, PWG, SGR and FI were significantly improved with dietary vitamin A levels up to 0.618 mg/kg and were significantly decreased with higher vitamin A levels. Fish fed the vitamin A 0.618 mg/kg diet showed significantly higher FE than fish fed the vitamin A-deficient control diet and the vitamin A 1.306 mg/kg diet.

Enteritis and enteritis morbidity

Fish fed the vitamin A-deficient control diet showed noticeable hypertrophy and haemorrhages after being challenged with *A. hydrophila*, as shown in Fig. 1. Meanwhile, the effect of vitamin A on fish enteritis morbidity is shown in Fig. 2. The highest enteritis morbidity (65.33%) was observed in fish fed the vitamin A-deficient control diet, and the morbidity decreased to a minimum (21.33%) with dietary vitamin A levels up to 0.618 mg/kg and then significantly increased with higher vitamin A levels.

Immune parameters in the three intestinal segments of fish

Significant effects of the vitamin A-deficient diet on fish LA and ACP activities as well as the C3 and C4 contents were observed in the PI, MI and DI of fish, as shown in Table 4.

In the PI, LA and ACP activities as well as C3 and C4 contents were all significantly lower in the vitamin A-deficient control group than in the other vitamin A-supplemented groups. Fish fed the vitamin A 0.618 mg/kg diet had significantly higher LA activity than the other groups. ACP activity was significantly increased with dietary vitamin A levels up to 0.416 mg/kg and plateaued with higher vitamin A levels. The C3 content gradually increased with dietary vitamin A levels up to 0.618 mg/kg and decreased thereafter. Fish fed the vitamin A 0.416 mg/kg diet had a significantly higher C4 content than the other groups.

Table 3. Initial body weight (IBW, g/fish), final body weight (FBW, g/fish), percentage of weight gain (PWG, %), specific growth rate (SGR, %/d), feed intake (FI, g/fish) and feed efficiency (FE, %) of young grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of vitamin A for 10 weeks (Mean values and standard deviations of three replicate groups, with thirty fish in each group)

Dietary vitamin A levels (mg/kg diet)...	Control		0.209		0.416		0.618		0.965		1.306	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IBW	262.11 ^a	0.30	262.11 ^a	0.38	261.55 ^a	0.69	262.33 ^a	0.58	261.67 ^a	0.33	262.33 ^a	0.33
FBW	836.09 ^a	24.54	888.01 ^b	23.41	939.54 ^c	13.34	996.67 ^d	32.18	906.17 ^{b,c}	3.69	848.89 ^a	16.68
PWG	218.98 ^a	9.52	238.80 ^b	9.26	259.21 ^c	4.52	279.93 ^d	12.51	246.31 ^{b,c}	1.12	223.60 ^a	6.55
SGR	1.66 ^a	0.04	1.74 ^b	0.04	1.83 ^c	0.02	1.91 ^d	0.05	1.77 ^{b,c}	0.01	1.68 ^a	0.03
FI	816.06 ^a	0.09	858.36 ^c	0.01	933.70 ^e	0.67	962.22 ^f	0.27	886.10 ^d	0.16	832.94 ^b	0.09
FE	70.33 ^a	3.02	72.92 ^{a,b}	2.76	72.61 ^{a,b}	1.42	76.32 ^b	3.36	72.73 ^{a,b}	0.38	70.42 ^a	2.02

a,b,c,d,e,f Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$; one-way ANOVA and Duncan's multiple-range tests).

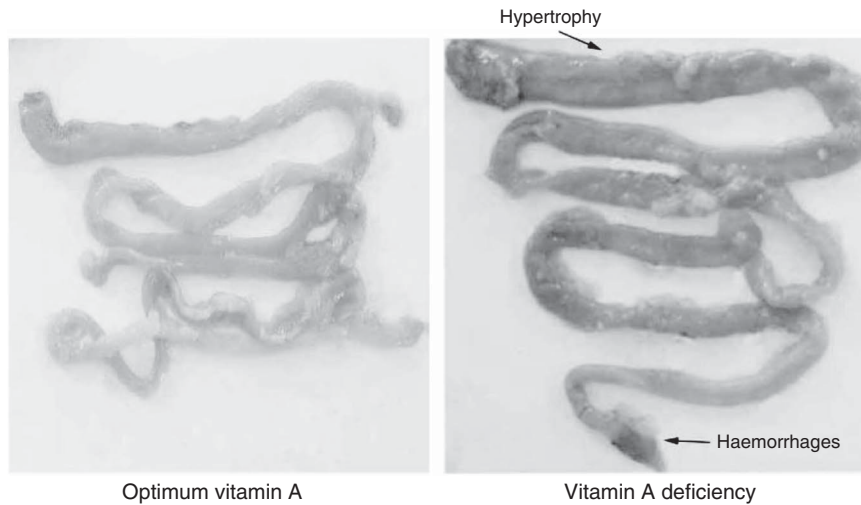


Fig. 1. Dietary vitamin A deficiency led to obvious enteritis after infection with *Aeromonas hydrophila* in young grass carp (*Ctenopharyngodon idella*). Noticeable hypertrophy and haemorrhages were observed in fish fed vitamin A-deficient diets after infection with *A. hydrophila*.

In the MI, LA and ACP activities as well as C3 content were all lower in fish fed the control diet compared with fish fed the vitamin A-supplemented diets. The C3 content was significantly increased with dietary vitamin A levels up to 0.416 mg/kg and gradually decreased with higher vitamin A levels. The C4 content was significantly increased with dietary vitamin A levels up to 0.416 mg/kg and then decreased with further increases in vitamin A levels.

In the DI, fish fed the control diet and the vitamin A 0.209 mg/kg diet showed significantly lower LA activity than fish in the other groups. ACP activity and C3 and C4 contents were all significantly decreased in the control group compared with fish fed the other vitamin A-supplemented diets. Fish fed the vitamin A 0.618, 0.965 and 1.306 mg/kg diets had significantly higher ACP activity than the other groups. The C3 content was significantly improved in fish fed the vitamin A 0.618 mg/kg diet and significantly decreased with higher vitamin A levels. Fish fed the vitamin A 0.416 mg/kg diet had significantly higher C4 content than fish in the other groups.

In fact, except for LA activity in the DI as well as the C4 content in the MI, the vitamin A-deficient group showed the lowest values for all parameters in all intestinal sections, which

suggested that vitamin A deficiency impaired immune function in fish intestine.

Gene expression in the intestine

mRNA expression of inflammatory cytokines and anti-microbial peptides (β -defensin-1, hepcidin, liver-expressed antimicrobial peptide 2A and liver-expressed antimicrobial peptide 2B) in the proximal intestine, mid intestine and distal intestine. In the PI, as shown in Fig. 3(A1) (pro-inflammatory cytokines) and (A2) (antimicrobial peptides and anti-inflammatory cytokines), *TNF- α* and *IL-15* mRNA levels were all significantly down-regulated in the vitamin A-supplemented diet groups compared with those in the control diet group, except for those in the vitamin A 0.209 mg/kg diet group. The interferon $\gamma 2$ (*IFN- $\gamma 2$*) and *IL-8* mRNA levels were significantly down-regulated in the vitamin A-supplemented diet groups compared with those in the control group, except for those in the vitamin A 1.306 mg/kg diet group. Fish fed the vitamin A 0.416 and 0.618 mg/kg diets had significantly lower *IL-1 β* mRNA levels than fish in the control group. Compared with fish fed the control diet, the relative mRNA expression of *IL-12p35* was significantly



down-regulated in fish fed the vitamin A-supplemented diet. In addition, *β-defensin-1* and *IL-4/13B* mRNA levels were all significantly up-regulated in fish fed the vitamin A-supplemented diet compared with those of fish fed the control diet, except for those in the vitamin A 0.209 mg/kg diet group. Fish fed the vitamin A 0.416, 0.618 and 0.965 mg/kg diets showed significantly higher *hepcidin* mRNA levels compared with the fish fed the control diet. Compared with fish fed the control diet, the liver-expressed antimicrobial peptide (*LEAP*)-2A and *LEAP*-2B mRNA levels were significantly up-regulated, except for those in fish in the vitamin A 1.306 mg/kg diet group. The relative mRNA expressions of *IL-11* and *IL-4/13A* were significantly higher in fish fed the vitamin A 0.618 mg/kg diet than those in fish fed the control diet. However, the relative mRNA expressions of *IL-6*, *IL-17D*, *IL-12p40*, *IL-10*, transforming growth factor (TGF)-*β1* and *TGF-β2* were not influenced by vitamin A levels in the PI.

In the MI, as shown in Fig. 3(B1) (pro-inflammatory cytokines) and (B2) (antimicrobial peptides and anti-inflammatory cytokines),

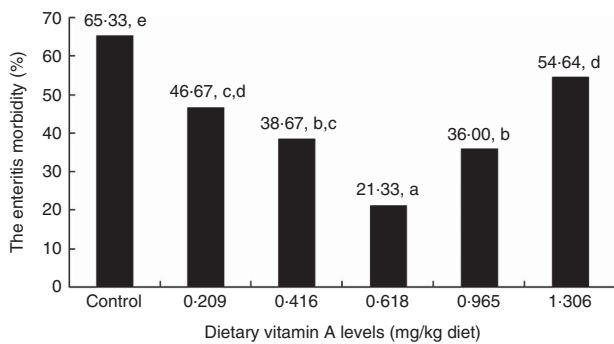


Fig. 2. Effects of dietary vitamin A on enteritis morbidity in fish fed diets containing different vitamin A levels for 10 weeks, and then challenged with *Aeromonas hydrophila* for 14 d. ^{a,b,c,d,e} Mean values with different letters were significantly different ($P < 0.05$; one-way ANOVA and Duncan's multiple-range tests).

a significant decrease in the mRNA levels of *TNF-α*, *IFN-γ2* and *IL-6* was observed in the vitamin A 0.416, 0.618 and 0.965 mg/kg diet groups compared with those of the control diet. Except for the vitamin A 1.306 mg/kg group, *IL-1β* and *IL-15* mRNA levels were significantly decreased compared with those of the control diet. Fish fed the vitamin A-supplemented diets exhibited a significant decrease in the mRNA levels of *IL-8* compared with fish fed the control diet. Fish fed the vitamin A 1209 and 0.618 mg/kg diets had significantly lower *IL-17D* and *IL-12p35* mRNA levels than fish of the control group. In addition, except for the vitamin A 1.306 mg/kg diet group, all the vitamin A-supplemented treatment groups had significantly higher mRNA levels of *β-defensin-1* than the control group. Compared with fish in the control group, fish fed the vitamin A 0.618 mg/kg diet exhibited significantly higher *hepcidin* and *TGF-β1* mRNA levels. The *LEAP*-2A and *IL-10* mRNA levels were all significantly up-regulated in fish fed the vitamin A 0.416, 0.618 and 0.965 mg/kg diets compared with fish in the control group. The relative mRNA levels of *LEAP*-2B and *TGF-β2* were significantly higher in fish fed the vitamin A 0.416 and 0.618 mg/kg diets compared with fish in the control group. All the vitamin A-supplemented diet groups had significantly higher mRNA levels of *IL-11* than the control groups. Compared with fish in the control group, fish fed the vitamin A 0.618, 0.965 and 1.306 mg/kg diets had higher *IL-4/13A* and *IL-4/13B* mRNA levels. However, vitamin A had no significant effects on *IL-12p40* mRNA levels in the MI.

In the DI, as shown in Fig. 3(C1) (pro-inflammatory cytokines) and (C2) (antimicrobial peptides and anti-inflammatory cytokines), *TNF-α* mRNA levels were significantly down-regulated in fish fed the vitamin A-supplemented diets compared with fish fed the control diet, except for those in the vitamin A 1.306 mg/kg diet group. Compared with fish in the control diet group, *IFN-γ2*, *IL-6* and *IL-17D* mRNA levels were significantly lower in fish fed the vitamin A 0.416 and 0.618 mg/kg diets. Fish fed the vitamin A 0.416, 0.618 mg and 0.965 mg/kg diets had significantly

Table 4. Lysozyme activity (LA, U/mg protein), acid phosphatase activity (ACP, U/g protein), complement 3 (C3, mg/g protein) and complement 4 (C4, mg/g protein) contents in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of young grass carp (*Ctenopharyngodon idella*) fed diets containing different vitamin A levels for 10 weeks, and then challenged with *Aeromonas hydrophila* for 14 days (Mean values and standard deviations of six replicates)

Dietary vitamin A levels (mg/kg diet)...	Control		0.209		0.416		0.618		0.965		1.306	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LA												
PI	91.48 ^a	5.09	122.76 ^b	10.35	122.46 ^b	10.04	147.47 ^c	8.64	130.19 ^b	4.54	132.59 ^b	8.87
MI	101.94 ^a	7.17	134.90 ^b	3.65	134.54 ^b	12.19	132.63 ^b	5.80	130.46 ^b	12.85	128.42 ^b	4.87
DI	111.94 ^a	7.51	111.79 ^a	7.08	192.80 ^b	5.23	184.76 ^b	6.20	185.58 ^b	6.75	187.35 ^b	5.09
ACP												
PI	173.80 ^a	113.39	192.45 ^b	15.35	211.28 ^c	13.23	214.69 ^c	17.99	217.51 ^c	5.95	217.48 ^c	13.64
MI	183.26 ^a	14.42	218.78 ^b	5.74	225.47 ^b	16.70	222.85 ^b	9.93	223.41 ^b	15.59	226.10 ^b	12.65
DI	214.30 ^a	11.61	258.25 ^b	8.83	273.95 ^b	13.22	381.59 ^c	13.21	386.79 ^c	18.33	388.31 ^c	18.37
C3												
PI	28.98 ^a	1.74	32.11 ^b	1.45	33.99 ^{b,c}	2.20	35.40 ^c	1.44	32.43 ^b	1.51	32.61 ^b	2.09
MI	32.42 ^a	1.43	34.92 ^b	2.40	38.02 ^c	1.63	36.64 ^{b,c}	1.31	35.10 ^b	1.77	34.69 ^b	1.57
DI	36.16 ^a	2.07	39.04 ^b	2.65	39.03 ^b	1.89	42.06 ^c	1.95	38.62 ^b	1.03	38.80 ^b	1.07
C4												
PI	1.96 ^a	0.10	2.51 ^b	0.20	3.06 ^c	0.15	2.37 ^b	0.11	2.32 ^b	0.20	2.43 ^b	0.16
MI	2.29 ^a	0.21	2.78 ^c	0.08	3.13 ^d	0.27	2.75 ^c	0.10	2.62 ^{b,c}	0.20	2.47 ^{a,b}	0.19
DI	2.31 ^a	0.14	3.20 ^d	0.12	3.69 ^e	0.13	2.81 ^c	0.09	2.76 ^c	0.10	2.60 ^b	0.15

^{a,b,c,d,e} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$; one-way ANOVA and Duncan's multiple-range tests).



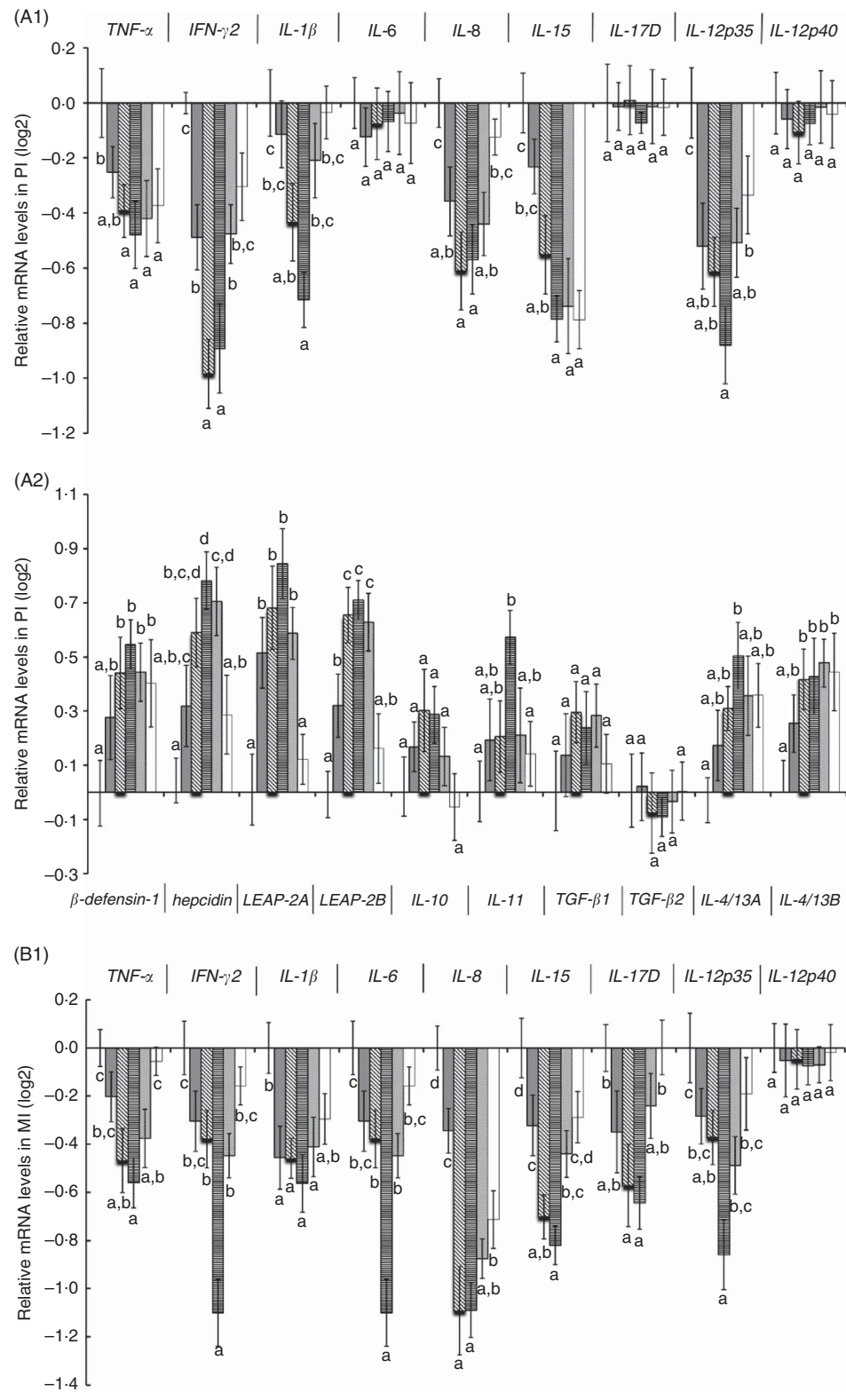


Fig. 3. (Continued on following page)

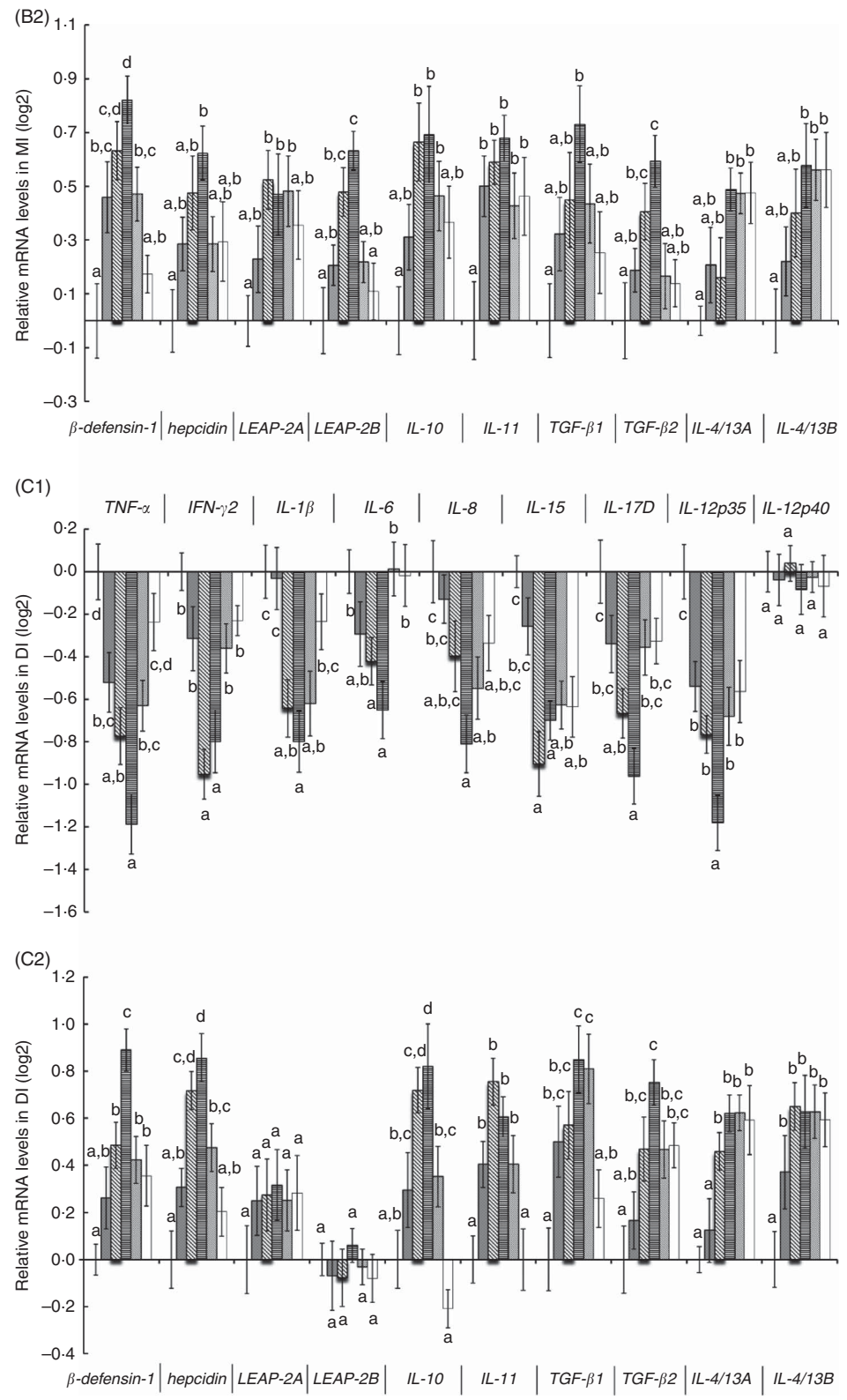


Fig. 3. (Continued from previous page) Effects of dietary vitamin A on relative mRNA levels of pro-inflammatory cytokines *TNF- α* , *IFN- γ 2*, *IL-1 β* , *IL-6*, *IL-8*, *IL-15*, *IL-17D*, *IL-12p35* and *IL-12p40* in the proximal intestine (PI) (A1), mid intestine (MI) (B1) and distal intestine (DI) (C1), and antibacterial peptides *β -defensin-1*, *hepcidin*, *LEAP-2A* and *LEAP-2B* as well as anti-inflammatory cytokines *IL-10*, *IL-11*, *TGF- β 1*, *TGF- β 2*, *IL-4/13A* and *IL-4/13B* in the PI (A2), MI (B2) and DI (C2) of fish fed diets containing different vitamin A levels for 10 weeks, and then challenged with *Aeromonas hydrophila* for 14 d. Expression results were normalised to *β -actin* expression, which did not change with treatment. Values are means (*n* 6 fish per treatment), with their standard errors represented by vertical bars. ^{a,b,c,d} Mean values were significantly different among the groups (*P* < 0.05; one-way ANOVA and Duncan's multiple-range tests). *IFN- γ 2*, interferon γ 2; *LEAP*, liver-expressed antimicrobial peptide; *TGF*, transforming growth factor; VA, vitamin A. ■, Control; □, VA 0.209; ▨, VA 0.416; ▩, VA 0.618; ▪, VA 0.965; ▫, VA 1.306 mg/kg diet.

lower *IL-1 β* mRNA levels than fish fed the control diet. *IL-8* mRNA level was significantly down-regulated in fish fed the vitamin A 0.618 and 0.965 mg/kg diets compared with that of fish fed the control diet. Except for those in the vitamin A 0.209 mg/kg diet group, fish in the vitamin A-supplemented groups had significantly higher *IL-15* mRNA levels than fish in the control group. Compared with fish in the control group, *IL-12p35* mRNA levels were significantly down-regulated in fish fed the vitamin A-supplemented diets. In addition, compared with the mRNA levels of fish in the control diet group, *β -defensin-1*, *TGF- β 2* and *IL-4/13A* were significantly improved in fish in all other groups, except for those in the vitamin A 0.209 mg/kg diet group. The mRNA levels of *hepcidin* were significantly up-regulated in fish fed vitamin A 0.416, 0.618 and 0.965 mg/kg diets compared with fish in the control group. Fish fed the vitamin A 0.416 and 0.618 mg/kg diets exhibited significant increases in the mRNA level of *IL-10*. Except for those in the vitamin A 1.306 mg/kg diet group, fish fed the vitamin A-supplemented diets had significantly higher *IL-11* and *TGF- β 1* mRNA levels than fish in the control diet group. Compared with the control group, all the vitamin A-supplemented diet groups showed significantly higher *IL-4/13B* mRNA levels. However, the *IL-12p40*, *LEAP-2A* and *LEAP-2B* mRNA levels in the DI were not affected by dietary vitamin A levels.

mRNA levels of immune-related signalling molecules in the proximal intestine, mid intestine and distal intestine. The effects of dietary vitamin A on *NF- κ B p65*, *NF- κ B p52*, *c-Rel*, *I κ B α* , *I κ B kinase α (IKK α)*, *IKK β* , *IKK γ* and *p38MAPK* gene expression levels are presented in Fig. 4. To the best of our knowledge, *NF- κ B p52*, *NF- κ B p65* and *c-Rel* are three members of the *NF- κ B* family that play a central role in regulating cytokines during inflammation in mammals⁽⁵³⁾. The results showed that the up-regulation of *NF- κ B p65* could up-regulate the mRNA levels of pro-inflammatory cytokines *IL-8* and *IFN- γ 2* as well as down-regulate the mRNA levels of anti-inflammatory cytokines *IL-10* and *TGF- β 1* in the intestine of grass carp⁽⁴²⁾. Meanwhile, Lui *et al.*⁽⁵⁴⁾ reported that the up-regulation of *p38MAPK* could increase pro-inflammatory cytokines *TNF- α* and *IL-6* in rats.

In the present study, as shown in Fig. 4(a), in the PI, *c-Rel* and *IKK γ* mRNA levels were significantly down-regulated in fish fed the vitamin A-supplemented diets compared with fish fed the control diet, except for those fed the vitamin A 0.209 mg/kg diet. Fish fed the vitamin A 0.618 mg/kg diet had significantly higher *I κ B α* mRNA levels than the control group. Compared with fish in the control group, *IKK β* mRNA levels were significantly down-regulated in fish fed the vitamin A 0.416 and 0.618 mg/kg diets. However, *NF- κ B p65*, *NF- κ B p52*, *IKK α* and *p38MAPK* mRNA levels in the PI were not significantly affected by dietary vitamin A levels.

In the MI, *NF- κ B p65* and *p38MAPK* mRNA levels were all significantly down-regulated in fish fed the vitamin A 0.416, 0.618 and 0.965 mg/kg diets compared with fish fed the control diet. Except for those fed the vitamin A 0.209 mg/kg diet, fish fed the vitamin A-supplemented diets had significantly lower *c-Rel* mRNA levels than fish in the control group. *I κ B α* mRNA levels were significantly enhanced in fish fed the vitamin A 0.618 mg/kg diet compared with fish fed the control diet. Both *IKK β* and *IKK γ* mRNA levels were significantly down-regulated in fish fed the vitamin A-supplemented diets compared with fish fed the control

diet, except for those fed the vitamin A 1.306 mg/kg diet. However, *NF- κ B p52* and *IKK α* mRNA levels in the MI were not affected by dietary vitamin A levels.

In the DI, compared with fish fed the control diet, *NF- κ B p65* mRNA levels were significantly down-regulated in fish fed the vitamin A 0.416 and 0.618 mg/kg diets. Fish fed the vitamin A-supplemented diets had significantly lower *c-Rel* mRNA levels than fish in the control group, except for fish fed the vitamin A 0.209 mg/kg diet. *I κ B α* mRNA level was significantly up-regulated in fish fed the vitamin A 0.209, 0.416 and 0.618 mg/kg diets and significantly down-regulated in fish fed the vitamin A 1.306 mg/kg diet compared with fish in the control group. Except for fish fed the vitamin A 1.306 mg/kg diet, fish fed the vitamin A-supplemented diets had significant lower *IKK β* mRNA levels than fish in the control group. Compared with fish fed the control diet, *IKK γ* mRNA levels were significantly down-regulated in fish fed the vitamin A 0.209, 0.416 and 0.618 mg/kg diets. *p38MAPK* mRNA levels were significantly lower in fish fed the vitamin A 0.416, 0.618 and 0.965 mg/kg diets compared with the control group. However, *NF- κ B p52* and *IKK α* mRNA levels in the DI were not affected by dietary vitamin A levels.

Furthermore, the correlation of inflammatory cytokines with *NF- κ B* and *p38MAPK* signalling in the fish PI, MI and DI are presented in Table 5. The mRNA levels of the pro-inflammatory cytokines *TNF- α* , *IFN- γ 2*, *IL-1 β* , *IL-6*, *IL-8*, *IL-15*, *IL-17D* and *IL-12p35* were positively related to *NF- κ B p65*, *c-Rel* and *p38MAPK* mRNA levels, whereas the mRNA levels of the anti-inflammatory cytokines *IL-10*, *IL-11*, *TGF- β 1*, *TGF- β 2*, *IL-4/13A* and *IL-4/13B* were negatively related to *NF- κ B p65*, *c-Rel* and *p38MAPK* mRNA levels in the different intestinal segments of fish. In addition, *NF- κ B p65* mRNA levels were negatively related to *I κ B α* mRNA levels in the MI and DI, and *c-Rel* mRNA levels were negatively related to *I κ B α* mRNA levels in the PI and MI, respectively, whereas *I κ B α* mRNA levels were negatively related to *IKK β* mRNA levels in the MI and to *IKK γ* mRNA levels in the PI, MI and DI.

Discussion

Vitamin A deficiency decreased fish growth performance and resistance to enteritis

The present study showed that compared with the use of optimum vitamin A levels, vitamin A deficiency remarkably decreased the PWG, SGR, FI and FE of fish (Table 3), suggesting that vitamin A deficiency significantly decreased fish growth performance. On the basis of PWG, the optimum vitamin A requirement of fish was estimated to be 0.664 mg/kg diet ($y = -115.914x^2 + 154.054x + 216.598$; $R^2 = 0.799$, $P < 0.01$) (Fig. 5). Generally, it is a common method to conduct a challenge trial by infecting fish with a widespread pathogenic bacterium after the feeding experiment, which can assess the nutritional enhancement of the disease-resistance abilities of the fish, as several studies have indicated^(55,56). To our knowledge, *A. hydrophila* is one of the most common pathogen causing bacterial enteritis in freshwater fish^(43,57). Song *et al.*⁽⁴⁵⁾ established an enteritis model in grass carp by anal intubation with *A. hydrophila*. Therefore, after the feeding trial, we infected fish with *A. hydrophila* to investigate the effect of vitamin A on



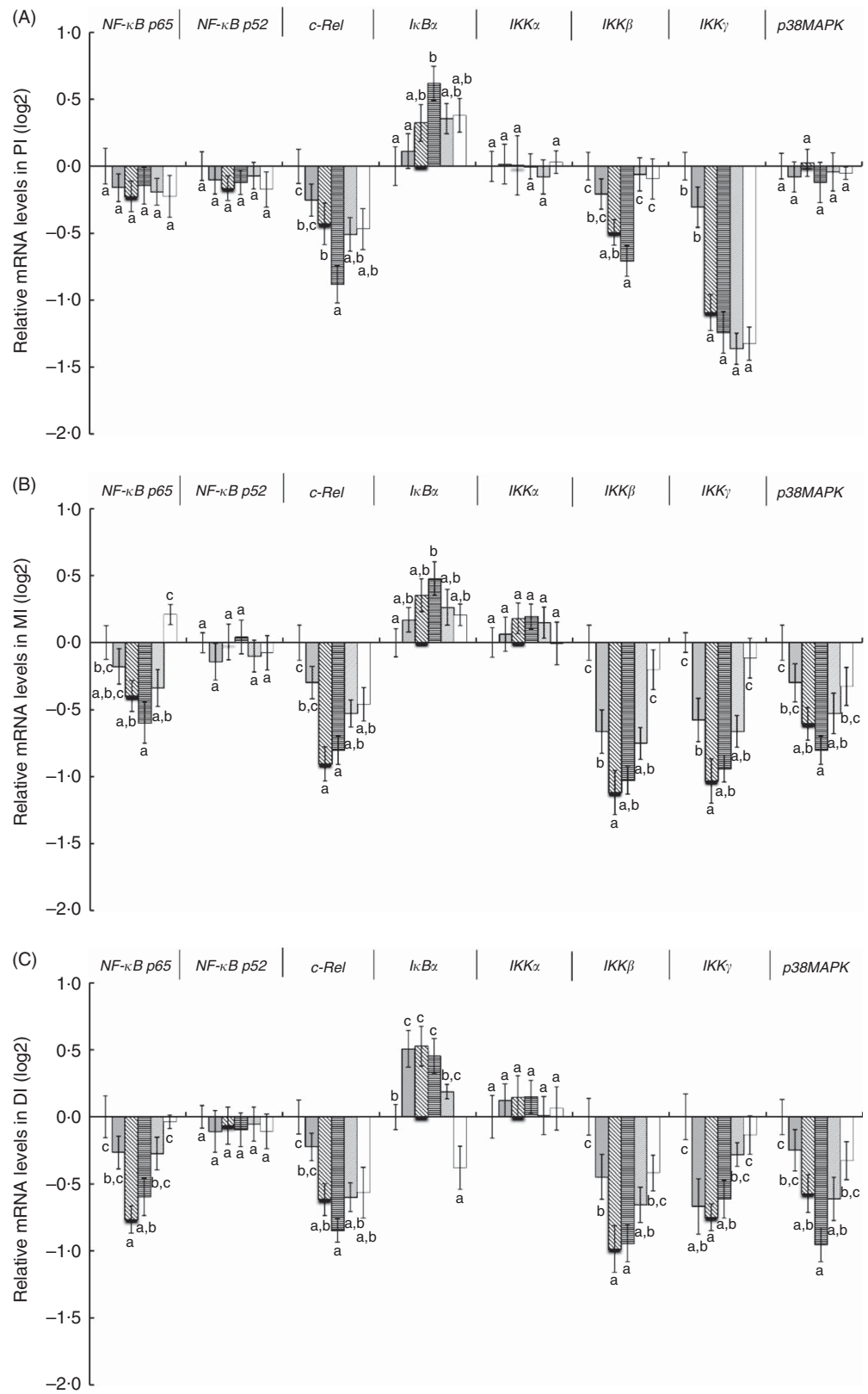


Fig. 4. Effects of dietary vitamin A on relative mRNA levels of signalling molecules *NF-κB p65*, *NF-κB p52*, *c-Rel*, *IκBα*, *IKKα*, *IKKβ*, *IKKγ* and *p38MAPK* in the proximal intestine (PI) (A), mid intestine (MI) (B) and distal intestine (DI) (C) of fish fed diets containing different vitamin A levels for 10 weeks, and then challenged with *Aeromonas hydrophila* for 14 d. Expression results were normalised to β -actin expression, which did not change with treatment. Values are means (*n* 6 fish per treatment), with their standard errors represented by vertical bars. ^{a,b,c} Mean values were significantly different among the groups (*P* < 0.05; one-way ANOVA and Duncan's multiple-range tests). *IκB*, inhibitor κ B; *IKK*, I κ B kinase; *p38MAPK*, p38 mitogen-activated protein kinase; VA, vitamin A. ■, Control; ▨, VA 0.209; ▩, VA 0.416; ▤, VA 0.618; ▥, VA 0.965; □, VA 1.306 mg/kg diet.

Table 5. Correlation coefficients of *TNF-α*, interferon $\gamma 2$ (*IFN-γ2*), *IL-1β*, *IL-6*, *IL-8*, *IL-15*, *IL-17D*, *IL-12p35*, *IL-10*, *IL-11*, transforming growth factor (*TGF*)- $\beta 1$, *TGF-β2*, *IL-4/13A* and *IL-4/13B* with *NF-κB p65*, *c-Rel* and p38 mitogen-activated protein kinase (*p38MAPK*); *NF-κB p65* and *c-Rel* with the inhibitor of $\kappa B\alpha$ (*IκBa*); *IκBa* with κB kinase (*IKK*) β and *IKKγ* in the proximal intestine (PI), mid intestine (MI) and distal intestine (DI) of fish

Dependent parameters	Independent parameters	PI		MI		DI	
		Correlation coefficients	P	Correlation coefficients	P	Correlation coefficients	P
<i>TNF-α</i>	<i>NF-κB p65</i>	+0.851	<0.05	+0.929	<0.01	+0.891	<0.05
	<i>c-Rel</i>	+0.934	<0.01	+0.865	<0.05	+0.821	<0.05
	<i>p38MAPK</i>	–	–	+0.938	<0.01	+0.931	<0.01
<i>IFN-γ2</i>	<i>NF-κB p65</i>	–	–	–	–	+0.978	<0.01
	<i>c-Rel</i>	+0.755	0.083	+0.916	<0.01	+0.806	0.053
	<i>p38MAPK</i>	–	–	+0.990	<0.01	+0.848	<0.05
<i>IL-1β</i>	<i>c-Rel</i>	+0.800	0.056	+0.854	<0.05	+0.905	<0.05
	<i>p38MAPK</i>	–	–	+0.903	<0.05	+0.945	<0.01
	<i>NF-κB p65</i>	–	–	+0.818	<0.05	+0.830	<0.05
<i>IL-6</i>	<i>c-Rel</i>	–	–	+0.758	0.081	–	–
	<i>p38MAPK</i>	–	–	+0.905	<0.05	–	–
	<i>c-Rel</i>	+0.739	0.093	+0.975	<0.01	+0.957	<0.01
<i>IL-8</i>	<i>p38MAPK</i>	–	–	+0.955	<0.01	+0.980	<0.01
	<i>NF-κB p65</i>	+0.762	0.078	+0.778	0.068	+0.740	0.093
	<i>c-Rel</i>	+0.914	<0.05	+0.966	<0.01	+0.945	<0.01
<i>IL-15</i>	<i>p38MAPK</i>	–	–	+0.986	<0.01	+0.857	<0.05
	<i>NF-κB p65</i>	–	–	+0.924	<0.01	+0.879	<0.05
	<i>c-Rel</i>	–	–	+0.762	0.078	+0.892	<0.05
<i>IL-17D</i>	<i>p38MAPK</i>	–	–	+0.826	<0.05	+0.929	<0.01
	<i>NF-κB p65</i>	–	–	+0.820	<0.05	+0.799	0.056
	<i>c-Rel</i>	+0.884	<0.05	+0.802	0.055	+0.937	<0.01
<i>IL-12p35</i>	<i>p38MAPK</i>	–	–	+0.939	<0.01	+0.951	<0.01
	<i>NF-κB p65</i>	–	–	–0.739	0.094	–0.946	<0.01
	<i>c-Rel</i>	–	–	–0.982	<0.01	–	–
<i>IL-10</i>	<i>p38MAPK</i>	–	–	–0.981	<0.01	–	–
	<i>NF-κB p65</i>	–	–	–	–	–0.984	<0.01
	<i>c-Rel</i>	–0.883	<0.05	–0.903	<0.05	–	–
<i>TGF-β1</i>	<i>p38MAPK</i>	–	–	–0.912	<0.05	–	–
	<i>c-Rel</i>	–	–	–0.860	<0.05	–	–
	<i>p38MAPK</i>	–	–	–0.962	<0.01	–0.914	<0.05
<i>TGF-β2</i>	<i>NF-κB p65</i>	–	–	–0.796	0.058	–	–
	<i>c-Rel</i>	–	–	–0.826	<0.05	–0.970	<0.01
	<i>p38MAPK</i>	–	–	–0.880	<0.05	–0.950	<0.01
<i>IL-4/13A</i>	<i>c-Rel</i>	–0.994	<0.01	–0.855	<0.05	–0.929	<0.01
<i>IL-4/13B</i>	<i>NF-κB p65</i>	–0.873	<0.05	–	–	–	–
	<i>c-Rel</i>	–0.876	<0.05	–0.802	0.055	–0.944	<0.01
	<i>p38MAPK</i>	–	–	–0.818	<0.05	–0.874	<0.05
<i>NF-κB p65</i>	<i>IκBa</i>	–	–	–0.776	0.069	–0.814	<0.05
<i>c-Rel</i>	<i>IκBa</i>	–0.879	<0.01	–0.935	<0.01	–	–
<i>IκBa</i>	<i>IKKβ</i>	–	–	–0.869	<0.05	–	–
	<i>IKKγ</i>	–0.832	<0.05	–0.856	<0.05	–0.902	<0.05

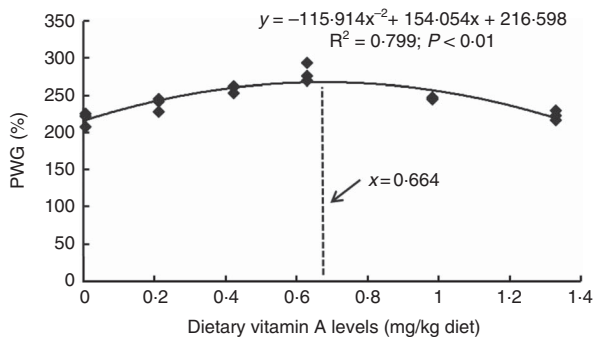


Fig. 5. Quadratic regression analysis of percentage of weight gain (PWG) in fish fed diets containing different vitamin A levels for 10 weeks.

fish resistance to enteritis in this study. The result showed that vitamin A deficiency caused the highest enteritis morbidity (65.33%), whereas optimum vitamin A levels significantly

decreased enteritis morbidity to 21.33% (Fig. 2), suggesting that vitamin A deficiency could impair fish resistance to enteritis, whereas optimum vitamin A levels could block this impairment. Meanwhile, the optimum vitamin A level for protecting fish against enteritis morbidity was estimated to be 0.707 mg/kg diet ($y = 78.309x^2 - 110.695x + 66.485, R^2 = 0.933, P < 0.05$). To our knowledge, the ability to resist disease is remarkably connected with immune function in fish⁽⁵⁸⁾. Thus, we next investigated the effects of vitamin A on the intestinal immune function of fish.

Vitamin A deficiency impaired fish innate immunity in the proximal intestine, mid intestine and distal intestine

Previous studies have shown that decreased innate humoral immune molecules such as complement (such as C3 and C4)⁽⁵⁹⁾, LA⁽⁶⁰⁾ and the antimicrobial peptide hepcidin⁽⁶¹⁾ could impair fish innate immunity. In the present study, compared with optimum vitamin A levels, dietary vitamin A deficiency significantly

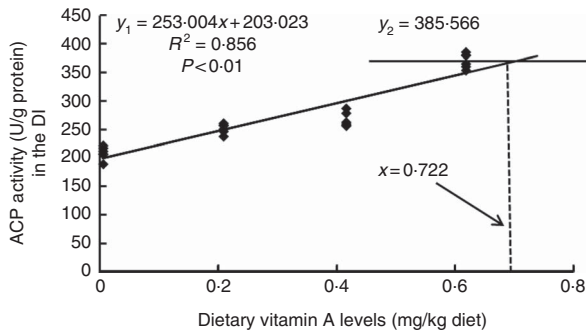


Fig. 6. Broken-line analysis of acid phosphatase (ACP) activity in fish fed diets containing different vitamin A levels for 10 weeks, and then challenged with *Aeromonas hydrophila* for 14 d. DI, distal intestine.

decreased LA and ACP activities, C3 and C4 contents and mRNA levels of β -defensin-1 and hepcidin in the PI, MI and DI, as well as *LEAP-2A* and *LEAP-2B* in the PI and MI, suggesting that vitamin A deficiency could impair fish intestinal innate immunity. On the basis of ACP activity in the DI, the optimum dietary vitamin A level for the fish was estimated to be 0.722 mg/kg (Fig. 6).

Interestingly, both *LEAP-2A* and *LEAP-2B* mRNA levels in the DI were not affected by vitamin A levels. Until now, no information is available regarding the effects of vitamin A on *LEAP-2* in animals. The reasons for the negligible effects of vitamin A on *LEAP-2A* and *LEAP-2B* mRNA levels in the DI might be partially explained by two factors. First, it could be attributed to the decrease in another antimicrobial peptide in the DI, *hepcidin* (*LEAP-1*), caused by vitamin A deficiency. To our knowledge, hepcidin (*LEAP-1*) shares large structural characteristics with *LEAP-2* in mammals⁽⁶²⁾. According to the study of Hocquellet *et al.*⁽⁶³⁾, the antimicrobial activity of *LEAP-2* may not be its main biological function. Thus, hepcidin (*LEAP-1*) might compensate for *LEAP-2* in the defence against pathogens. The present study showed that compared with the optimum vitamin A level vitamin A deficiency significantly decreased the mRNA levels of *hepcidin* (*LEAP-1*) in fish DI. Therefore, vitamin A might be operating through *hepcidin* (*LEAP-1*) rather than *LEAP-2* to defend against pathogens in the DI. Second, it could be partly associated with *LEAP-2* expression in different intestinal segments of the fish. One study reported that *LEAP-2B* mRNA expression in the DI was significantly lower than that in the PI of common carp⁽⁶⁴⁾. These might be a few reasons for the down-regulation of *LEAP-2A* and *LEAP-2B* mRNA levels in the PI and MI caused by vitamin A deficiency, whereas both of them were not affected by vitamin A levels in the DI. However, the mechanisms remain unclear. It is well known that fish innate immunity is closely associated with inflammation, which is largely mediated by cytokines⁽⁶⁵⁾. Therefore, we next examined the effects of vitamin A on intestinal inflammation in the fish.

Vitamin A deficiency aggravated fish intestinal inflammation and was partly connected to the NF- κ B canonical signalling pathway and the p38 mitogen-activated protein kinase signalling pathway

Available studies have shown that the inflammatory process is initiated upon the up-regulation of pro-inflammatory cytokines

such as *IFN- γ* , *TNF- α* , *IL-1 β* and *IL-12* and the down-regulation of anti-inflammatory cytokines such as *IL-4/13A*, *IL-4/13B*, *IL-10* and *TGF- β 1* in fish⁽⁶⁵⁾. In the present study, compared with optimum vitamin A levels, vitamin A deficiency significantly up-regulated the mRNA levels of pro-inflammatory cytokines *TNF- α* , *IFN- γ 2*, *IL-1 β* , *IL-8*, *IL-15* and *IL-12p35* and down-regulated the mRNA levels of anti-inflammatory cytokines *IL-11*, *IL-4/13A* and *IL-4/13B* in the PI, MI and DI, indicating that vitamin A deficiency aggravated fish intestinal inflammation. Previous studies have reported that inflammatory cytokines could be regulated by the signalling molecule NF- κ B in Jian carp (*Cyprinus carpio* var. Jian)⁽⁶⁶⁾ and the signalling molecule *p38MAPK* in rats⁽⁶⁷⁾. Thus, we next investigated the effect of vitamin A on NF- κ B and *p38MAPK* signalling pathways in the three intestinal segments of fish.

To the best of our knowledge, NF- κ B p52, NF- κ B p65 and *c-Rel* are three members of the NF- κ B family that play a central role in regulating cytokines during inflammation in mammals⁽⁵³⁾. The results showed that the up-regulation of NF- κ B p65 could up-regulate pro-inflammatory cytokines *IL-8* and *IFN- γ 2* as well as down-regulate the mRNA levels of anti-inflammatory cytokines *IL-10* and *TGF- β 1* in the intestine of grass carp⁽⁴²⁾. In the present study, compared with the optimum vitamin A levels, vitamin A deficiency significantly up-regulated *c-Rel* mRNA levels in the PI, MI and DI and NF- κ B p65 mRNA levels in the MI and DI, whereas NF- κ B p52 mRNA levels were not affected by vitamin A. The analysis showed that the mRNA levels of the pro-inflammatory cytokines *TNF- α* , *IFN- γ 2*, *IL-1 β* , *IL-6*, *IL-8*, *IL-15*, *IL-17D* and *IL-12p35* were positively correlated with NF- κ B p65 or *c-Rel* mRNA levels, whereas the mRNA levels of the anti-inflammatory cytokines *IL-10*, *IL-11*, *TGF- β 1*, *TGF- β 2*, *IL-4/13A* and *IL-4/13B* were negatively correlated with NF- κ B p65 or *c-Rel* mRNA levels in the different intestine segments of fish (Table 5), indicating that vitamin A deficiency might enhance NF- κ B p65 or *c-Rel* (rather than NF- κ B p52) mRNA levels to aggravate fish intestinal inflammation. The reason for the negligible effect of vitamin A on NF- κ B p52 mRNA might be partially associated with the different effects of vitamin A on different NF- κ B signalling pathways. To our knowledge, NF- κ B signalling can be elicited in two activation pathways: the canonical signalling pathway and the non-canonical signalling pathway. Canonical signalling depends on the activation of *I κ B* kinase (*IKK α* , *IKK β* , *IKK γ*) and the degradation of the inhibitory protein *I κ B α* , which leads to the translocation of NF- κ Bs (*p65/p50/c-Rel*) to the nucleus. In contrast, non-canonical NF- κ B activation is dependent on the activation of *IKK α* (but not *IKK β* and *IKK γ*) to induce the processing of *p52/RelB* dimers in cancer cells⁽⁶⁸⁾. The present study showed that compared with optimum vitamin A levels, vitamin A deficiency significantly down-regulated *I κ B α* mRNA levels and up-regulated *IKK β* and *IKK γ* mRNA levels in the PI, MI and DI. However, *IKK α* mRNA level was not affected by vitamin A levels. Correlation analysis showed that NF- κ B p65 mRNA levels were negatively related to *I κ B α* mRNA levels in the MI and DI, and that *c-Rel* mRNA levels were negatively related to *I κ B α* mRNA levels in the PI and MI, whereas *I κ B α* mRNA levels were negatively related to *IKK β* in the MI and to *IKK γ* mRNA levels in the PI, MI and DI (Table 5). These results suggested that vitamin A regulated these cytokines might be partly through the NF- κ B canonical signalling

Immune responses and signalling pathways in carp

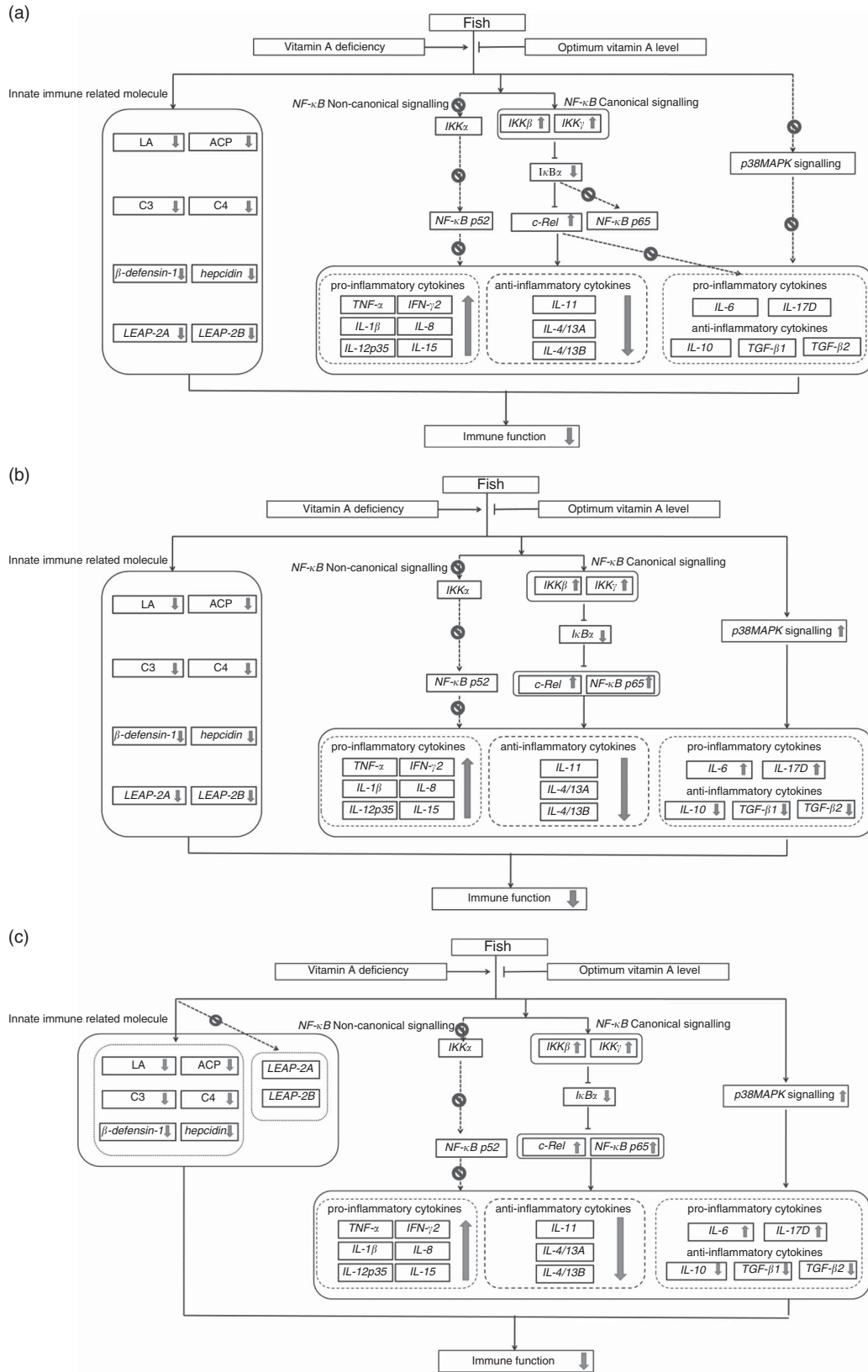


Fig. 7. General summary of the effects of vitamin A on immune function and its potential signalling pathways in the proximal intestine (PI) (a), mid intestine (MI) (b) and distal intestine (DI) (c) of young grass carp (*Ctenopharyngodon idella*). LA, lysozyme; ACP, acid phosphatase; C3, complement 3; C4, complement 4; LEAP, liver-expressed antimicrobial peptide; IKK, I κ B kinase; I κ B, inhibitor κ B; p38MAPK, p38 mitogen-activated protein kinase; TGF, transforming growth factor. \cdots \ominus \rightarrow , Not through; \cdots \ominus , inhibit; \cdots \rightarrow , might through.



pathway rather than the non-canonical signalling pathway in the PI, MI and DI. However, the underlying mechanism requires further investigation. Furthermore, Liu *et al.*⁽⁶⁷⁾ reported that the up-regulation of *p38MAPK* could increase the pro-inflammatory cytokines *TNF- α* and *IL-6* in rats. In the present study, compared with the optimum vitamin A levels, vitamin A deficiency caused significant up-regulation of *p38MAPK* mRNA levels in the MI and DI. Further correlation analysis indicated that the mRNA levels of the pro-inflammatory cytokines *TNF- α* , *IFN- γ 2*, *IL-1 β* , *IL-8*, *IL-15*, *IL-17D* and *IL-12p35* in the MI and DI and *IL-6* in the MI were positively correlated with *p38MAPK* mRNA levels. In addition, the mRNA levels of the anti-inflammatory cytokines *IL-10* and *IL-11* in the MI and *TGF- β 1*, *TGF- β 2* and *IL-4/13B* in the MI and DI were negatively related to *p38MAPK* mRNA levels (Table 5), suggesting that *p38MAPK* also participated in regulating cytokine production in the MI and DI of fish.

Interestingly, the effects of vitamin A on inflammation in the PI showed some differences compared with the MI and DI in this study. Our results showed that compared with optimum vitamin A levels, vitamin A deficiency caused significant up-regulation in the mRNA levels of the pro-inflammatory cytokines *IL-6* and *IL-17D* and the down-regulation of the mRNA levels of the anti-inflammatory cytokines *IL-10*, *TGF- β 1* and *TGF- β 2* in the MI and DI, whereas all these cytokines were not affected by dietary vitamin A levels in the PI. The reason might be partly associated with *NF- κ B p65* and *p38MAPK* signalling pathways. The present study showed that vitamin A deficiency could significantly up-regulate *NF- κ B p65* and *p38MAPK* mRNA in the MI and DI. Correlation analysis suggested that *IL-6*, *IL-17D*, *IL-10*, *TGF- β 1* and *TGF- β 2* mRNA levels in the MI and DI were significantly correlated with *NF- κ B p65* or *p38MAPK* mRNA levels (Table 5), suggesting that *IL-6*, *IL-17D*, *IL-10*, *TGF- β 1* and *TGF- β 2* mRNA levels could be regulated by *NF- κ B p65* or *p38MAPK* in the MI and DI. However, both *NF- κ B p65* and *p38MAPK* were not affected by vitamin A levels in the PI, which could be a potential reason for the negligible effects of dietary vitamin A on *IL-6*, *IL-17D*, *IL-10*, *TGF- β 1* and *TGF- β 2* mRNA levels in the PI. Furthermore, the reason for the non-significant impact of vitamin A on *NF- κ B p65* and *p38MAPK* mRNA levels in the PI may be partly associated with the different effects of vitamin A on the different intestinal segments in fish. In general, specific subsets of intestinal DC and macrophages could constitutively express RA-synthesising enzymes, in which vitamin A (retinol) could be metabolised to RA to exert most of its functions including immunological function in animals^(23,24). To the best of our knowledge, the PI is the main nutrient-absorbing region and contains typically lower numbers of bacteria, which showed a relatively weaker inflammatory response than the posterior intestine in teleosts⁽⁶⁹⁾. Thus, vitamin A may play a greater immune function in the posterior intestine than in the PI. This might be a part of the reason why vitamin A did not affect *NF- κ B p65* and *p38MAPK* mRNA levels in the PI. However, the underlying mechanisms deserve further investigation.

Comparison of the optimal vitamin A levels depending on different indicators

According to our study, the optimum dietary vitamin A level was effective in promoting growth and enhancing intestinal

immune function in fish, which clearly varied with different indicators. The optimum vitamin A levels for PWG and protection against enteritis morbidity in grass carp (262.02–996.67 g) were estimated to be 0.664 and 0.707 mg/kg in the diet, respectively. In addition, based on ACP activity, the optimum vitamin A levels were estimated to be 0.722 mg/kg in the diet. Apparently, the intestinal immune requirements of fish were slightly higher than the growth requirement, indicating that more vitamin A might be required for the development of fish intestinal immune response and to enhance disease resistance. The results were similar to Zhou *et al.*⁽³⁰⁾, who observed that the requirement of vitamin E based on plasma LA activity was higher than the requirement based on WG in the cobia (*Rachycentron canadum*).

Conclusion

The results of this study demonstrated for the first time that vitamin A deficiency could decrease fish growth, resistance to enteritis and immune function in the PI, MI and DI, and different immune responses in the PI, MI and DI were mediated at least in part by the *NF- κ B* canonical signalling pathway rather than the *NF- κ B* non-canonical signalling pathway. Meanwhile, vitamin A deficiency might also mediate *p38MAPK* signalling to disturb the inflammatory cytokines in the MI and DI, but it did not appear to regulate inflammatory cytokines in the PI (see in Fig. 7 for details). In addition, based on the PWG, to protect fish against enteritis morbidity and ACP activity, the optimum dietary vitamin A levels required for optimal young grass carp growth, enteritis resistance and immune function were estimated to be 0.664, 0.707 and 0.722 mg/kg, respectively.

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The authors declare that there are no conflicts of interest.



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