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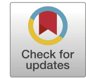
Original Research Article

Lysine deficiency impaired growth performance and immune response and aggravated inflammatory response of the skin, spleen and head kidney in grown-up grass carp (*Ctenopharyngodon idella*)



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ABSTRACT

This dissertation was primarily focused on the immune response, inflammatory response and molecular mechanisms in the skin, head kidney and spleen of grown-up grass carp (*Ctenopharyngodon idella*). Six iso-nitrogen diets differing in lysine concentrations (5.6, 8.5, 11.6, 14.4, 17.5 and 20.7 g/kg) were fed to 540 grass carp (164.85 ± 0.79 g) for 60 d. After that, grass carp were challenged by *Aeromonas hydrophila* for 6 d. This study revealed that lysine deficiency (1) suppressed the growth performance of the fish and decreased their ability to resist skin lesion morbidity, (2) impaired the immune organ's immune response by decreasing the gene expressions of mucin, liver-expressed antimicrobial peptide (*LEAP*)*-2B*, β-defensin-1 and *LEAP-2A* and the production of antibacterial compounds of grown-up grass carp, and (3) aggravated the inflammatory response of immune organs in the fish by increasing the gene expressions of pro-inflammatory cytokines (interferon γ2 [*IFN-γ2*], tumor necrosis factor α [*TNF-α*], interleukin [*IL*]*-15*, *IL-17D*, *IL-12p40*, *IL-6* and *IL-8*) and down-regulating anti-inflammatory cytokines (*IL-11*, transforming growth factor β1 [*TGF-β1*], *IL-10* and *IL-4/13A*), which were tightly correlated with signal transducer and activator of transcription (STAT)1 and STAT3 signaling pathway, respectively. The different phenomenon in the skin, spleen and head kidney of fish may be correlated with the difference in gene subtype. In addition, using quadratic regression analysis of percent weight gain (PWG), skin lesion morbidity, and the lysozyme activities in the spleen and head kidney, the dietary lysine requirements for grown-up grass carp were estimated to be 13.58, 13.51, 14.56 and 14.18 g/kg, respectively.

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1. Introduction

In fish, lysine is an essential amino acid (NRC, 2011). One previous study observed that lysine deficiency caused growth retardation and feed utilization reduction of sub-adult grass carp (Cai et al., 2018). Intensive aquaculture increases the stress of fish and increases the risk of infection (Wang et al., 2018). Improving immunity is crucial for the prevention and control of diseases in fish (Pohlenz et al., 2014). The immune organ's immune function (e.g., skin, spleen and head kidney) guarantees the immunity of fish (Ni et al., 2016). One previous study reviews adverse effects of arginine (essential amino acid) deficiency on the immune organ's immune

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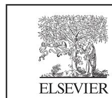

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function of fish (Chen et al., 2015). In grass carp, lysine enhanced the growth performance and intestinal antioxidant capacity of fish (Li et al., 2016a,b; Li et al., 2013). Fish growth is often correlated with the immune organ's immune function (Liu et al., 2018). The fish immune organ's immune function is closely related to the production of antibacterial compounds and inflammatory factors (Liu et al., 2018), which could be regulated by Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) (e.g., STAT3). However, to date, no research has investigated the impacts of lysine on the immune organ's immune function in fish. In the amygdala in rats, lysine decreased the secretion of 5-hydroxytryptamine (Smriga et al., 2002). Meanwhile, in peritoneal macrophages, an associated increase in 5-hydroxytryptamine could be linked to an up-regulation in the activity of acid phosphatase (ACP) (Kondomerkos et al., 2003). Niijima et al. (1998) reported that lysine increased the vagal efferent firing rate to the thymus. In mice, stimulation of the vagus nerve activated intestinal macrophages *STAT3* (de Jonge et al., 2005). The above studies illustrate that there may be a probable correlation between lysine levels and the production of antibacterial compounds and inflammatory factors, as well as the related signaling pathway (*JAK/STAT*) of the immune organs in fish.

In recent years, nutritional immunology (especially essential amino acids) has obtained global concern (Li et al., 2007). So far, there have been only fragmentary studies concerning the impacts of essential amino acids on the immune organ's immune function in fish. Further, studies of essential amino acids on fish spleen and head kidney immunity have lacked systematic structure and depth. In fish, apart from the previous study on arginine and isoleucine from our lab (Chen et al., 2015; Zhao et al., 2013), most other studies have mainly focused on the contents of complement (C)3/C4 and the activity of lysozyme, and have not investigated the involved mechanisms (Giri et al., 2015; Machado et al., 2018). Moreover, previous research on immunity and its molecular mechanism between essential amino acids and the immune organs of fish was mainly focused on the gut and gills (Jiang et al., 2015; Luo et al., 2014). Simultaneously, the influences of essential amino acids on fish immune response may seem to vary across immune organs. For instance, in the immune response of gill, threonine played an important regulatory role on the c-rel signaling pathway, while in the intestine, threonine had no effect on this pathway (Dong et al., 2017, 2018). Besides, different amino acids may regulate the immune function of fish immune organs through different signaling pathways. For example, in the immune response of proximal intestine, phenylalanine played an important regulatory role on the nuclear factor (*NF*)-*κB* signaling pathway, however, leucine had no effect on this pathway (Feng et al., 2015; Jiang et al., 2015). Hence, we should lucubrate the effects of other essential amino acids on the immune organ's immune function in fish.

For the first time, this research was undertaken to investigate the impacts of lysine deficiency on the production of antibacterial compounds, inflammatory factors and the possible signaling of STAT signaling pathway, which might partially be used as theoretical evidence for the impacts of lysine on fish immune organ's immune response and molecular mechanisms. Grass carp is the major component of global aquaculture (Zhong et al., 2019). One previous study evaluated the lysine requirement in grown-up grass carp based on PWG alone (Li et al., 2016a,b). However, the requirement of essential amino acids (such as arginine) varies with different indices (Chen et al., 2015). Therefore, we also elevated the optimal lysine levels based on immune-related indicators, which will provide a theoretical basis for commercial diet preparation to ensure healthy aquaculture of grown-up grass carp.

2. Materials and methods

2.1. Feed preparation and experimental design

This project was approved by the Sichuan Agricultural Animal Care Advisory Committee. Tests were conducted in 2018 in Sichuan province Dayi County Hanchang test base town. Table 1 summarizes the formulation of the experimental diet. We evenly mixed all the ingredients, lysine premix, mineral premix, vitamin premix and proper water to produce pellets using experimental feed twin-screw. Six experimental diets with graded lysine concentrations of 5.8 (un-supplemented control), 8.8, 11.8, 14.8, 17.8 and 20.8 g/kg diet were formed by adding L-lysine hydrochlorideto to the basal diet. To make all diets iso-nitrogenous, as the method of Dong et al. (2017), we adjusted the contents of crystalline glycine and glutamic acid in the diets. The feeding stuff were stored at 4 °C until used, as the method of Dong et al. (2017). The lysine concentration in the prepared diets was measured to be 5.6, 8.5, 11.6, 14.4, 17.5 and 20.7 g/kg diet by using high-performance liquid chromatography, as the method of Li et al. (2013).

Table 1
Composition and nutrient contents of the basal diet (air-dry basis, %).

Ingredient[1]	Content	Nutrient content	Content
Fish meal	6.00	Crude protein[7]	28.74
Rice gluten meal	12.00	Crude lipid[7]	4.88
Gelatin	3.40	n-3[8]	1.04
Amino acid mix[2]	15.22	n-6[8]	0.96
Lysine premix[3]	5.00	Available phosphorus[9]	0.40
Corn starch	22.14		
Alpha-starch	22.00		
Fish oil	2.61		
Soya bean oil	1.12		
Trace mineral premix[4]	2.00		
Vitamin premix[5]	1.00		
$Ca(H_2PO_4)_2$	1.46		
Choline chloride premix [6]	1.00		
Microcrystalline cellulose	5.00		
Ethoxyquin (30%)	0.05		

[1] Ingredients were obtained from: cellulose (Linghu Xinwang Chemical Co., LTD., Huzhou, China); fish oil (CAI. Pesquera Camanchaca S.A., Santiago, Chile); gelatin (Henan zhongruo Biological Technology Co. LTD, Henan, China); corn starch and fish meal (Meishan Wangjiahao Feed co. LTD, Sichuan, China); soybean oil (National Golden dragon fish co. LTD, Sichuan, China); ethoxyquin, L-lysine hydrochlorideto, choline chloride, mineral premix, vitamin premix, $Ca(H_2PO_4)_2$ and α-starch (Animal Nutrition Institute, Sichuan Academy of Animal Science, China).

[2] Amino acid mix (g/kg diet): arginine, 2.19; histidine, 5.61; isoleucine, 3.36; leucine, 5.38; methionine, 3.06; cysteine, 1.20; phenylalanine, 4.12; tyrosine, 3.55; threonine, 7.10; tryptophan, 2.36; valine, 6.48; glutamic acid, 61.46; glycine, 46.33.

[3] L-lysine hydrochloride was added to obtain graded level of lysine. Per kilogram of lysine premix composition from diet 1–6 was as follows (g/kg): L -lysine hydrochloride 0.00, 76.40, 152.80, 229.4, 305.8, 382.2 and glycine 311.40, 249.00, 186.80, 124.60, 62.20, 0.00, respectively and corn starch 688.6, 674.60, 660.40, 646.00, 632.00, 617.8, respectively.

[4] Mineral premix (g/kg): $MnSO_4 \cdot H_2O$ (31.8% Mn), 2.6590; $MgSO_4 \cdot H_2O$ (15.0% Mg), 256.7933; $FeSO_4 \cdot H_2O$ (30.0% Fe), 12.6083; $ZnSO_4 \cdot H_2O$ (34.5%Zn), 8.8700; $CuSO_4 \cdot 5H_2O$ (25.0% Cu), 0.9560; Ca $(IO_3)_2$ (3.2% I), 1.5625; Na_2SeO_3 (44.7% Se), 0.0611. All ingredients were diluted with corn starch to 1 kg.

[5] Vitamin premix (g/kg): retinyl acetate (1,000,000 IU/g), 0.193; cholecalciferol (500,000 IU/g), 0.204; D, L-a-tocopherol acetate (50%), 23.23; me-nadione (96%), 1.979; thiamine nitrate (98%), 0.09; calcium-D-pantothenate (90%), 4.19; pyridoxine hydrochloride (98%), 0.45; cyanocobalamin (1%), 0.94; niacin (99%), 3.44; D-biotin (2%), 0.75; meso-inositol (97%), 28.53; folic acid (95%), 0.17; riboflavin (80%), 0.73; ascorhyl acetate (95%), 9.77. All ingredients were diluted with corn starch to 1 kg.

[6] Choline chloride premix: choline chloride (50%), 216.90. The ingredient was diluted with corn starch to 1 kg.

[7] Crude protein and crude lipid were measured values.

[8] n-3 and n-6 contents were referenced to Zeng et al. (2016) and calculated according to NRC (2011).

[9] Available phosphorus was referenced to Wen et al. (2015) and calculated according to NRC (2011).

After the fish were acquired from a local fishery (Sichuan, China), a 4 week acclimatized period in natural light cycle environment (approximately 11 h light, 13 h darkness) was undertaken, as described by Tie et al. (2019). Then, 540 healthy fish were kept in 18 (1.4 m × 1.4 m × 1.4 m) net cages (30 fish in each cage) and completely immersed in the outdoor tank. In addition, 18 net cages were randomly divided into 6 treatments, with 3 replicates per treatment. A tray was hung (1 m) in each cage to count the leftovers after 30 min of feeding according to the Tie et al. (2019). Simultaneously, fish were fed 4 times per day, as the method Zhong et al. (2019). During the period of the trial, water quality was taken daily (except for ammonia nitrogen content, which was measured once in 2 d). Water parameters were temperature 28.3 ± 3.2 °C, pH 7.69 ± 0.1, dissolved oxygen greater than 6.0 mg/L (6.11 ± 0.33). Feed replenished was determined by amount consumed the day before. After the 60 d growth trial, fish were starved for 1 d and then weighed to determine the growth parameters. After that, to determine the plasma ammonia content, 6 h after the last feeding, we collected the blood samples from the fish caudal vein, removed and stored the plasma, which was same as the method described in previous study of Chen et al. (2015). Twelve grass carps were caught stochastically from each treatment to euthanized by benzocaine (50 mg/L), which is based on Geraylou et al. (2012). After that, we quickly dissected the grass carp and took out the skin, spleen and head kidney for computation of the index of the spleen (SI) and head kidney (HKI) and assay the activities of glutamate-pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT) in the hepatopancreas. Finally, the skin, spleen and head kidney were soaked with liquid nitrogen and refrigerated at −80 °C based on Liu et al. (2019).

2.2. Challenge test

The injected bacterial number was determined by conducting a pre-experiment. We randomly put 90 healthy grown-up grass carp into 6 treatments. Fish were starved for 24 h, and then injected 1 mL *Aeromonas hydrophila* at concentrations of 0, 1.5×10^6, 1.5×10^7, 1.5×10^8, 1.5×10^9 and 1.5×10^{10} CFU/mL, respectively. Six days later, the skin of fish from each treatment was scored based on the hemorrhage and ulcerates as the method of Seguin et al. (2006). At the end of 60 d of feeding, 24 healthy fish of close weight from each group were administered an intraperitoneal injection of 1.0 mL 1.6×10^8 CFU/mL *A. hydrophila*. The dose was selected to induce distinct inflammation with 100% survival on the basis of our preliminary test, and the days relied on previous work Zhong et al. (2019). After the 6-d challenge test, the grass carp were euthanasia with a benzocaine bath (50 mg/L), and the skin, spleen and head kidney were removed immediately. Samples were kept at −80 °C until analysis, as the method of Wu et al. (2010). Our score of the skin was mainly based on the area and degree of skin bleeding and ulceration, which mainly occurred in the abdomen.

2.3. Histological examination

After sampling, the tissues (spleen, head kidney) of 3 fish were reserved in 4% paraformaldehyde and then embedded in paraffin, as discussed by Su et al. (2018). Four-micrometer thick sections were cut and fixed in fixatives for 24 h, and then dyed with hematoxylin and eosin (H & E), as discussed by Fischer et al. (2008). The histological sections were examined by a Nikon TS100 light microscope. The spleen and head kidney of 3 fish were scored based on the symptoms as the method of Liu et al. (2016). The specific details are listed as follows: (a) 10 images were chosen at random from each section in per treatment (b) standard for evaluation: not observed = 0, low frequency (1 to 3), moderate frequency (4 to 6) and high frequency (≥7).

2.4. Biochemical analysis

Referring to AOAC, we examined the approximate dietary composition, as the method of Horwitz et al. (1975). The activities of GPT and GOT in hepatopancreas, and plasma ammonia content were determined as discussed by Dong et al. (2017). We homogenized the samples of skin, spleen and head kidney in 10 volumes of ice-cold physiological saline and centrifuged at 6,000 × *g* for 20 min at 4 °C for the subsequent analysis on enzyme activity. Commercial test kits (Nanjing Jiancheng Bioengineering Institute) were used to detect immune organs immunoglobulin M (IgM), complement 4 (C4), complement 3 (C3), ACP and lysozyme levels, as the methods of Takemura et al. (1993), Yang et al. (2015) and Jiang et al. (2016), and the data were expressed as mean ± standard. The specific procedure was according to the manufacturer's guidelines.

2.5. Real-time quantitative PCR (RT-qPCR)

We used pre-sterilized and pre-cooled mortar to grind the sample (50 to 100 mg) into a fine powder, then added the sample powder to the 1.5 mL EP tube with TRIZOL added in advance. Next, we added chloroform and isopropanol to extract and precipitate total RNA, respectively, following the manufacturer's guidelines. We used 1% agarose gel to determine RNA quality (28S:18S rRNA bands was approximate 2:1). Whereafter, RNA was reverse transcribed using cDNA synthesis kit, as discussed by Torrecillas et al. (2015). Specific primers for DNA amplification are outlined in Table 2. According to the evaluation of our preliminary experiment, we used β-actin as a housekeeping gene, as discussed by He et al. (2019). The target and housekeeping gene were computed based on the dissolution curve generated from serial 10-fold serial dilution. As discussed by Livak et al. (2001), when amplification efficiency of the primer reached about 100%, we used $2^{-\Delta\Delta CT}$ method to process the result.

2.6. Western blot

The protein homogenates of skin, spleen and head kidney antibodies and western blotting were conducted as the method of Jiang et al. (2015). Shortly thereafter, we used a BCA assay kit (Beyotime Biotechnology Inc., China) to determine the concentrations of protein. SDS-PAGE were used to separate protein samples, and then shift to PVDF membrane, as discussed by Hemre et al. (2004). At room temperature, sealed PVDF membrane was blocked for 1 point 5 h in 5% BSA, and subsequently incubated with primary antibodies overnight at 4 °C. The p-STAT3 Tyr705 (ET1603-40, 1:1,000 dilution) antibodies were purchased from HUABIO. *STAT3* and β-actin antibodies were selected as the method of Hu et al. (2015). Finally, the PVDF membrane was incubated with a secondary antibody (A0208, 1:8,000) for 1.5 h. We selected ECL kits (Beyotime Biotechnology Inc.) to visualize immune complexes. Quantification of protein bands was determined by Image Lab (NIH, USA).

2.7. Statistical analysis

Data were analyzed by one-way ANOVA statistical analysis using SPASS 18.0 (SPSS Inc., Chicago, IL, USA) software, as discussed by Day et al. (1989). *P*-values < 0.05 were considered statistically significant. When significant differences were found ($P < 0.05$), Duncan's multiple range tests were applied to determined marked differences among 6 treatment groups. Data were presented as

Table 2
Real-time quantitative PCR primer sequences.

Target gene	Primer sequence, forward (5′→3′)	Primer sequence, reverse (5′→3′)	Temperature, °C	Accession number
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	59.3	JQ246442.1
LEAP-2A	TGCCTACTGCCAGAACCA	AATCGGTTGGCTGTAGGA	59.3	FJ390414
LEAP-2B	TGTGCCATTAGCGACTTCTGAG	ATGATTCGCCACAAAGGGG	59.3	KT625603.1
β-defensin-1	TTGCTTGTCCTTGCCGTCT	AATCCTTTGCCACAGCCTAA	58.4	KT445868.1
Mucin2	GAGTTCCCAACCCAACACAT	AAAGGTCTACACAATCTGCCC	60.4	KT625602
IL-1β	AGAGTTTGGTGAAGAAGAGG	TTATTGTGGTTACGCTGGA	57.1	JQ692172
IL-6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	62.3	KC535507.1
IL-8	ATGAGTCTTAGAGGTCTGGGT	ACAGTGAGGGCTAGGAGGG	60.3	JN663841
IL-12p35	TGGAAAAGGAGGGGAAGATG	AGACGGACGCTGTGTGAGTGTA	55.4	KF944667.1
IL-12p40	ACAAAGATGAAAAACTGGAGGC	GTGTGTGGTTTAGGTAGGAGCC	59.0	KF944668.1
IL-15	CCTTCCAACAATCTCGCTTC	AACACATCTTCCAGTTCTCCTT	61.4	KT445872.1
IL-17D	GTGTCCAGGAGAGCACCAAG	GCGAGAGGCTGAGGAAGTT T	62.3	KF245426.1
TNF-α	CGCTGCTGTCTGCTTCA	CCTGGTCCTGGTTCACTC	58.4	HQ696609
IFN-γ2	TGTTTGATGACTTTGGGATG	TCAGGACCCGCAGGAAGAC	60.4	JX657682
IL-4/13A	CTACTGCTCGCTTTCGCTGT	CCCAGTTTTCAGTTCTCTCAGG	55.9	KT445871.1
IL-4/13B	TGTGAACCAGACCCTACATAACC	TTCAGGACCTTTGCTGCTTG	55.9	KT625600.1
IL-10	AATCCCTTTGATTTTGCC	GTGCCTTATCCTACAGTATGTG	61.4	HQ388294
IL-11	GGTTCAAGTCTCTTCCAGCGAT	TGCGTGTTATTTTGTTCAGCCA	57.0	KT445870.1
TGF-β1	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	55.9	EU099588
TGF-β2	TACATTGACAGCAAGGTGGTG	TCTTGTTGGGGATGATGTAGTT	55.9	KM279716
JAK2	AGAGGCCATCGAGAGCTACT	TCATACGCCCCAACTGCAA	59.7	JF825474.1
TYK2	TTCGCCGTGTGTTTGCAAA	ACGCCAAAATGAGGAGCCA	59.7	KT724353.1
STAT1	TGGCACTTTGGAACCAGCT	AGGAGTTCATGGAGCGCA	59.5	KU508677.1
STAT3a	ACATTCCTGCTGCGCTTCA	ACGAGGATGTTGGTGGCAT	59.8	KC978890
STAT3b1	TCAACATGGCCCAGTGGAA	AGCGTTGCGTGAGATTCCT	59.4	KU559609
STAT3b2	TCAACATGGCCCAGTGGAA	AGCGTTGCGTGAGATTCCT	59.4	KU559610
TOR	TCCCACTTTCCACCAACT	ACACCTCCACCTTCTCCA	61.4	JX854449
β-actin	GGCTGTGCTGTCCCTGTA	GGGCATAACCCTCGTAGAT	61.4	M25013

LEAP-2 = liver expressed antimicrobial peptide 2; *IL* = interleukin; *TNF-α* = tumor necrosis factor α; *IFN-γ2* = interferon γ2; *TGF-β* = transforming growth factor β; *JAK2* = Janus kinase 2; *TYK2* = tyrosine kinase 2; *STAT* = signal transducers and activators of transcription; *TOR* = target of rapamycin.

mean ± SEM. A quadratic regression model was used to estimate the requirement of lysine level for grown-up grass carp, as described by Zhang et al. (2017).

SI (%) = [Spleen weight (g)/Body weight (g)] × 100;

HKI (%) = [Head kidney weight (HKW, g)/Body weight (g)] × 100;

Specific growth rate (SGR, %/d) = ln [Final body weight (FBW, g/fish)/Initial body weight (IBW, g/fish)]/days × 100;

Feed efficiency (FE, %) = [FBW (g/fish) − IBW (g/fish)]/Feed intake (FI, g/fish) × 100;

Percent weight gain (PWG, %) = [FBW (g/fish) − IBW (g/fish)]/IBW (g/fish) × 100.

The above calculation methods are as described by Du et al. (2005), Xu et al. (2016), Shi et al. (2017) and Pan et al. (2017). Values are means ± SD for 3 replicate groups, with 30 fish in each group, and different superscripts in the same row are significantly different ($P < 0.05$).

3. Results

3.1. The growth performance and immune organs growth of grown-up grass carp

Growth performance related indices are summarized in Table 3, the FE, FI, FBW, SGR, PWG, SI, HKI, HKW and spleen weight were all markedly increased during the starter period ($P < 0.05$), and then began declining. Based on the PWG, skin lesion morbidity and the activities of lysozyme in the spleen and head kidney for grown-up grass carp, the requirements of lysine were estimated to be 13.58, 13.51, 14.56 and 14.18 g/kg diet (Fig. 1 and Table 4), respectively. In the hepatopancreas, significant differences were found among groups in GOT and GPT activities, the GPT activity markedly increased when lysine compositions ranged from 5.6 to 11.6 g/kg of the diet ($P < 0.05$), and after that became declining, and the GOT activity markedly increased when lysine compositions ranged from 5.6 to 14.4 g/kg of the diet ($P < 0.05$), then became declining. Additionally, the plasma ammonia content markedly decreased when lysine compositions ranged from 5.6 to 14.4 g/kg of the diet ($P < 0.05$), and after that became increasing (Table 5).

3.2. The histologic changes of immune organs in grown-up grass carp

This research indicated that lysine deficiency markedly increased ($P < 0.05$) the skin lesion morbidity and hemorrhage in grown-up grass carp (Fig. 2). As exhibited in Fig. 1B, the estimated optimal dietary lysine requirement was 13.51 g/kg diet (47.01 g/kg of dietary protein) in grown-up grass carp, which was based on skin lesion morbidity by quadratic regression model. In addition, in the head kidney, endothelial cell swelling and defluxion, blood vessel wall incrassating, increased macrophage numbers and extravasated blood were observed in fish fed 5.6 g/kg lysine diets (Fig. 3A), and endothelial cell swelling and defluxion, blood vessel wall incrassating and haemorrhage were observed in fish fed 20.7 g/kg lysine diets (Fig. 3C). In the spleen, the haemorrhage, increased macrophage numbers and vacuolization were observed in fish fed 5.6 g/kg lysine diets (Fig. 3D), and haemorrhage, increased macrophage numbers and endothelial cell swelling and defluxion were observed in fish fed 20.7 g/kg lysine diets (Fig. 3F). We provided the evaluation scores for morphological changes in Table 6.

Table 3
Growth performance, head kidney and spleen weight, index of grown-up grass carp (*Ctenopharyn-godon idellus*) fed diets containing graded levels of lysine for 60 d.

Item	Dietary lysine level, g/kg diet					
	5.6	8.5	11.6	14.4	17.5	20.7
IBW[1], g/fish	164.33 ± 0.33[a]	164.89 ± 1.07[a]	165.00 ± 0.88[a]	165.44 ± 0.38[a]	164.89 ± 1.02[a]	164.56 ± 1.02[a]
FBW[1], g/fish	403.33 ± 3.53[a]	485.33 ± 3.72[c]	581.33 ± 4.00[f]	549.78 ± 13.23[e]	515.33 ± 5.92[d]	442.22 ± 10.12[b]
PWG[1], %	144.20 ± 2.11[a]	192.62 ± 3.36[c]	250.01 ± 2.80[f]	230.21 ± 8.24[e]	210.59 ± 4.13[d]	167.22 ± 5.56[b]
SGR[1], %/d	1.49 ± 0.01[a]	1.79 ± 0.02[c]	2.09 ± 0.01[f]	1.99 ± 0.04[e]	1.89 ± 0.02[d]	1.64 ± 0.03[b]
FI[1], g/fish	454.47 ± 0.30[a]	527.02 ± 0.28[c]	606.50 ± 0.25[f]	579.18 ± 0.30[e]	541.55 ± 0.26[d]	471.06 ± 0.30[b]
FE[1], %	0.52 ± 0.01[a]	0.61 ± 0.01[b]	0.68 ± 0.01[d]	0.66 ± 0.02[cd]	0.65 ± 0.01[c]	0.59 ± 0.02[b]
HKW[2], g	0.71 ± 0.01[a]	0.96 ± 0.05[b]	1.29 ± 0.06[d]	1.23 ± 0.02[d]	1.05 ± 0.04[c]	0.88 ± 0.06[b]
HKI[2], %	0.18 ± 0.00[a]	0.20 ± 0.01[ab]	0.22 ± 0.02[cd]	0.23 ± 0.00[d]	0.21 ± 0.01[bc]	0.20 ± 0.01[ab]
SW[2], g	0.55 ± 0.03[a]	0.80 ± 0.03[b]	1.00 ± 0.02[c]	0.99 ± 0.08[c]	0.82 ± 0.03[b]	0.63 ± 0.01[a]
SI[2], %	0.14 ± 0.01[a]	0.17 ± 0.01[cd]	0.17 ± 0.02[cd]	0.18 ± 0.01[d]	0.16 ± 0.00[bc]	0.14 ± 0.00[ab]
Regressions						
$Y_{PWG} = -1.495X^2 + 40.617X - 36.919$					$R^2 = 0.970$	$P < 0.05$
$Y_{SGR} = -0.008X^2 + 0.231X + 0.463$					$R^2 = 0.956$	$P < 0.01$
$Y_{FE} = -0.211X^2 + 5.916X + 26.061$					$R^2 = 0.957$	$P < 0.01$
$Y_{HKI} = -0.001X^2 + 0.018X + 0.096$					$R^2 = 0.909$	$P < 0.05$
$Y_{SI} = -0.001X^2 + 0.019X + 0.055$					$R^2 = 0.933$	$P < 0.05$

IBW = Initial body weight; FBW = final body weight; PWG = percent weight gain; SGR = specific growth rate; FI = feed intake; FE = feed efficiency; HKW = head kidney weight; HKI = head kidney index; SW = spleen weight; SI = spleen index.
[a to f] Values with different letter superscripts are significantly different ($P < 0.05$).
[1] Values are means ± SD for 3 replicate groups, with 30 fish in each group.
[2] Values are means ± SD ($n = 12$).

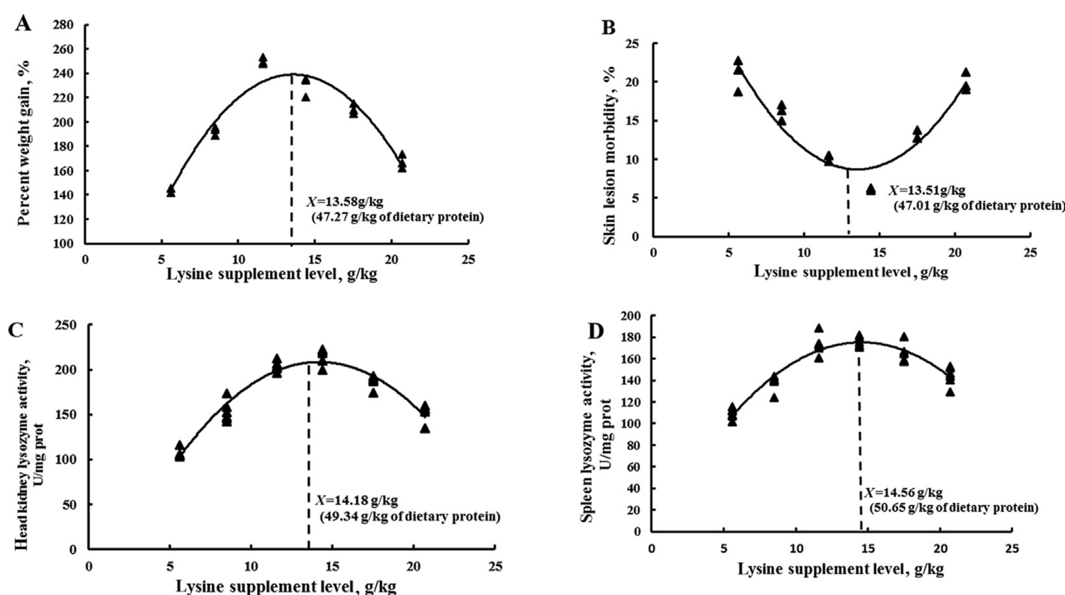

Fig. 1. Quadratic regression analysis of percent weight gain (PWG) (A), skin lesion morbidity (B) and lysozyme activity in head kidney (C) and spleen (D) for grown-up grass carp fed diets containing graded levels of lysine (g/kg) for 60 d.

3.3. The immune function of immune organs in grown-up grass carp

3.3.1. IgM, C4 and C3 contents, ACP and lysozyme activities in the skin, spleen and head kidney

As exhibited in Table 7, the levels of production of IgM, C4 and lysozyme in the skin, spleen and head kidney of grown-up grass carp increased as dietary lysine level increased to 14.4 g/kg ($P < 0.05$), after which, they became declining. The contents of C3 in the head kidney and spleen increased with dietary lysine supplements up to 14.4 g/kg ($P < 0.05$), and the content of C3 in the skin increased with dietary lysine supplements up to 11.6 g/kg ($P < 0.05$), then all decreased. In the skin and spleen, as lysine supplements neared 11.6 g/kg, the activities of ACP were decreased

Table 4
The optimal dietary lysine levels based on different indices for grown-up grass carp (*Ctenopharyngodon idella*).

Item	Regressive equation	R^2	*P*-value	Optimal dietary lysine level, g/kg
PWG	$Y = -1.495X^2 + 40.618X - 36.922$	0.931	<0.05	13.58
Skin lesion morbidity	$Y = 0.215X^2 - 5.81X + 47.947$	0.888	<0.05	13.51
Head kidney lysozyme activity	$Y = -1.435X^2 + 40.7X - 80.103$	0.936	<0.01	14.18
Spleen lysozyme activity	$Y = -0.849X^2 + 24.717X - 4.611$	0.899	<0.01	14.56

Table 5
Glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) activities in the hepatopancreas and plasma ammonia contents of grown-up grass carp (*Ctenopharyngodon idella*) fed with diets containing graded levels of lysine for 60 d.[1]

Item	Dietary lysine level, g/kg diet					
	5.6	8.5	11.6	14.4	17.5	20.7
GOT activities in hepatopancreas, U/g tissue	1,733.90 ± 82.87[a]	1,968.90 ± 23.41[c]	2,146.61 ± 43.95[d]	2,390.82 ± 68.24[e]	1,945.12 ± 49.07[c]	1,816.86 ± 36.88[b]
GPT activities in hepatopancreas, U/g tissue	577.81 ± 14.90[b]	605.94 ± 21.83[c]	635.98 ± 13.47[d]	611.26 ± 42.16[cd]	559.26 ± 15.24[b]	514.83 ± 16.16[a]
Plasma ammonia contents, μmol/L	236.38 ± 16.89[c]	197.24 ± 10.95[b]	151.88 ± 12.47[a]	150.79 ± 8.02[a]	192.77 ± 14.39[b]	220.48 ± 20.49[c]
Regressions						
$Y_{\text{GOT activity in the hepatopancreas}} = -8.394X^2 + 225.544X + 708.163$					$R^2 = 0.798$	$P = 0.091$
$Y_{\text{GPT activity in the hepatopancreas}} = -1.326X^2 + 31.224X + 450.609$					$R^2 = 0.951$	$P < 0.05$
$Y_{\text{Plasma ammonia contents}} = -1.330X^2 - 35.768X + 396.743$					$R^2 = 0.927$	$P < 0.05$

[a to d] Values with different letter superscripts are significantly different ($P < 0.05$).
[1] Values are means ± SD ($n = 6$).

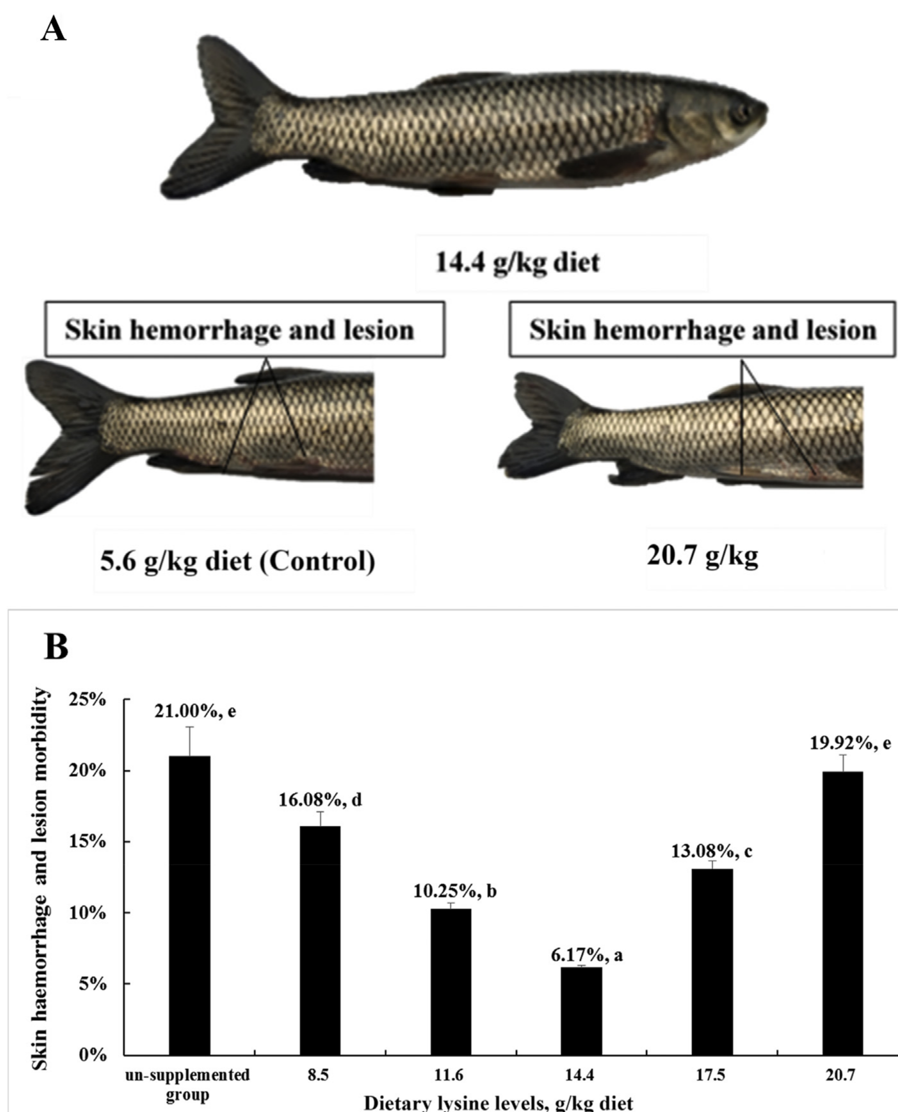

Fig. 2. Effects of dietary lysine (g/kg) on the skin hemorrhage and lesion morbidity of grown-up grass carp (*Ctenopharyngodon idella*) after infection of *Aeromonas hydrophila*. (A) Low or excess levels of dietary lysine led to obvious skin haemorrhage and lesions, compared to optimal dietary lysine level in on-growing grass carp (*Ctenopharyngodon idella*). (B) The skin haemorrhage and lesion morbidity in grown-up grass carp (*Ctenopharyngodon idella*). Data represent means of 24 fish in each group, error bars indicate S.D. All values are presented as the means ± SD, ($n = 3$ replicates, and each replicate with 8 fish). Values marked with different letters (a to e) are significantly different ($P < 0.05$).

($P < 0.05$), after that became increasing ($P < 0.05$). In the head kidney, the activities of ACP were decreased with lysine levels up to 14.4 g/kg ($P < 0.05$), after that became increasing.

3.3.2. Cytokines, STAT1, STAT3 gene expression and STAT3 protein expression in the skin, spleen and head kidney

Compared to control group (5.6 g/kg lysine diet), the β-defensin-1, hepcidin, mucin*2* and antimicrobial peptide (*LEAP*)-*2A* mRNA

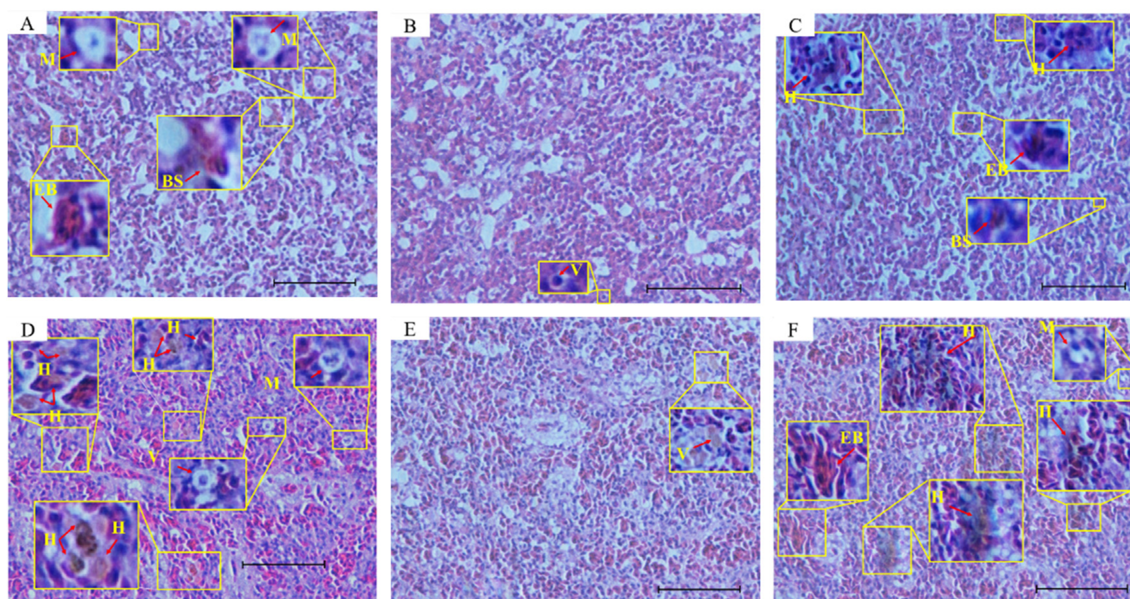

Fig. 3. The histological observation in the head kidney and spleen of grown-up grass carp (*Ctenopharyngodon idella*) fed diets containing graded levels of lysine for 60 d, and then infected with *Aeromonas hydrophila* for 6 d. (A) The head kidney of un-supplemented group. (B) The head kidney of 14.4 g/kg diet group. (C) The head kidney of 20.7 g/kg diet group. (D) The spleen of un-supplemented group. (E) The spleen of 14.4 g/kg diet group. (F) The spleen of 20.7 g/kg diet group. V: vacuolization; H: hemosiderin; BS: blood vessel wall incrassating and endothelial cell swelling and defluxion; M: macrophage; EB: extravasated blood. The sections were stained with haematoxylin and eosin (H & E) and observed at 400× magnification.

Table 6
Head kidney and spleen morphological changes in different groups of grown-up grass carp fed diets containing different levels of lysine for 60 d after infection with the *Aeromonas hydrophila* for 6 d.[1]

Item	Dietary lysine levels, g/kg diet		
	5.6	14.4	20.7
Head kidney			
Macrophage numbers	1	0.33	0.67
Blood vessel wall incrassating	1.3	0	1.33
Endothelial cell swelling and defluxion	0.67	0	0.67
Haemorrhage	0.67	0	0.33
Extravasated blood	0.67	0	0.67
Vacuolization	0.33	0.33	1
Column totals	4.64[b]	0.66[a]	4.67[b]
Spleen			
Macrophage numbers	1.67	0	1
Blood vessel wall incrassating	0.33	0.33	0.67
Endothelial cell swelling and defluxion	1	0.33	0.67
Haemorrhage	1.33	0.33	1.67
Extravasated blood	1	0.67	0.67
Vacuolization	0.67	0.33	1
Column totals	6[b]	1.99[a]	5.68[b]

[a, b] Values with different letter superscripts are significantly different ($P < 0.05$).
[1] Values are means ($n = 3$).

levels of skin, spleen and head kidney were significantly enhanced when lysine compositions ranged from 5.6 to 14.4 g/kg of the diet ($P < 0.05$), after that became declining. In the spleen and head kidney, the *LEAP-2B* gene expressions were significantly increased in the fish fed 14.4 g/kg diet as compared to control group ($P < 0.05$), and then decreased. The gene expression of *LEAP-2B* in the skin of fish was markedly increased as lysine levels were raised to 11.6 g/kg ($P < 0.05$), and after that became declining (Fig. 4).

Relative to the control group, optimal lysine level markedly decreased the gene expressions of tyrosine kinase 2 (*TYK2*), *STAT1*, interleukin (*IL*)*-17D*, *IL-15*, *IL-12p40*, interferon γ2 (*IFN-γ2*), tumor necrosis factor α (*TNF-α*), *IL-8*, *IL-6* and *IL-1β* and markedly increased the gene expressions of *JAK2*, *STAT3b1*, *STAT3b2*, target of rapamycin (*TOR*), transforming growth factor (*TGF*)*-β2*, *TGF-β1*, *IL-11*, *IL-4/13A* and *IL-10* in the 3 immune organs of fish ($P < 0.05$). Meanwhile, lysine deficiency showed no influence on the gene expressions of *STAT3a*, *IL-12p35* and *IL-4/13B* in the 3 immune organs of grown-up grass carp ($P > 0.05$).

Relative to the control group, the STAT3 Tyr705 protein levels of spleen, head kidney and skin were significantly enhanced when lysine compositions ranged from 5.6 to 14.4 g/kg of the diet ($P < 0.05$), after that became declining ($P < 0.05$) (Fig. 5).

4. Discussion

4.1. Lysine deficiency deteriorated growth performance of fish

In our study, we found that lysine deficiency decreased the growth performance (such as FE, FI and PWG), immune organs size (such as spleen weight and head kidney weight) and the amino utilization (indicated by the decreases the GPT and GOT activities in the hepotopancreas, as well as increase of plasma ammonia content) in fish. The dietary lysine requirement was estimated to be 47.27 g/kg of dietary protein for maximum of PWG. This value was similar to our previous research for on-grown grass carp, which was 47.30 g/kg of dietary protein (Li et al., 2016a,b). Additionally, previous studies have indicated that challenge with *A. hydrophila* could lead to histopathological change (Abdelhamed et al., 2017) and skin haemorrhage and lesions in fish (Wang et al., 2018).

4.2. Lysine deficiency aggravated skin haemorrhage and lesion and histopathological damage in fish

In fish, skin plays a significant role in the mucosal immune system (Lazado et al., 2014). There is no research until now that has investigated the impacts of lysine deficiency on skin lesions in fish. The optimal lysine markedly decreased the lesion morbidity in

Table 7
Effects of graded levels of lysine on immune related parameters in the spleen, head kidney and skin of grown-up grass carp (*Ctenopharyngodon idella*).[1]

Item	Dietary lysine levels, g/kg diet					
	5.6	8.5	11.6	14.4	17.5	20.7
Spleen						
Lysozyme, U/mg protein	109.21 ± 4.79^{a}	137.97 ± 7.13^{b}	173.06 ± 8.93^{d}	176.40 ± 4.34^{d}	164.18 ± 9.02^{c}	144.29 ± 8.65^{b}
ACP, U/mg protein	184.69 ± 5.96^{abc}	183.19 ± 17.43^{abc}	170.46 ± 9.99^{a}	173.88 ± 13.58^{ab}	186.35 ± 11.64^{bc}	191.23 ± 9.20^{c}
C3, mg/g protein	29.49 ± 2.81^{a}	33.62 ± 1.46^{b}	40.38 ± 3.35^{de}	42.32 ± 2.49^{e}	37.77 ± 3.59^{cd}	35.47 ± 2.36^{bc}
C4, mg/g protein	8.17 ± 0.28^{a}	10.12 ± 0.79^{b}	13.45 ± 1.11^{de}	14.18 ± 1.25^{e}	12.76 ± 1.20^{cd}	12.07 ± 0.65^{c}
IgM, mg/g protein	42.72 ± 3.59^{a}	57.99 ± 5.38^{b}	68.56 ± 4.22^{cd}	72.19 ± 4.26^{d}	70.09 ± 3.04^{cd}	65.61 ± 2.64^{c}
Head kidney						
Lysozyme, U/mg protein	106.40 ± 4.78^{a}	153.10 ± 11.42^{b}	203.72 ± 5.26^{d}	213.57 ± 8.55^{d}	186.95 ± 6.53^{c}	149.08 ± 11.43^{b}
ACP, U/mg protein	194.62 ± 9.49^{ab}	184.40 ± 10.27^{a}	182.88 ± 6.83^{a}	179.39 ± 10.59^{a}	194.54 ± 12.96^{ab}	203.61 ± 18.14^{b}
C3, mg/g protein	18.08 ± 1.41^{a}	19.83 ± 1.81^{a}	25.80 ± 1.79^{cd}	26.45 ± 1.70^{d}	24.00 ± 1.66^{c}	21.84 ± 1.48^{b}
C4, mg/g protein	8.33 ± 0.54^{a}	10.09 ± 0.46^{b}	12.45 ± 0.80^{c}	12.63 ± 0.93^{c}	9.44 ± 0.29^{b}	8.43 ± 0.51^{a}
IgM, mg/g protein	46.43 ± 4.15^{a}	55.01 ± 4.05^{b}	61.93 ± 4.61^{c}	62.15 ± 4.78^{c}	60.57 ± 3.81^{c}	58.66 ± 4.28^{bc}
Skin						
Lysozyme, U/mg protein	86.54 ± 5.67^{a}	90.18 ± 8.23^{ab}	113.09 ± 9.49^{c}	115.52 ± 8.91^{c}	95.94 ± 1.98^{b}	87.32 ± 2.89^{a}
ACP, U/mg protein	171.88 ± 12.97^{b}	170.11 ± 13.18^{b}	152.59 ± 13.26^{a}	172.65 ± 12.76^{b}	184.46 ± 16.89^{bc}	192.99 ± 6.64^{c}
C3, mg/g protein	21.37 ± 1.14^{a}	24.62 ± 1.18^{c}	28.48 ± 2.39^{d}	28.31 ± 0.95^{d}	23.41 ± 1.45^{bc}	21.97 ± 2.12^{ab}
C4, mg/g protein	4.85 ± 0.41^{a}	6.12 ± 0.29^{b}	7.90 ± 0.52^{d}	8.00 ± 0.26^{d}	6.61 ± 0.42^{c}	5.81 ± 0.33^{b}
IgM, mg/g protein	71.75 ± 7.69^{a}	92.40 ± 4.32^{b}	122.93 ± 5.54^{d}	123.90 ± 7.51^{d}	104.90 ± 10.03^{c}	90.19 ± 8.86^{b}

ACP = acid phosphatase; C = complement; IgM = immunoglobulin M.
[a to e] Values with different letter superscripts are significantly different ($P < 0.05$).
[1] Values are means ± SD ($n = 6$).

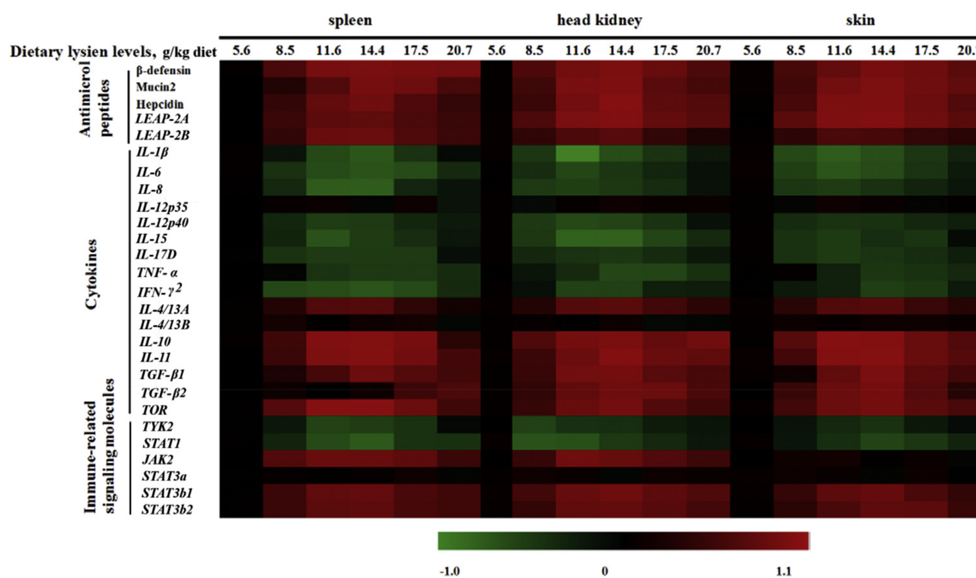

Fig. 4. Heat-map of the mRNA levels of antimicrobial peptides, cytokines, STAT signalling pathway-related molecules in the spleen, head kidney and skin of grown-up grass carp fed graded levels of lysine. *LEAP-2* = liver expressed antimicrobial peptide 2; *IL* = interleukin; *TNF-α* = tumor necrosis factor α; *IFN-γ2* = interferon γ2; *TGF-β* = transforming growth factor β; *TOR* = target of rapamycin; *TYK2* = tyrosine kinase 2; *STAT* = signal transducers and activators of transcription; *JAK2* = Janus kinase 2. The values of upregulation (red) and downregulation (green) (i.e. from 1.1 to −1.0) represent log2 fold changes, as compared to group 1. $n = 6$ for each lysine level. The fold changes is based on the mRNA expression levels of different genes.

grown-up grass carp, which is in relation to reduced immune organ's immune function and histopathological changes in fish (He et al., 2019). However, no previous research had successfully been conducted to study the impacts of lysine deficiency on histopathological damage in the spleen and head kidney of fish. Our results showed that lysine deficiency impaired the structure of mucosal immune organ (such as skin) and systemic immune organs (such as spleen and head kidney). Guo et al. reported that the integrity of the immune organ structure guarantees its capacity of immune response (Guo et al., 2018).

4.3. Lysine deficiency reduced immune response of immune organs in fish

Research in our lab shows that antibacterial compounds (such as immunoglobulins, antibacterial peptides, complement system, ACP and lysozyme) play crucial roles in the immune organ's immune function in fish (Zheng et al., 2018). However, to date, no research involving the impacts of lysine on immune function of immune organs was reported in fish. Our research is the first to show that lysine deficiency restrained the immune response of immune

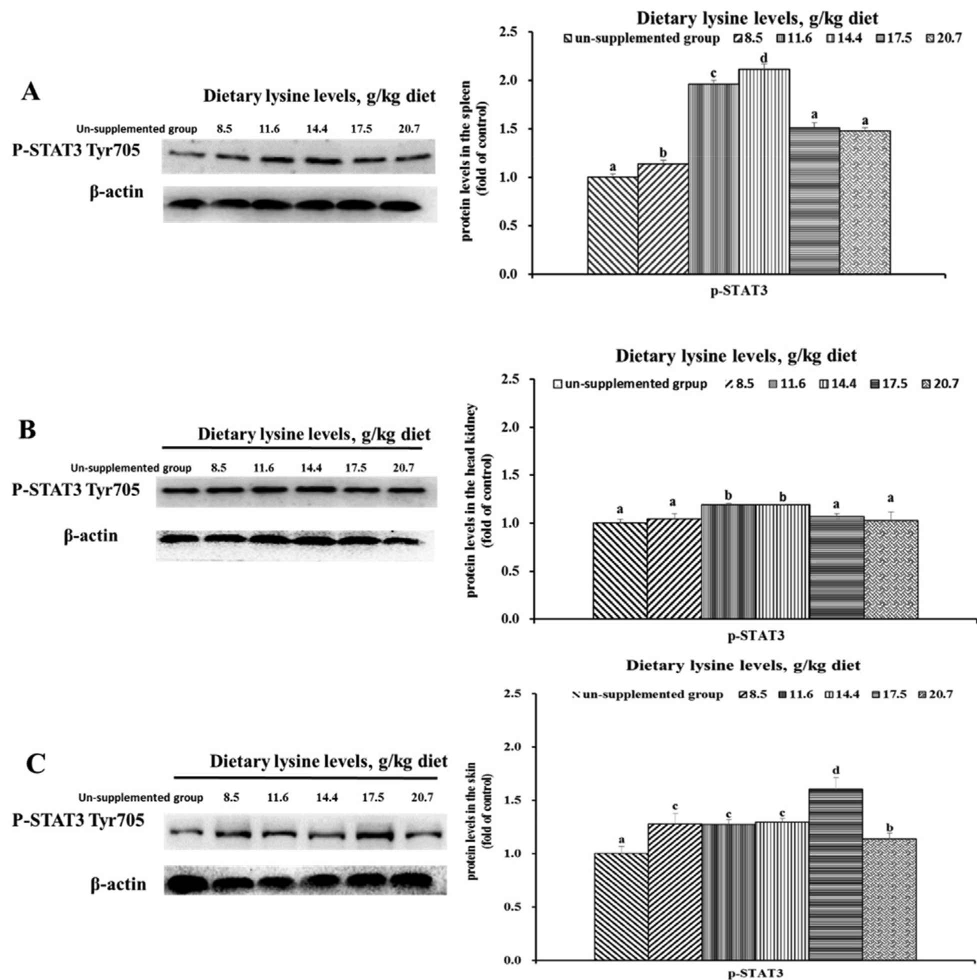

Fig. 5. Western blot analysis of signal transducers and activators of transcription 3 (STAT3) phosphorylation at Tyr705 proteins in the spleen (A), head kidney (B) and skin (C) of grown-up grass carp fed diets containing graded levels of lysine for 60 d, and then infected with *Aeromonas hydrophila* for 6 d. Data represent means ($n = 3$ replicates in each group), error bars indicate SD. All values are presented as the means ± SD. Values marked with different letters (a to d) are significantly different ($P < 0.05$).

organs in fish. Simultaneously, based on the activity of lysozyme in the head kidney for grown-up grass carp (Fig. 1C), the dietary lysine requirement was estimated to be 14.18 g/kg diet (49.34 g/kg protein of diet), which was higher than the optimal lysine requirement for growth (47.27 g/kg). This phenomenon might be explained by the requirement of more lysine to accomplish the immune response in fish, as indicated by Chen et al. (2015).

However, we found that lysine deficiency increased the ACP activity in the skin, spleen and head kidney of grown-up grass carp, which is in relation to inducible nitric oxide synthase (iNOS). Carter et al. (2004) reported that lysine deficiency raised iNOS activity in neonatal pigs, and Chen et al. (2015) reported that iNOS could increase the production of nitric oxide (NO) in Jian carp. One study reported that NO could increase ACP activity in mice macrophages (Garica et al., 2000). These results support our hypothesis and need further research. In addition, the inflammatory response is also closely linked to the immune response of immune organs in fish (Wang et al., 2018).

4.4. Lysine deficiency aggravated inflammatory response referring to STAT signaling pathway in the head kidney, spleen and skin of fish

Morimoto et al. (2016) observed that a decrease in pro-inflammatory cytokines and an increase in the anti-inflammatory cytokines gene expressions could attenuate inflammatory response of immune organs in fish. However, no prior research had successfully been studied on the impacts of lysine deficiency on inflammatory response of immune organs in fish. The present research was for the first time investigating that lysine deficiency elevated the gene levels of pro-inflammatory cytokines (except for *IL-12p40*) and decreased the gene levels of anti-inflammatory (except for *IL-4/13B*) in the skin, spleen and head kidney of grown-up grass carp. All the above results indicated that lysine deficiency aggravated the inflammatory response of immune organs in fish.

We found that lysine deficiency only increased the gene expression of *IL-4/13A* (rather than *IL-4/13B*) and *IL-12p40* (rather than *IL-12p35*) in the spleen, head kidney and skin of grown-up grass carp. The different results and probable causes are discussed as follows. First, one previous study indicated that *IL-1β* could increase the transcript levels of *IL-12p40*, but not *IL-12p35* in Atlantic salmon (*Salmo salar*) HK cells (Wang et al., 2014). Simultaneously, the present study indicated that lysine deficiency increased the gene expressions of *IL-1β* in the skin, spleen and head kidney of grown-up grass carp, supporting our assumption. Second, one study observed that the activation of mammalian target of rapamycin (mTOR) could enhance GATA binding protein 3 (*GATA-3*) mRNA levels in mice CD4^{+} T cells (Cook et al., 2010). Ohtani et al.

Table 8
Correlation coefficient of parameters in the head kidney, spleen and skin.

Independent parameters	Dependent parameters	Spleen		Head kidney		Skin	
		Correlation coefficients	*P*-value	Correlation coefficients	*P*-value	Correlation coefficients	*P*-value
STAT1	*IL-1β*	0.858	<0.05	0.392	0.442	0.768	0.075
	IL-6	0.942	<0.01	0.910	<0.05	0.782	0.066
	IL-8	0.902	<0.05	0.976	<0.0.1	0.689	0.130
	IL-12p40	0.944	<0.01	0.947	<0.01	0.839	<0.05
	IL-15	0.901	<0.05	0.864	<0.05	0.689	0.130
	IL-17D	0.770	0.073	0.896	<0.05	0.805	0.053
	TNF-α	0.832	<0.05	0.438	0.384	0.906	<0.05
	IFN-γ2	0.887	<0.05	0.627	0.183	0.963	<0.01
	TYK2	0.817	<0.05	0.980	<0.01	0.831	<0.05
STAT3b1	*IL-10*	0.914	<0.05	0.968	<0.01	0.984	<0.01
	IL-11	0.967	<0.01	0.981	<0.01	0.987	<0.01
	IL-4/13A	0.905	<0.0.5	0.952	<0.01	0.994	<0.01
	TGF-β1	0.889	<0.05	0.984	<0.01	0.902	<0.05
	TGF-β2	0.109	0.837	0.971	<0.01	0.985	<0.01
	JAK2	0.960	<0.01	0.968	<0.01	0.767	0.075
STAT3b2	*IL-10*	0.909	<0.05	0.959	<0.01	0.905	<0.05
	IL-11	0.964	<0.01	0.960	<0.01	0.934	<0.01
	IL-4/13A	0.897	<0.05	0.959	<0.01	0.893	<0.05
	TGF-β1	0.896	<0.05	0.990	<0.01	0.920	<0.01
	TGF-β2	0.124	0.815	0.940	<0.01	0.903	<0.05
	JAK2	0.961	<0.01	0.982	<0.01	0.748	0.087
STAT3 protein level	*IL-10*	0.855	<0.05	0.855	<0.05	0.626	0.183
	IL-11	0.910	<0.05	0.910	<0.05	0.595	0.213
	IL-4/13A	0.917	<0.05	0.917	<0.05	0.558	0.250
	TGF-β1	0.884	<0.05	0.884	<0.05	0.522	0.289
	TGF-β2	–	–	–	–	0.636	0.175
	JAK2	0.782	0.066	0.782	0.066	0.508	0.303

STAT = signal transducers and activators of transcription; *IL* = interleukin; *TNF-α* = tumor necrosis factor α; *IFN-γ2* = interferon γ2; *TYK2* = tyrosine kinase 2; *TGF-β* = transforming growth factor β; *JAK2* = Janus kinase 2.

(2008) found that the presence of a *GATA-3* binding motif in teleost *IL-4/13A* (rather than *IL-4/13B*) gene promoters. Our result showed that lysine deficiency depressed the transcript levels of *TOR* in the skin, spleen and head kidney of grown-up grass carp, supporting our hypothesis. Additionally, cytokines transcription was closely related to *STAT* signaling pathway in humans (Coskun et al., 2013).

STAT can be activated by the upstream signaling molecule Janus kinases (e.g., TYK2, JAK2) (Coskun et al., 2013) (de Jonge et al., 2005). One study observed that activated STAT1 could aggravate inflammatory responses in mice macrophages (Lu et al., 2014). In this study, we found that lysine deficiency increased the gene expressions of *TYK2* and *STAT1* in the skin, spleen and head kidney of fish. The results of correlation analysis (Table 8) indicated that pro-inflammatory cytokines (*IFN-γ2*, *IL-17D*, *IL-15*, *IL-12p40*, *IL-6* and *IL-8*) were positively related to the gene expressions of *STAT1* in the spleen and head kidney of fish. Our data showed that a strengthened pro-inflammatory effect of lysine deficiency was likely to be relevant to TYK2/STAT1 signaling in the skin, spleen and head kidney of fish.

One study observed that *STAT3* signaling pathway serves a vital function in promoting the gene expressions of anti-inflammatory cytokines in humans (Murray et al., 2006). In humans, phosphorylation of STAT3 on Tyr705 proteins is routinely performed to detect the activated STAT3 signaling pathway (Schuringa et al., 2000). There is no research until now that has investigated the impacts of lysine deficiency on *STAT3* signaling pathway. We first investigated that lysine deficiency down-regulated the gene expressions of *JAK2*, *STAT3b1*, *STAT3b2* (rather than *STAT3a*), and p-STAT3 Tyr705 protein levels in the skin, spleen and head kidney, of grown-up grass carp (Fig. 5). The results of correlation analysis (Table 8) indicated that there was a positive correlation between anti-inflammatory cytokines (TGF-β2, TGF-β1, IL-4/13A, IL-11 and IL-10) and the p-STAT3 Tyr705 protein levels in the spleen and head kidney of grown-up grass carp. Our data showed that a weakened anti-inflammatory effect of lysine deficiency may be partly related to JAK2/STAT3 signaling in the skin, spleen and head kidney of grown-up grass carp.

We found that lysine deficiency decreased the gene expressions of *STAT3b1*, *STAT3b2* (rather than *STAT3a*) in the skin, spleen and head kidney of grown-up grass carp, which may be related to arginine and colony stimulating factor. In chickens, lack of lysine can inhibit the utilization of arginine (Fisher et al., 1960). Rutherford reported that arginine deficiency can inhibit granulocyte macrophage colony stimulating factor activity in mice macrophages (Rutherford et al., 1992). Simultaneously, in human myeloid cells, macrophage colony stimulating factor could activate STAT3b (rather than STAT3a) (Chakraborty et al., 1996). Therefore, we speculated that lysine deficiency down-regulated *STAT3b1/b2* rather than *STAT3a* gene expressions in the skin, spleen and head kidney may be caused by the decrease of arginine utilization and the activity of colony stimulating factor.

4.5. Lysine excess impaired growth performance and deteriorated immune function of immune organs in fish

Excess lysine impaired the growth performance, immune response and aggravated inflammatory response. The particular mechanisms are displayed as follows. First, the poor growth performance resulting from and excess of lysine might be partly explained by the fact that excess lysine inhibits the absorption of other amino acids, thereby affecting protein synthesis. For example, a previous study indicated that lysine and arginine utilize the same transport mechanism for getting into the cells, thus, excessive lysine could decrease intracellular availability of arginine (Wu et al., 2002). Second, the immune response is connected with the structural integrity and the development and growth of

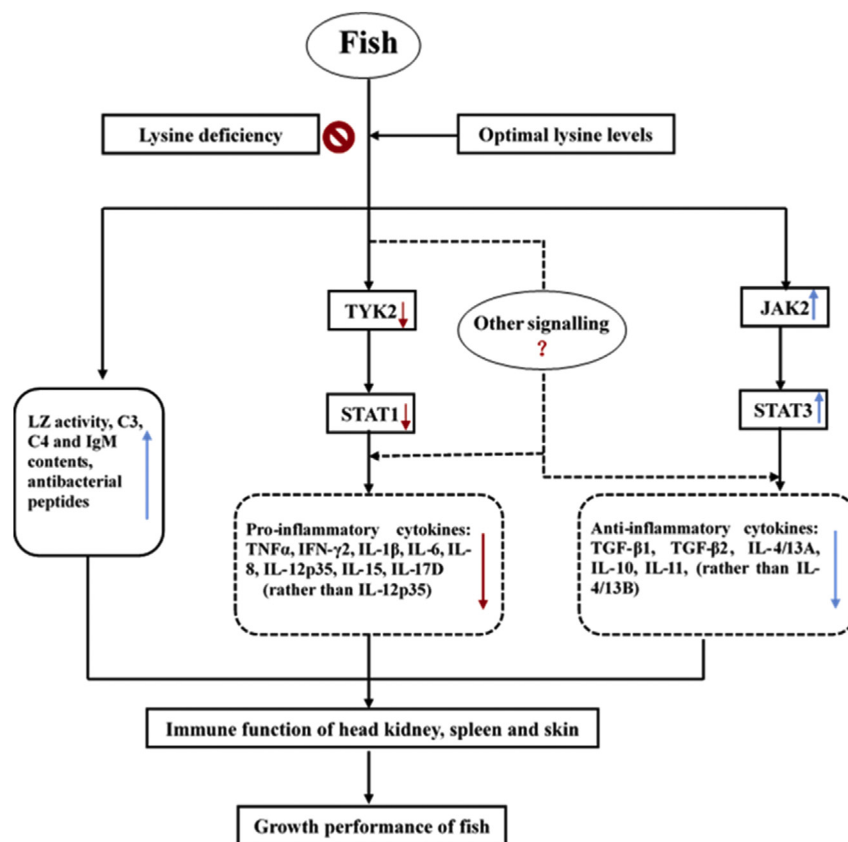

Fig. 6. The potential pathways about the effects of lysine on immune function in the head kidney, spleen and skin of fish. TYK2 = tyrosine kinase 2; JAK2 = Janus kinase 2; STAT = signal transducers and activators of transcription; *LZ* = lysozyme; C3 = complement 3; C4 = complement 4; IgM= immunoglobulin M; TNF-α = tumor necrosis factor α; *IFN-γ2* = interferon γ2; IL = interleukin; *TGF-β* = transforming growth factor β.

immune organs. Our conclusion showed that lysine deficiency increased the skin lesion morbidity and decreased the growth of immune organs in grown-up grass carp, which provides support for our hypothesis.

Third, an aggravated inflammatory response associated with lysine excess might be partly correlated with the increased malonaldehyde (MDA) levels in the skin, spleen and head kidney of fish. Research on fish shows that inflammatory response of immune organs is tightly correlated with its oxidative damage (Zheng et al., 2018). The MDA content is used as a biomarker to measure the level of oxidative damage (Wang et al., 2010). Our previous study reported that lysine excess could increase the content of MDA levels in grass carp intestine (Li et al., 2016a,b). These results help to explain our findings.

5. Conclusions

In general (Fig. 6), this research observed that lysine deficiency reduced growth performance of grown-up grass carp. Our research is the first to show that lysine deficiency impaired immune response and aggravated inflammatory response in the skin, spleen and head kidney of fish. The particular mechanisms are displayed as follows: (1) lysine deficiency impaired fish immune response by depressing the production of antibacterial compounds (except for ACP); (2) lysine deficiency aggravated fish inflammatory response by up-regulating pro-inflammation cytokines (except for *IL-12p40*) and down-regulating anti-inflammation cytokines (except for IL-4/13B) mRNA abundances in part relevant to TYK2/STAT1 and JAK2/*STAT3* signaling pathway, respectively. (3) compared with the optimal lysine group, lysine excess decreased the growth performance, immune response and aggravated inflammatory response in fish, while compared to lysine deficiency group, excessive lysine improved indicators mentioned above. (4) based on the PWG, the lysozyme activities in the head kidney and spleen and skin morbidity, the dietary lysine requirements for grown-up grass carp (164.33 to 581.33 g) were estimated to be 13.51 to 14.55 g/kg.

Author contributions

Yangyang Hu conducted the animal experiments, lab analysis, statistical analysis and wrote the manuscript. **Xiaoqiu Zhou**, **Lin Feng**, **Weidan Jiang**, **Pei Wu** and **Yang Liu** contributed to the study design, conducted guide experiments, revised the paper and laying out experiment scheme. **Shengyao Kuang** and **Ling Tan**g provision the experiment site and partial experimental materials. All authors carefully read and approved the final revision of the manuscript.

Conflict of interest

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

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References

Abdelhamed H, Ibrahim I, Baumgartner W, Lawrence ML, Karsi A. Characterization of istopathological and ultrastructural changes in channel Catfish experimentally infected with virulent *Aeromonas hydrophila*. Front Microbiol 2017;8:1–15.

Cai W, Fang, Liu W, Bin, Jiang G, Zhen, Wang K, Zhuo, Sun C, Xin, Li X, Fei. Lysine supplement benefits the growth performance, protein synthesis, and muscle development of Megalobrama amblycephala fed diets with fish meal replaced by rice protein concentrate. Fish Physiol Biochem 2018;44:1159–74.

Carter Jr BW, Chicoine LG, Nelin LD. L-lysine decreases nitric oxide production and increases vascular resistance in lungs isolated from lipopolysaccharide-treated neonatal pigs. Pediatr Res 2004;55:979–87.

Chakraborty B,A, White S,M, Schaefer T,S, Ball E,D, Dyer K,F, Tweardy D,J. Granulocyte colony-stimulating factor Activation of Stat3a and Stat3b in immature normal and Leukemic human myeloid cells. Rapid Commun 1996;88:2442–9.

Chen GF, Liu Y, Jiang J, Jiang WD, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng L. Effect of dietary arginine on the immune response and gene expression in head kidney and spleen following infection of Jian carp with *Aeromonas hydrophila*. Fish Shellfish Immunol 2015;44:195–202.

Cook KD, Miller J. TCR-dependent translational control of GATA-3 enhances Th2 differentiation. J Immunol 2010;185:3209–16.

Coskun M, Salem M, Pedersen J, Nielsen OH. Involvement of JAK/STAT signaling in the pathogenesis of inflammatory bowel disease. Pharmacol Res 2013;76:1–8.

Day RW. Comparisons of tretments after an analysis of variance in ecology. Ecol Monogr 1989;59.

de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ, Berthoud HR, Uematsu S, Akira S, van den Wijngaard RM, Boeckxstaens GE. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. Nat Immunol 2005;6:844–51.

Dong YW, Feng L, Jiang WD, Liu Y, Wu P, Jiang J, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ. Dietary threonine deficiency depressed the disease resistance, immune and physical barriers in the gills of juvenile grass carp (*Ctenopharyngodon idella*) under infection of Flavobacterium columnare. Fish Shellfish Immunol 2018;72:161–73.

Dong YW, Jiang WD, Liu Y, Wu P, Jiang J, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng L. Threonine deficiency decreased intestinal immunity and aggravated inflammation associated with NF-kappaB and target of rapamycin signalling pathways in juvenile grass carp (*Ctenopharyngodon idella*) after infection with Aeromonas hydrophila. Br J Nutr 2017;118:92–108.

Du Z, Yu, Liu Y, Jian, Tian L, Xia, He J, Guo, Cao J, Ming, Liang G, Ying. The influence of feeding rate on growth, feed efficiency and body composition of juvenile grass carp (*Ctenopharyngodon idella*). Aquacult Int 2005;14:247–57.

Feng L, Li W, Liu Y, Jiang WD, Kuang SY, Jiang J, Tang L, Wu P, Tang WN, Zhang YA, Zhou XQ. Dietary phenylalanine-improved intestinal barrier health in young grass carp (*Ctenopharyngodon idella*) is associated with increased immune status and regulated gene expression of cytokines, tight junction proteins, antioxidant enzymes and related signalling molecules. Fish Shellfish Immunol 2015;45:495–509.

Fischer A H, Jacobson K,A, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections, vol. 3. Cold Spring Harbor Laboratory Press; 2008.

Fisher H, Griminger P, Leveille G A, Shapiro R. Quantitative aspects of lysine deficiency and amino acid imbalance 1960;71:213–20.

Garica I, Guler R, Vesin D, Olleros M,L, Vassalli P, Chvatchko Y, Jacobs M, Bernhard R. Lethal Mycobacterium bovis Bacillus calmette guerin infection in nitric oxide synthase 2-deficient mice: cell-mediated immunity requires nitric oxide synthase 2. Lab Invest 2000;80:1385–97.

Geraylou Z, Souffreau C, Rurangwa E, D'Hondt S, Callewaert L, Courtin CM, Delcour JA, Buyse J, Ollevier F. Effects of arabinoxylan-oligosaccharides (AXOS) on juvenile Siberian sturgeon (*Acipenser baerii*) performance, immune responses and gastrointestinal microbial community. Fish Shellfish Immunol 2012;33:718–24.

Giri SS, Sen SS, Chi C, Kim HJ, Yun S, Park SC, Sukumaran V. Effect of dietary leucine on the growth parameters and expression of antioxidant, immune, and inflammatory genes in the head kidney of Labeo rohita fingerlings. Vet Immunol Immunopathol 2015;167:36–43.

Guo YL, Wu P, Jiang WD, Liu Y, Kuang SY, Jiang J, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng L. The impaired immune function and structural integrity by dietary iron deficiency or excess in gill of fish after infection with Flavobacterium columnare: regulation of NF-kappaB, TOR, JNK, p38MAPK, Nrf2 and MLCK signalling. Fish Shellfish Immunol 2018;74:593–608.

He P, Jiang WD, Liu XA, Feng L, Wu P, Liu Y, Jiang J, Tan BP, Yang QH, Kuang SY, Tang L, Zhou XQ. Dietary biotin deficiency decreased growth performance and impaired the immune function of the head kidney, spleen and skin in on-growing grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol 2019;97:216–34.

Hemre G, Ingunn, Deng D, Fang, Wilson RP, Berntssen M,HG. Vitamin A metabolism and early biological responses in juvenile sunshine bass (*Morone chrysops*×*M. saxatilis*) fed graded levels of vitamin A. Aquaculture 2004;235:645–58.

Horwitz W. Official methods of analysis of the association of official analytical chemists. J Pharm Sci 1975;60:414.

Hu K, Zhang JX, Feng L, Jiang WD, Wu P, Liu Y, Jiang J, Zhou XQ. Effect of dietary glutamine on growth performance, non-specific immunity, expression of cytokine genes, phosphorylation of target of rapamycin (TOR), and antioxidative system in spleen and head kidney of Jian carp (*Cyprinus carpio var.* Jian). Fish Physiol Biochem 2015;41:635–49.

Jiang W-D, Deng Y-P, Liu Y, Qu B, Jiang J, Kuang S-Y, Tang L, Tang W-N, Wu P, Zhang Y-A, Zhou X-Q, Feng L. Dietary leucine regulates the intestinal immune status, immune-related signalling molecules and tight junction transcript abundance in grass carp (*Ctenopharyngodon idella*). Aquaculture 2015;444:134–42.

Jiang WD, Liu Y, Jiang J, Wu P, Feng L, Zhou XQ. Copper exposure induces toxicity to the antioxidant system via the destruction of Nrf2/ARE signaling and caspase-3-regulated DNA damage in fish muscle: amelioration by myo-inositol. Aquat Toxicol 2015;159:245–55.

Jiang WD, Wen HL, Liu Y, Jiang J, Kuang SY, Wu P, Zhao J, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng L. The tight junction protein transcript abundance changes and oxidative damage by tryptophan deficiency or excess are related to the modulation of the signalling molecules, NF-kappaB p65, TOR, caspase-(3,8,9) and Nrf2 mRNA levels, in the gill of young grass carp (*Ctenopharyngodon idellus*). Fish Shellfish Immunol 2015;46:168–80.

Jiang WD, Wen HL, Liu Y, Jiang J, Wu P, Zhao J, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng L. Enhanced muscle nutrient content and flesh quality, resulting from tryptophan, is associated with anti-oxidative damage referred to the Nrf2 and TOR signalling factors in young grass carp (*Ctenopharyngodon idella*): avoid tryptophan deficiency or excess. Food Chem 2016;199:210–9.

Kondomerkos DJ, Kalamidas SA, Kotoulas OB. In vitro effects of hormones and autacoids on the activity of acid phosphatase in the lysates of endotoxin-activated rat peritoneal and bronchoalveolar macrophages. Histol Histopathol 2003;18:1103–13.

Lazado CC, Caipang CMA. Probiotics–pathogen interactions elicit differential regulation of cutaneous immune responses in epidermal cells of Atlantic cod Gadus morhua. Fish Shellfish Immunol 2014;36:113–9.

Li P, Yin YL, Li D, Kim SW, Wu G. Amino acids and immune function. Br J Nutr 2007;98:237–52.

Li XY, Liu Y, Jiang WD, Jiang J, Wu P, Zhao J, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng L. Co- and post-treatment with lysine protects primary fish enterocytes against Cu-induced oxidative damage. PloS One 2016;11:1–18.

Li XY, Liu Y, Jiang WD, Jiang J, Wu P, Zhao J, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng L. Co- and post-treatment with lysine protects primary fish enterocytes against Cu-induced oxidative damage. PloS One 2016;11:e0147408.

Li XY, Tang L, Hu K, Liu Y, Jiang WD, Jiang J, Wu P, Chen GF, Li SH, Kuang SY, Feng L, Zhou XQ. Effect of dietary lysine on growth, intestinal enzymes activities and antioxidant status of sub-adult grass carp (*Ctenopharyngodon idella*). Fish Physiol Biochem 2013;40:659–71.

Liu HX, Zhou XQ, Jiang WD, Wu P, Liu Y, Zeng YY, Jiang J, Kuang SY, Tang L, Feng L. Optimal alpha-lipoic acid strengthen immunity of young grass carp (*Ctenopharyngodon idella*) by enhancing immune function of head kidney, spleen and skin. Fish Shellfish Immunol 2018;80:600–17.

Liu T, Zhou J, Li W, Rong X, Gao Y, Zhao L, Fan Y, Zhang J, Ji C, Ma Q. Effects of sporoderm-broken spores of Ganoderma lucidum on growth performance, antioxidant function and immune response of broilers. Animal Nutr 2019. https://doi.org/10.1016/j.aninu.2019.11.005.

Liu W, Ran C, Liu Z, Gao Q, Xu S, Ringo E, Myklebust R, Gu Z, Zhou Z. Effects of dietary Lactobacillus plantarum and AHL lactonase on the control of *Aeromonas hydrophila* infection in tilapia. MicrobiologyOpen 2016;5:687–99.

Livak K,J, Schmittgen T,D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle CT}$ Method. Methods 2001;25:402–8.

Lu B, Antoine D J, Kwan K, Lundback P, Wahamaa H, Schierbeck H, Robinson M. JAK/STAT1 signaling promotes HMGB1 hyperacetylation and nuclear translocation. 2014.

Luo JB, Feng L, Jiang W D, Liu Y, Wu P, Jiang J, Kuang S,Y, Tang L, Zhang Y,A, Zhou X,Q. The impaired intestinal mucosal immune system by valine deficiency for young grass carp (*Ctenopharyngodon idella*) is associated with decreasing immune status and regulating tight junction proteins transcript abundance in the intestine. Fish Shellfish Immunol 2014;40:197–207.

Machado M, Azeredo R, Fontinha F, Fernandez-Boo S, Conceicao LEC, Dias J, Costas B. Dietary methionine improves the European seabass (*Dicentrarchus labrax*) immune status, inflammatory response, and disease resistance. Front Immunol 2018;9.

Morimoto T, Biswas G, Kono T, Sakai M, Hikima J-i. Immune responses in the Japanese pufferfish (Takifugu rubripes) head kidney cells stimulated with particulate silica. Fish Shellfish Immunol 2016;49:84–90.
Murray P J. Understanding and exploiting the endogenous interleukin 10/STAT3-mediated anti-inflammatory response. Elsever 2006;6:379–86.
Ni PJ, Jiang WD, Wu P, Liu Y, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ, Feng L. Dietary low or excess levels of lipids reduced growth performance, and impaired immune function and structure of head kidney, spleen and skin in young grass carp (*Ctenopharyngodon idella*) under the infection of *Aeromonas hydrophila*. Fish Shellfish Immunol 2016;55:28–47.
Niijima A, Meguid M M. Influence of systemic arginine-lysine on immune organ function: an electrophysiological study. Brain Res Bull 1998;45:437–41.
NRC. Nutrient requirements of fish and shrimp. 2011.
Ohtani M, Hayashi N, Hashimoto K, Nakanishi T, Dijkstra JM. Comprehensive clarification of two paralogous interleukin 4/13 loci in teleost fish. Immunogenetics 2008;60:383–97.
Pan F, Yu, Wu P, Feng L, Jiang W, Dan, Kuang S, Yao, Tang L, Tang W, Neng, Zhang Y, An, Zhou X, Qiu, Liu Y. Methionine hydroxy analogue improves intestinal immunological and physical barrier function in young grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol 2017;64:122–36.
Pohlenz C, Gatlin DM. Interrelationships between fish nutrition and health. Aquaculture 2014;431:111–7.
Rutherford M,S, Schook L,B. Macrophage function in response to PGE2, L-arginine deprivation, and activation by colony-stimulating factors is dependent on hematopoletic stimulus 1992;52:228–35.
Schuringa J, Wierenga A,T, Kruijer W, Vellenga E. Constitutive Stat3, Tyr705, and Ser727 phosphorylation in acute myeloid leukemia cells caused by the autocrine secretion of interleukin-6 2000;95:37–55.
Seguin M,J, Barney D, Widowski T,M. Assessment of a group-housing system for gestating sows: effects of space allowance and pen size on the incidence of superficial skin lesions, changes in body condition, and farrowing performance. Orig Res 2006;14:89–96.
Shi XC, Jin A, Sun J, Yang Z, Tian JJ, Ji H, Yu HB, Li Y, Zhou JS, Du ZY, Chen LQ. alpha-lipoic acid ameliorates n-3 highly-unsaturated fatty acids induced lipid peroxidation via regulating antioxidant defenses in grass carp (*Ctenopharyngodon idellus*). Fish Shellfish Immunol 2017;67:359–67.
Smriga M, Kameishi M, Uneyama H, Torii K. Dietary L-lysine deficiency increases stressn induced anxiety and fecal excretion in rats. Nutr Eurosci—Res Commun 2002;132:3744–6.
Su YN, Wu P, Feng L, Jiang WD, Jiang J, Zhang YA, Figueiredo-Silva C, Zhou XQ, Liu Y. The improved growth performance and enhanced immune function by DL-methionyl-DL-methionine are associated with NF-kappaB and TOR signalling in intestine of juvenile grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol 2018;74:101–18.
Takemura A. Changes in an immunoglobulin M (IgM)-like protein during larval stages in tilapia, Oreochromis mossambicus. Aquaculture 1993;115:233–41.
Tie H, Mao, Wu P, Jiang W, Dan, Liu Y, Kuang S, Yao, Zeng Y, Yun, Jiang J, Tang L, Zhou X, Qiu, Feng L. Dietary nucleotides supplementation affect the physicochemical properties, amino acid and fatty acid constituents, apoptosis and antioxidant mechanisms in grass carp (*Ctenopharyngodon idellus*) muscle. Aquaculture 2019;502:312–25.
Torrecillas S, Montero D, Caballero M,J, Robaina L, Zamorano M,J, Sweetman J, Lzquierdo M. Effects of dietary concentrated mannan oligosaccharides supplementation on growth, gut mucosal immune system and liver lipid metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. Fish Shellfish Immunol 2015;42:508–16.
Wang P, Powell SR. Decreased sensitivity associated with an altered formulation of a commercially available kit for detection of protein carbonyls. Free Radic Biol Med 2010;49:119–21.
Wang T, Husain M. The expanding repertoire of the IL-12 cytokine family in teleost fish: identification of three paralogues each of the p35 and p40 genes in salmonids, and comparative analysis of their expression and modulation in Atlantic salmon Salmo salar. Dev Comp Immunol 2014;46:194–207.
Wang XZ, Jiang WD, Feng L, Wu P, Liu Y, Zeng YY, Jiang J, Kuang SY, Tang L, Tang WN, Zhou XQ. Low or excess levels of dietary cholesterol impaired immunity and aggravated inflammation response in young grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol 2018;78:202–21.
Wen J, Jiang W, Feng L, Kuang S, Jiang J, Tang L, Zhou X, Liu Y. The influence of graded levels of available phosphorus on growth performance, muscle antioxidant and flesh quality of young grass carp (*Ctenopharyngodon idella*). Anim Nutr 2015;1:77–84.
Wu C,L, Zhang W, bing, Mai K,S, Liang X,F, Xu w, Wang J, Ma H,M. Molecular cloning, characterization and mRNA expression of selenium-binding protein in abalone (*Haliotis discus hannai* Ino): response to dietary selenium, iron and zinc. Fish Shellfish Immunol 2010;29:117–25.
Wu G,Y, Meininger C,J. Regulation of nitric oxide synthesis by dietary factors. Annu Rev Nutr 2002;22:61–86.
Xu H, Mu Y, Zhang Y, Li J, Liang M, Zheng K, Wei Y. Graded levels of fish protein hydrolysate in high plant diets for turbot (*Scophthalmus maximus*): effects on growth performance and lipid accumulation. Aquaculture 2016;454:140–7.
Yang Q,H, Tan B, ping, Dong X, hui, Chi S,Y, Liu H,Y. Effects of different levels of Yucca schidigera extract on the growth and nonspecific immunity of Pacific white shrimp (*Litopenaeus vannamei*) and on culture water quality. Aquaculture 2015;439:39–44.
Zeng Y,Y, Jiang W,D, Liu Y, Wu P, Zhao J, Jiang J, Kuang S,Y, Tang L, Tang W,N, Zhang Y,A, Zhou X,Q, Feng L. Optimal dietary alpha-linolenic acid/linoleic acid ratio improved digestive and absorptive capacities and target of rapamycin gene expression of juvenile grass carp (*Ctenopharyngodon idellus*). Aquacult Nutr 2016;22:1251–66.
Zhang L, Feng L, Jiang WD, Liu Y, Wu P, Kuang SY, Tang L, Tang WN, Zhang YA, Zhou XQ. Vitamin A deficiency suppresses fish immune function with differences in different intestinal segments: the role of transcriptional factor NF-kappaB and p38 mitogen-activated protein kinase signalling pathways. Br J Nutr 2017;117:67–82.
Zhao J, Liu Y, Jiang J, Wu P, Jiang W,D, Li S,H, Tang L, Kuang S,Y, Feng l, Zhou X,Q. Effects of dietary isoleucine on the immune response, antioxidant status and gene expression in the head kidney of juvenile Jian carp (*Cyprinus carpio var. Jian*). Fish Shellfish Immunol 2013;35:572–80.
Zheng L, Feng L, Jiang WD, Wu P, Tang L, Kuang SY, Zeng YY, Zhou XQ, Liu Y. Selenium deficiency impaired immune function of the immune organs in young grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol 2018;77:53–70.
Zhong JR, Feng L, Jiang WD, Wu P, Liu Y, Jiang J, Kuang SY, Tang L, Zhou XQ. Phytic acid disrupted intestinal immune status and suppressed growth performance in on-growing grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol 2019;92:536–51.