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Alterations on growth performance, antioxidant responses and lipid metabolism in liver for juvenile hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *Epinephelus lanceolatus*) fed dietary vitamin E

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ABSTRACT

The theme of present study was to estimate the alterations on growth performance, antioxidant responses and lipid metabolism in liver of juvenile hybrid grouper (♀ *Epinephelus fuscoguttatus* × ♂ *Epinephelus lanceolatus*) fed dietary vitamin E (VE). The 540 fish (initial weight 8.79 ± 0.06 g) were distributed into 18 fiberglass tanks and fed isonitrogenous and isolipidic diets with VE at different levels (11, 43, 98, 193, 404, and 789 mg/kg) for 7 weeks. Results showed that weight gain rate (WGR), specific growth rate, protein efficiency ratio, and condition factor were significantly improved in fish fed dietary VE levels. The triglyceride levels in serum, and the number and size of lipid droplets and the content of malondialdehyde in the liver of the treatment groups significantly decreased. In addition, dietary VE improved the activities of hepatic antioxidant (catalase (CAT), superoxide dismutase, and total antioxidant capacity) and lipid metabolism enzymes (hormone sensitive lipase (HSL)) and regulated the expressions of antioxidant (CAT, hepatic copper/zinc superoxide dismutase, and nuclear factor erythroid-2-related factor 2) and lipid metabolism related genes (HSL, fatty acid synthase, lipoprotein lipase, peroxisome proliferator activated receptor-γ and carnitine palmitoyl transferase-1). The appropriate VE supplementation was assessed at 133.45 mg/kg according to the WGR broken line analysis model. Overall, dietary VE could enhance the antioxidant capacity and reduce the hepatic lipid deposition of hybrid grouper, thereby improving its growth performance.

1. Introduction

Vitamin E (VE) is a class of biologically active phenolic compounds with fat-soluble property, among which alpha-tocopherol has the highest activity (NRC, 2011). Given their deficiency to synthesize VE, fish are usually fed diets supplemented with VE in the form of dl-a-tocopherol acetate (Li et al., 2018b; Jiang et al., 2020). VE participates in several physiological processes, including growth performance, antioxidant capacity, lipid metabolism, immune function, reproductive development, and hematopoiesis (Pan et al., 2017; Zhang et al., 2017; Erdogan and Arslan, 2019). In addition, VE can improve muscular degeneration by increasing oxidative stability (Jittinandana et al., 2006). VE deficiency in fish causes growth retardation, liver degeneration, anemia, erythrocyte fragility, lipid oxidation, and abnormal lipid deposition (Qiang et al., 2019; Kocabas and Gatlin III, 1999). Thus, the suitable VE requirement for fish should be identified to ensure normal growth and health.

VE is an important part of the antioxidant defense system of fish, whose main role is to prevent damage caused by lipid peroxidation to cells and tissues (Hamre, 2011). The regulation of antioxidant response is associated with oxidative stress activation of the nuclear factor erythroid-2-associated factor 2 (Nrf2) (Li and Kong, 2009). VE not only can directly induce the expression of Nrf2 and its transferral to the nucleus (Li and Kong, 2009) but also indirectly protect cells against inhibitors of Nrf2 (Ernst et al., 2013). Previous studies found that the optimal VE supplementation of diet in various fish species could increase

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activity of antioxidant enzyme and expression of related gene, and decrease malondialdehyde (MDA) content (Shahkar et al., 2018; Pan et al., 2018; Huang et al., 2020). The influence of VE on the antioxidant system was further explored by mediating the signaling molecule Nrf2 in fish. The liver is a crucial organ for VE storage and lipid metabolism, and VE plays an important role in lipid metabolism (He et al., 2019; Li et al., 2009). Lipogenic enzymes (such as fatty acid synthase [FAS]) and lipolytic enzymes (carnitine palmitoyl transferase 1 [CPT-1], lipoprotein lipase [LPL], hormone sensitive lipase [HSL], and hepatic lipase [HL]) are involved in lipid metabolism and can effectively regulate abnormal lipid deposition. (Zhuo et al., 2018; Tian et al., 2013; Perret et al., 2002). Additionally, peroxisome proliferator-activated receptors (PPARs) are pivotal transcriptional agents in the regulation of lipid homeostasis, which can mediate the expression of genes related to lipid metabolism (Lee et al., 2003). At present, relatively few reports focused on the interaction of VE with genes and transcription factors related to lipid metabolism in fish. Thus, this research gap should be filled to elucidate the underlying mechanisms of lipid metabolism.

Fish flesh is an important and popular source of animal protein for humans because it is rich in polyunsaturated fatty acids, high-quality proteins, essential amino acids, and micronutrients (vitamins and minerals) (FAO, 2020). Grouper is a carnivorous fish that ranked second in farmed production of marine fish in China (Ministry of Agriculture and Rural Affairs, 2020). The breeding prospect for hybrid species of the brown-marbled grouper and giant grouper (♀ *Epinephelus fuscoguttatus* × ♂ *E. lanceolatus*) is largely owing to its advantages of fast growth (Liang et al., 2020b), stronger resistance than its parents, and excellent taste (Fan et al., 2018). Currently, studies on different fish species found that VE is closely related to fatty acid composition and lipid peroxidation (Peng et al., 2009; Fatima et al., 2019; Zhang et al., 2017). Nevertheless, nutritional VE requirements and influence of VE on the lipid metabolism of juvenile hybrid grouper remain unknown. Thus, the study estimated the influence of dietary VE on the growth performance, serum biochemical indexes, oxidation ability, and lipid metabolism of juvenile hybrid grouper fed diets with VE supplementation and to determine the optimal VE concentration.

2. Materials and methods

The processes of study were performed in accordance with the relevant guidelines and regulations and agreed by the Animal Care Advisory Committee of Guangdong Ocean University.

2.1. Design and preparation of experimental diets

A basic diet (Table 1) was formulated using white fish meal, casein and gelatin as protein sources (about 48% crude protein), and fish oil, soybean lecithin and corn oil as lipid sources (about 13% ether extract). The levels of protein and lipid in diet could satisfy the growth of juvenile hybrid grouper according to previous study (Rahimnejad et al., 2015). Different doses of VE (dl-α-tocopherol acetate, Zhejiang Langbo Pharmaceutical Co., Ltd, China) were supplemented into basic diets to create graded levels of 0, 50, 100, 200, 400 and 800 mg/kg, respectively. The real VE concentrations in diet were detected to be 11 (control group, without supplementation), 43, 98, 193, 404, 789 mg/kg, by high-performance liquid chromatography respectively. The basic diet without addition of VE was referred to as the control diet. All raw materials were ground into fine powder and passed 60 screen meshes and the process of mixing raw materials referred to the method of Liang et al. (2020b). The pellets (2.0 and 2.5 mm) were produced through a pelletizer. The two kinds of pellets were dried under air conditioning at 20 °C, and stored in bags at − 20 °C until feeding.

2.2. Feeding procedure

Juvenile hybrid groupers were bought from a local hatchery in Donghai Island (Zhanjiang, China), which were fed for 2 weeks under experimental conditions (dissolved oxygen: > 7 mg/L; salinity: 26–28; water temperature: 29–31 °C). After 10 days of feeding the control diet, a total of 540 healthy fish (mean weight 8.79 ± 0.06 g) were randomly selected and placed into 18 fiberglass tanks (1000 L), a total of six groups with three replicates each. The fish of six groups were fed experimental diets to apparent satiation twice (8:00 and 16:00) daily (Tang et al., 2018) for 7 weeks. Approximately 50% of the water in tanks was changed every day and siphoning was used to remove feces from tanks. All fish were reared under natural light and dark cycle.

Table 1
Formulation and nutrient levels of basic diet (%, dry weight).

ingredient	%
White fish meal	18.00
Casein (vitamin free)[a]	28.00
Gelatin	7.00
Wheat flour	23.00
Fish oil	4.00
Corn oil	4.00
Soy lecithin	2.30
Vitamin premix[b]	1.00
Mineral premix[c]	0.50
choline chloride	0.50
Calcium dihydrogen phosphate	1.00
vitamin C	0.05
Ethoxyquin	0.05
Microcrystalline Cellulose	9.60
Attractant	1.00
Total	100
Nutrient levels	
Moisture	7.76
Crude protein	47.81
Ether extract	12.92
Crude ash	6.59
VE basic content (mg/kg)	11

[a] Casein (vitamin free), Sigma Chemical Co., Ltd.
[b] Vitamin Premix (g/kg mixture): vitamin A, 0.62 g; vitamin D_3, 0.60 g; vitamin K_3, 1.18 g; vitamin B_1, 2.55 g; vitamin B_2, 5.63 g; calcium pantothenate, 6.12 g; nicotinic acid, 20.20 g; vitamin B_6, 2.04 g; biotin, 7.50 g; vitamin B_{12}, 5.00 g; inositol, 50.51 g; folic acid, 1.19 g. All ingredients were diluted with corn starch to 1 kg.
[c] Mineral premix, Qingdao Master Biotechnology Co., Ltd.

2.3. Sample collection

At the end of the feeding trail, fish of each tank were fasted for 24 h (Wang et al., 2018) before capture, and thereafter were anesthetized by using diluted eugenol (1:10,000; Shanghai, China). Then fish were counted and weighed for assessment of specific growth rate (SGR), weight gain rate (WGR), survival rate (SR), protein efficiency ratio (PER) and feed conversion ratio (FCR). Three fish per tank were randomly selected, then weighed viscera and liver and measured length for calculation of morphological indicators including condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI). Three additional fish were randomly captured in each tank and frozen at − 20 °C for proximate composition analysis of whole body. The blood samples were drawn from the caudal vein of six fish per tank using 1 mL syringes, and subsequently clotted at 4 °C overnight. After centrifugation (4000 rpm for 10 min) at 4 °C, obtained serum samples were separated and stored at − 80 °C for subsequent analysis of biochemical indexes (Liang et al., 2020a). For histological analysis (Oil red O staining in liver), livers of two fishes from each tank were sampled, placed in 4% paraformaldehyde solution, frozen-sectioned and stained with oil red solution, counter-stained with hematoxylin solution, and sealed with glycerin before examination under an optical microscope.

The ten fishes sampled from each tank were rapidly dissected, and livers were collected, immediately stored in liquid nitrogen, followed by

storage at − 80 °C. The liver samples were used for analysis of antioxidant-related parameters, lipid metabolism enzymes, VE concentration and RNA extraction.

2.4. Analysis and measurement of Sample

2.4.1. Composition analysis

The proximate composition of fish samples and diets were analyzed using the standard procedures (AOAC, 2000). Moisture content was measured via samples dried in a ventilation drying oven at 105 °C until constant weight. The crude lipid content was tested by the ether extraction method and crude ash content was evaluated through combustion of samples in a muffle furnace at 550 °C for 6 h. The crude protein content (N × 6.25) was determined by the Kjeldahl method. VE concentration in liver was determined with a colorimetric method by using the assay kit, which was similar to Huang et al. (2020).

2.4.2. Biochemical parameters in serum

Total cholesterol (TCHO), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) contents of serum were analyzed with kits of Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.4.3. Hepatic antioxidant and lipid metabolism indexes

The liver samples were taken out from − 80 °C ultrafreezer and thereafter accurately weighed and homogenized with ice-cold saline in the proportion of 1:9 (w/v). The supernatant was collected by centrifugation at 4 °C (3000 rpm for 15 min) until analysis. The superoxide dismutase (SOD) and catalase (CAT) activities, total antioxidant capacity (T-AOC) and MDA content were tested with kits of Nanjing Jiancheng Bioengineering Institute (Nanjing, China) following operating instructions. The detailed test methods have been introduced by Li et al. (2018b).

The FAS and HSL activities were measured with enzyme linked immunosorbent assay kits of Shanghai Enzyme linked Biotechnology Co., Ltd (Shanghai, China). Optical density of solution in the microplate was determined at 450 nm wavelength.

2.5. RNA extraction and gene expression analysis in liver

Total RNA was isolated from liver with TransZol Up Plus RNA Kit of Beijing TransGen Biotech Co., Ltd (Beijing, China) as the manufacturer's instructions. The integrity of total RNA was confirmed by denaturing agarose gel electrophoresis at 1%. The purity and concentration of total RNA was evaluated by spectrophotometric analysis (A260:280 nm), and absorbance ratio ranged from 1.80 to 2.00. Subsequently, the RNA of each sample was reverse transcribed into complementary DNA (cDNA) for real-time quantitative PCR (RT-qPCR) using PrimeScript™ RT eagent Kit (Takara, Japan) in accordance with kit protocol. The cDNA was thereafter stored at − 20 °C for future analysis. Gene sequences obtained from Gen Bank were used to designed gene-specific primers via Primer 5 software (Table 2). The β-actin was used as a reference gene to normalize cDNA loading (An et al., 2020). Reaction conditions for RT-qPCR of target gene were introduced in detail by Liang et al. (2020b). The relative gene expression levels was calculated by using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.6. Calculations and statistical analysis

The standard formulas (NRC, 2011) were used to calculate indictors of growth performance (WGR, SGR and SR), feed utilization (PER and FCR) and morphology (HSI, VSI and CF).

The data were assessed for homogeneity of variances before one-way analysis of variance (ANOVA) via SPSS 21.0 software. Tukey's multiple comparison test was selected for significance comparison in different groups. Statistically significance was declared at $P < 0.05$. All results were expressed as the means ± SD. The suitable VE level of diet for juvenile hybrid groupers was assessed via broken-line regression analysis.

3. Results

3.1. Growth performance

Table 3 showed that the WGR and SGR of fish increased with increasing dietary VE levels and then stabilized. The WGR and SGR of the groups fed the diets with 98–789 mg/kg VE were significantly enhanced compared with those of the control group ($P < 0.05$). The PER of the group fed the diet with 193 mg/kg VE was significantly higher than that of the control group ($P < 0.05$). The CF of the groups fed the diets with 193 and 404 mg/kg VE was significantly high compared with that of the control group ($P < 0.05$). The FCR, SR, HSI, and VSI were not significantly affected by dietary VE levels ($P > 0.05$). The suitable VE level of diet was 133.45 mg/kg diet on the basis of the broken-line regression analysis model of WGR (Fig. 1).

3.2. Body composition and VE concentration in liver

As shown in Table 4, contents of moisture, crude protein, crude lipid, and crude ash in whole fish had no significant differences ($P > 0.05$). The VE concentration in the liver increased with increasing dietary VE level to 404 mg/kg and then stabilized (Fig. 2). The VE concentration in the liver significantly decreased and was lowest in control group ($P < 0.05$).

3.3. Serum biochemical parameters

Table 5 showed that there were no significant differences in the LDL, HDL, and TCHO levels of serum. The TG levels were significantly lower in the serum of the group fed the diets with 43–789 mg/kg VE compared

Table 2
Primers used for real-time PCR in study.

Gene	Forward sequence	Reverse sequence	Accession number
CuZnSOD	CAGTGGGACCGTGTATTTTGAG	CAGTCACATTTCCCAGGTCTCC	AY735008.1
CAT	TGTTTCGGACCCCTCGTTC	TTGAAGGCGGTGATGGAGA	KT884509.1
Nrf2	AGCACCAACTGAACGAAGCC	CTCACTCAACAGACGCTCCTTC	KU892416.1
FAS	GCTTTCTTTGGAGCGAGGAG	ACGACTTGAACCCGACCATC	FJ196231.1
HSL	CGCCATCAGTCAGGAATAGGT	TGGCGTGGATGTCATACTGG	KF049203.1
LPL	ACATCAACAAGGTCCGTCCG	CCTTCACTCCGAGCATTCCAT	EU683732.1
HL	TGGCTCACCAGCACTACCCTA	TCCAATCTTCTCCGAACCCTC	EU683733.1
CPT-1	GGATGTTTTATGATGGACGGC	ACCACGGCTGAAGAAGGCT	HM037343.1
PPAR-γ	CCGCAGCACGAAGAACAACT	TGGACGCCATAGTGAAACCC	KM052849.1
β-actin	GGCTACTCCTTCACCACCACA	TCTGGGCAACGGAACCTCT	AY510710.2

CuZnSOD, copper/zinc superoxide dismutase; CAT, catalase; Nrf2, nuclear factor erythroid-2-related factor 2; FAS, fatty acid synthase; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; HL, hepatic lipase; CPT-1, carnitine palmitoyl transferase-1; PPAR-γ, peroxisome proliferator activated receptor-γ.

Table 3
Growth performance of hybrid grouper fed experimental diets.

	Dietary vitamin E levels / (mg/kg diet)					
Items	11	43	98	193	404	789
IBW (g)	8.79 ± 0.29	8.81 ± 0.21	8.81 ± 0.34	8.79 ± 0.29	8.81 ± 0.33	8.78 ± 0.31
WGR (%)	432.70 ± 9.92^{a}	442.59 ± 13.90^{ab}	468.87 ± 17.08^{bc}	484.54 ± 17.48^{c}	475.46 ± 7.97^{bc}	474.84 ± 11.15^{bc}
SGR (%/d)	3.41 ± 0.04^{a}	3.45 ± 0.05^{ab}	3.55 ± 0.07^{bc}	3.60 ± 0.06^{c}	3.57 ± 0.03^{bc}	3.57 ± 0.04^{bc}
FCR	0.84 ± 0.02	0.81 ± 0.02	0.79 ± 0.05	0.76 ± 0.03	0.76 ± 0.05	0.77 ± 0.03
PER	2.51 ± 0.04^{a}	2.56 ± 0.05^{ab}	2.65 ± 0.16^{ab}	2.81 ± 0.04^{b}	2.76 ± 0.16^{ab}	2.71 ± 0.07^{ab}
SR (%)	78.89 ± 5.09	81.11 ± 1.92	78.89 ± 10.18	81.11 ± 6.94	77.78 ± 3.85	78.89 ± 3.85
HSI (%)	2.15 ± 0.18	1.99 ± 0.11	2.48 ± 0.11	2.64 ± 0.23	2.37 ± 0.30	2.35 ± 0.42
VSI (%)	9.62 ± 0.63	9.79 ± 0.83	10.41 ± 0.75	10.21 ± 1.27	9.59 ± 0.56	9.42 ± 0.89
CF (g/cm^3)	2.67 ± 0.30^{a}	2.86 ± 0.21^{ab}	2.86 ± 0.15^{ab}	3.08 ± 0.19^{b}	3.02 ± 0.23^{b}	2.93 ± 0.14^{ab}

Data are means ± SD. Different superscript letters in each row represent significant differences (P < 0.05).
IBW, initial mean body weight; WGR, weight gain rate; SGR, specific growth rate; PER, protein efficiency ratio; FCR, feed conversion ratio; SR, survival rate; VSI, viscerosomatic index; CF, condition factor; HSI, hepatosomatic index.

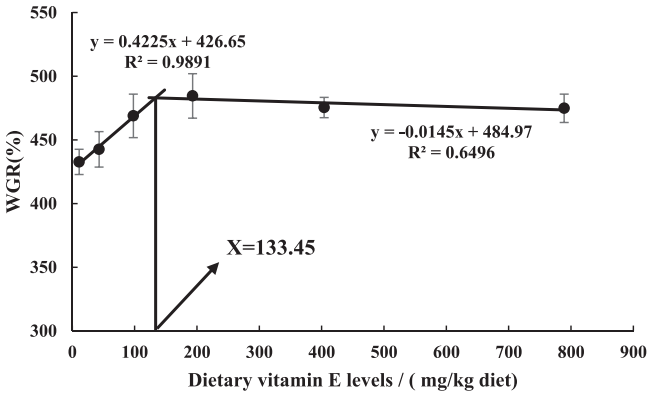

Fig. 1. The broken-line regression analysis of WGR in hybrid grouper. The optimal dietary VE level was 133.45 mg/kg diet.

with that of the control group in serum ($P < 0.05$), and the lowest TG level was observed in the group fed the diet with 193 mg/kg VE.

3.4. Lipid metabolism and antioxidant capacity in liver

As shown in Table 6, the SOD activity in liver of the group fed the diet with 193 mg/kg VE was significantly improved compared with that of the control group in liver ($P < 0.05$). The CAT activity in the liver of the groups fed the diets with 43–193 mg/kg VE was significantly higher than that in the liver of the control group ($P < 0.05$). The groups fed the diets with 98, 193, and 789 mg/kg VE showed significantly higher T-AOC than the control group and the group fed the diet with 43 mg/kg VE ($P < 0.05$). The MDA content of the liver significantly decreased with increasing VE level in the treatment groups than the control group ($P < 0.05$).

The FAS activity of the liver significantly decreased in the groups fed the diets with 43, 193, and 404 mg/kg VE. The HSL activity of the liver increased with the dietary increasing VE level to 193 mg/kg and then decreased. The HSL activity of the liver significantly enhanced in the groups fed the diets with 43–789 mg/kg VE than that in control group ($P < 0.05$).

3.5. Oil red O staining in liver

Oil red O staining in the liver was used to further evaluate the lipid distributions (Fig. 3). Plenty of large lipid droplets were displayed in the control group, reflecting high lipid content in the liver. The number and size of lipid droplets significantly decreased with increasing VE levels in the diet. Few lipid droplets were found in the group fed the diets with 193–789 mg/kg VE.

3.6. Lipid metabolism and antioxidant-related genes expression in liver

As shown in Figs. 4 and 5, the expression of the copper/zinc superoxide dismutase (CuZnSOD) gene was significantly higher in the groups fed the diets with 193–789 mg/kg VE than in control group ($P < 0.05$). The groups fed the diets with 43–404 mg/kg VE showed significantly higher CAT gene expression than the groups fed the diets with 11 and 789 mg/kg VE ($P < 0.05$). The expression of the Nrf2 gene was significantly higher in the groups fed the diets with 43–193 mg/kg VE than in control group ($P < 0.05$).

The expression of the FAS gene was significantly lower in the groups fed the diets with 43–193 and 789 mg/kg VE than in control group ($P < 0.05$). The expression of the HSL gene in the groups fed the diets with 43–789 mg/kg VE was significantly higher than that in control group ($P < 0.05$). Compared with the groups fed the diets with 11–43 and 404–789 mg/kg VE, the groups fed the diets with 98 and 193 mg/kg VE showed significantly lower expression of the LPL gene ($P < 0.05$). The expression of the HL gene was significantly higher in the groups fed the diets with 98 and 404 mg/kg VE than in control group ($P < 0.05$), and it was the highest in group fed the diet with 98 mg/kg VE. The expression of the CPT-1 and PPAR-γ genes significantly decreased in the groups fed the diets with 98–789 mg/kg VE than in control group ($P < 0.05$).

4. Discussion

VE is an important vitamin involved in many important

Table 4
Proximate composition in whole body of hybrid grouper fed experimental diets.

	Dietary vitamin E level (mg/kg diet)					
Items	11	43	98	193	404	789
Moisture (%)	72.41 ± 0.90	72.23 ± 0.28	72.16 ± 0.87	72.06 ± 1.08	71.65 ± 1.12	72.11 ± 0.45
Crude protein (%)	16.76 ± 0.77	16.72 ± 0.50	16.47 ± 0.71	16.59 ± 0.74	17.25 ± 0.62	17.11 ± 0.46
Crude lipid (%)	6.66 ± 0.36	7.06 ± 0.62	7.57 ± 0.55	7.28 ± 0.70	7.62 ± 0.77	6.76 ± 0.41
Crude ash (%)	4.14 ± 0.04	4.19 ± 0.30	3.99 ± 0.09	3.93 ± 0.08	4.14 ± 0.33	4.18 ± 0.24

Data are means ± SD. Different superscript letters in each row represent significant differences (P < 0.05).

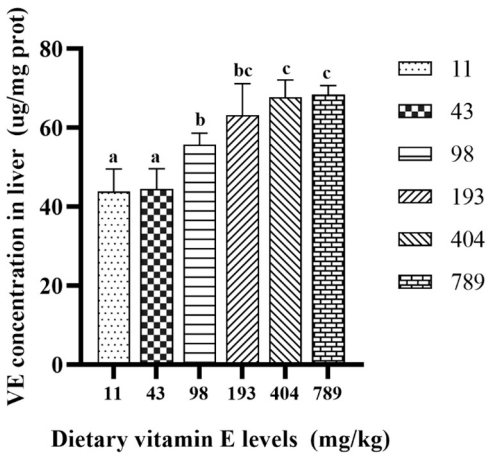

Fig. 2. Hepatic vitamin E concentration of hybrid grouper fed experimental diets. Data are means ± SD. The different letters above bar graphs indicate significant difference ($P < 0.05$). The concentration of VE in the liver first increased and then kept stable with the increase of VE level in the diet.

physiological processes. It is also a critical nutrient in fish feeds not only to maintain fish health but also to promote fish growth (Pan et al., 2017). The results of the current study found that the WGR and SGR were improved with increasing VE levels in diet and then stabilized. This result agrees with those observed in blunt snout bream (*Megalobrama amblycephala*) (Zhang et al., 2017), largemouth bass (*Micropterus salmoides*) (Li et al., 2018a), channel catfish (*Ictalurus punctatus*) (He et al., 2017), beluga (*Huso huso*) (Amlashi et al., 2011), and sea cucumber (*Apostichopus japonicus*) (Li et al., 2020). Thus, dietary VE at an appropriate level is conducive to improve the growth of aquatic animals, but excess dietary VE may not have a significant negative effect on growth. VE deficiency may induce the oxidative rancidity of many unsaturated fatty acids in feeds (He et al., 2017), and suitable VE can enhance the antioxidant capacity (Pan et al., 2018) and lipid metabolism (Qiang et al., 2019) of fish to maintain healthy growth. Wang et al. (2019) found that excess VE in diet can suppress the growth of yellow drum (*Nibea albiflora*) owing to the imbalance and accumulation of VE radicals, which might function as prooxidants (Li et al., 2013). Nevertheless, the present results indicated that no significant reduction in growth was observed in the group fed the diet with high VE levels, which agree with the findings obtained in grouper (*Epinephelus malabaricus*) (Lin and Shiau, 2005). This phenomenon may be due to strong tolerance of grouper to the toxic effects of dietary VE, but further study is required. The current study showed that the PER of fish was high with supplementation of VE, suggesting that dietary VE improves feed utilization and elevates fish growth. In the present study, the HSI and VSI did not differ significantly in all groups, but CF in the control group was significantly reduced than in the treatment groups. This result may be ascribed to the fact that VE deficiency is vulnerable to oxidative stress, which is an important factor in muscle atrophy in humans and animals (Barker and Traber, 2007). Similar results were found in beluga (Amlashi et al., 2011) and golden shiner (*Notemigonus crysoleucas*) (Chen et al., 2004).

In the current study, the SR was low in all groups. This may be attributed to the fact that grouper is a carnivorous fish, and the low content of fishmeal in the diet caused poor palatability of the diet, resulting in a lower feed intake of hybrid grouper and thus leading to their death in response to overall malnutrition state. In addition, grouper have the characteristic of cannibalism, and some of the better-growing fish constantly try to prey on some of malnourished fish, which was conducive to reducing SR.

Based on regression analysis using the broken-line model for WGR (Fig. 1), we concluded that the optimal dietary VE level was 133.45 mg/kg diet, which supported the healthy growth for hybrid grouper. The VE requirement of hybrid grouper is higher than those of grouper (104–115 mg/kg diet) (Lin and Shiau, 2005), Nile tilapia (*Oreochromis niloticus*) (92.06 mg/kg diet) (Qiang et al., 2019), and blunt snout bream (55.53 mg/kg diet) (Zhang et al., 2017) but lower than those of meagre (*Argyrosomus regius*) (451 mg/kg diet) (Lozano et al., 2017), Japanese eel (*Anguilla japonica*) (212.9 mg/kg diet) (Shahkar et al., 2018), and fingerling (*Channa punctatus*) (140–169 mg/kg diet) (Abdel-Hameid et al., 2012). The discrepancy of VE requirement in fish is primarily attributed to fish species and size, development stage, evaluation indicators, culture environment, and VE sources in diet (Lu et al., 2016; Li et al., 2018a). Moreover, dietary lipid levels and source, as well as the presence of other antioxidants in the diet effectively influence the

Table 5
Serum biochemical parameters of hybrid grouper fed experimental diets.

	Dietary vitamin E level (mg/kg diet)					
Items	11	43	98	193	404	789
LDL (mmol/L)	0.34 ± 0.13	0.48 ± 0.12	0.37 ± 0.08	0.38 ± 0.05	0.53 ± 0.08	0.33 ± 0.03
HDL (mmo/L)	1.37 ± 0.25	1.47 ± 0.12	1.39 ± 0.28	1.34 ± 0.10	1.32 ± 0.16	1.56 ± 0.22
TCHO (mmol/L)	1.70 ± 0.23	1.61 ± 0.33	1.46 ± 0.26	1.34 ± 0.09	1.74 ± 0.18	1.37 ± 0.12
TG (mmo/L)	1.08 ± 0.01^{d}	0.62 ± 0.06^{c}	0.47 ± 0.05^{b}	0.33 ± 0.01^{a}	0.69 ± 0.03^{c}	0.34 ± 0.01^{a}

Data are means ± SD. Different superscript letters in each row represent significant differences ($P < 0.05$).
LDL, Low-density lipoprotein; HDL, high-density lipoprotein; TCHO, total cholesterol; TG, triglyceride.

Table 6
Hepatic antioxidant and lipid metabolism indexes of hybrid grouper fed experimental diets.

	Dietary vitamin E level (mg/kg diet)					
Items	11	43	98	193	404	789
Oxidative status and antioxidant indexes						
SOD (U/mg prot)	51.39 ± 2.30^{a}	50.58 ± 1.79^{a}	66.14 ± 4.41^{ab}	69.56 ± 5.47^{b}	60.36 ± 11.37^{ab}	50.60 ± 7.45^{a}
CAT (U/mg prot)	8.16 ± 0.30^{a}	10.73 ± 0.87^{b}	11.18 ± 1.13^{bc}	13.26 ± 1.05^{c}	9.97 ± 0.38^{ab}	8.89 ± 1.29^{ab}
T-AOC (mmol/g prot)	22.79 ± 3.97^{a}	21.02 ± 0.96^{a}	48.02 ± 5.05^{c}	44.65 ± 4.60^{bc}	32.81 ± 3.44^{ab}	38.10 ± 7.84^{bc}
MDA (nmol/mg prot)	4.65 ± 0.08^{e}	4.51 ± 0.06^{e}	2.97 ± 0.03^{c}	3.47 ± 0.05^{d}	2.39 ± 0.03^{b}	0.92 ± 0.12^{a}
Lipid metabolism indexes						
FAS (U/mg prot)	1.54 ± 0.17^{c}	1.31 ± 0.09^{ab}	1.45 ± 0.04^{bc}	1.07 ± 0.05^{a}	1.16 ± 0.21^{ab}	1.55 ± 0.23^{c}
HSL (U/mg prot)	0.86 ± 0.22^{a}	1.14 ± 0.13^{b}	1.15 ± 0.06^{b}	1.42 ± 0.20^{c}	1.27 ± 0.15^{bc}	1.08 ± 0.13^{b}

Data are means ± SD. Different superscript letters in each row represent significant differences ($P < 0.05$).
SOD, superoxide dismutase; CAT, catalase; T-AOC, total antioxidant capacity; MDA, malondialdehyde; FAS, fatty acid synthetase; HSL, hormone-sensitive lipase.

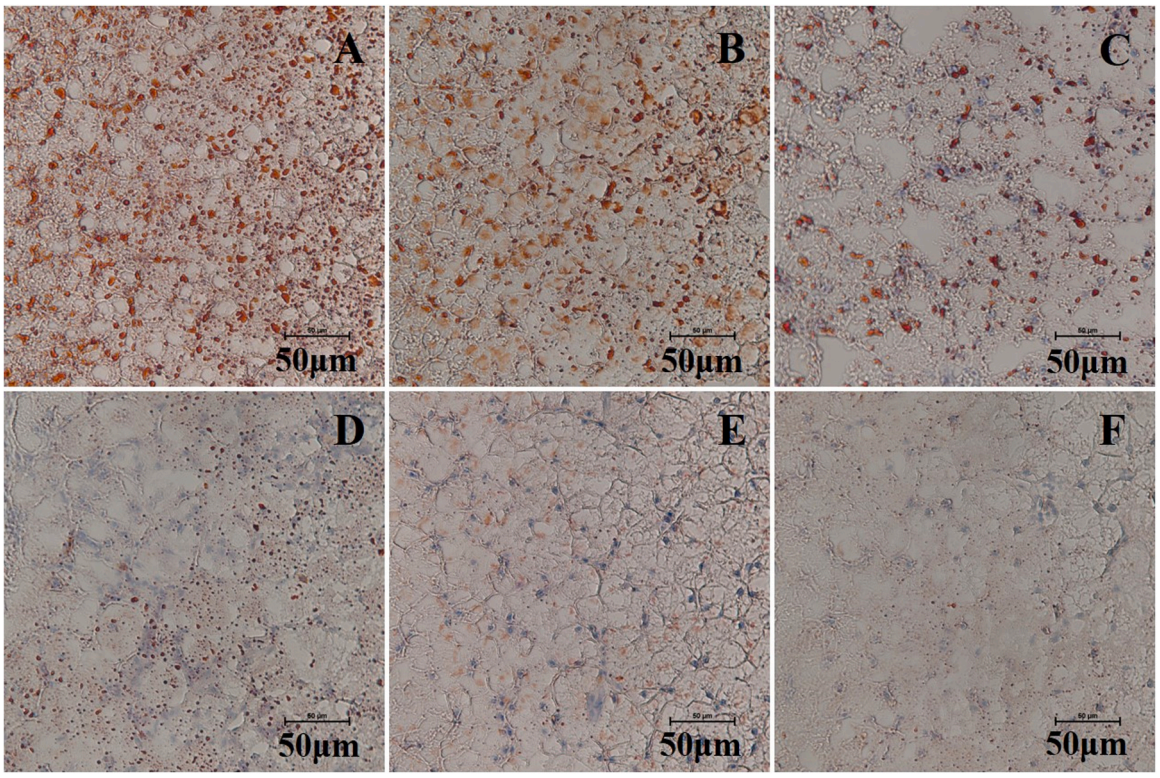

Fig. 3. The lipid distributions in livers (Oil red O×200, scale 50 μm) of hybrid grouper fed experimental diets. (A) 11 mg/kg diet, (B) 43 mg/kg diet, (C) 98 mg/kg diet, (D) 193 mg/kg diet, (E) 404 mg/kg diet, (F) 789 mg/kg diet.

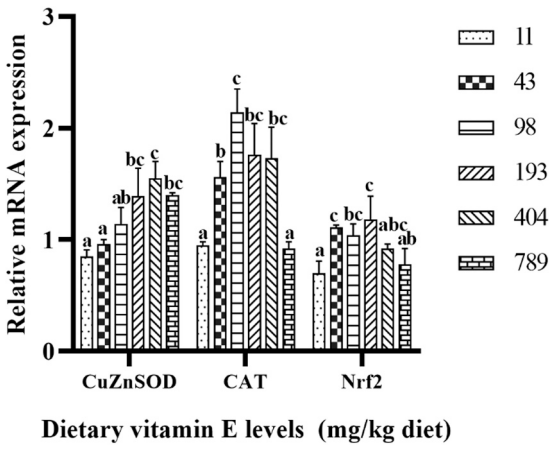

Fig. 4. Relative mRNA expression of antioxidant-related genes in the liver, respectively, of hybrid grouper fed experimental diets. Data are means ± SD. The same letters above the bars present no significant differences ($P > 0.05$). CuZnSOD, copper/zinc superoxide dismutase; CAT, catalase; Nrf2, nuclear factor erythroid-2-related factor 2.

requirement of VE (Lin and Shiau, 2005; Moniruzzaman et al., 2017).

The VE concentration in the tissue is an important indicator for evaluating the VE requirement (Shahkar et al., 2018). VE can only be synthesized by photosynthetic organisms (Quadrana et al., 2013), and the liver probably is the primary site of VE storage (Hamre, 2011). Thus, within a certain range, the VE concentration in the liver was high significantly with increasing VE level in diet. The current study found that the VE concentration in the liver initially increased and then remained stable with the increasing VE level in the diet, similar to the changes in WGR in this study. Similar findings were recorded in yellow drum (Wang et al., 2019), hybrid snakehead (*Channa argus* × *Channa maculata*) (Zhao et al., 2018), and largemouth bass (Li et al., 2018a). However, Huang et al. (2020) and Lin and Shiau (2005) found that consumption of the highest VE level in the diet increases hepatic VE concentration, whereas Bai and Lee (1998) observed the opposite change in the liver. This result may be ascribed to the fish species and VE gradient used in these studies. In addition, the present study showed that VE level in the diet exerted no significant effect on body composition, which gives consent to results of Bae et al. (2013) and Zhou et al. (2013). Nevertheless, some studies demonstrated that dietary supplementation of VE could affect the body composition of grass carp (*Ctenopharyngodon idellus*) (Li et al., 2014) and largemouth bass (Li et al., 2018a). This difference deserves further investigation.

The lipid compounds of serum in fish (e.g. LDL, HDL, TCHO, and TG) are biochemical parameters that reflect the level of lipid metabolism and are important indicators of the health status of the organism (Mensinger et al., 2005). The rise and fall of TG levels in the serum of mice are consistent with changes in cholesterol level (He et al., 2019). The present results indicated that the TG levels in the serum of the treatment groups were significantly reduced compared with those in the serum of the control group, and the changes between increased and decreased levels of TG and TCHO in serum were similar, indicating that VE deficiency may hinder lipid metabolism in the serum. Similarly, previous studies illustrated that the supplementation of dietary VE could reduce the TG and TCHO levels of serum in tilapia (Qiang et al., 2019) and manchurian trout (*Brachymystax lenok*) (Chang et al., 2017). These results may be due to the following factors: first, VE can increase the activity of the enzyme that metabolizes TG and TCHO in the serum and promote transport and excretion (Qiang et al., 2019). Second, it is partially due to the fact that VE can regulate the expression of related genes concerning lipid metabolism and activates the Nrf2/CES1 signaling pathway, thereby reducing the synthesis of TG and TCHO (He et al., 2019). Oil red O, a dye that is soluble in lipids, was used to stain the liver in the study to measure lipid accumulation in the liver (Sen et al., 2001). The lipid content of the liver was significantly lower with

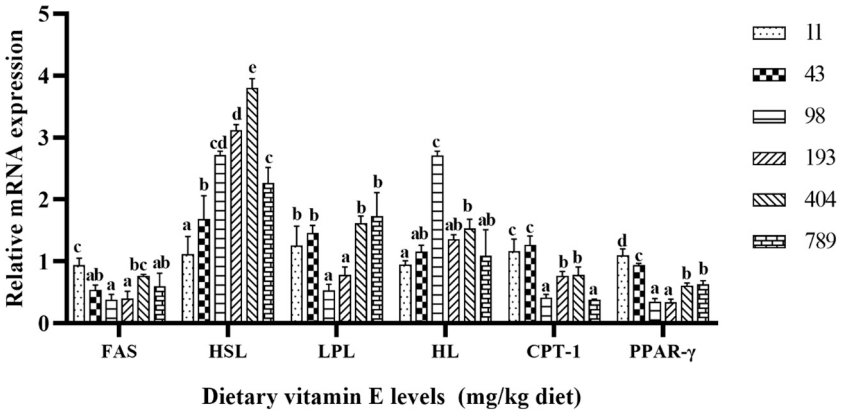

Fig. 5. Relative mRNA expression of lipid metabolism related genes in the liver, respectively, of hybrid grouper fed experimental diets. Data are means ± SD. The same letters above the bars present no significant differences ($P > 0.05$). FAS, fatty acid synthase; HSL, hormone sensitive lipase; LPL, lipoprotein lipase; HL, hepatic lipase; CPT-1, carnitine palmitoyl transferase-1; PPAR-γ, peroxisome proliferator activated receptor-γ.

supplementation of VE, which is similar to the change of TG in serum. This result further supported the above-mentioned assumption that VE deficiency suppresses lipid metabolism.

To prevent damage caused by reactive oxygen species (ROS) and maintain good health, fish have developed an antioxidant system that includes enzymatic antioxidants (such as SOD, CAT, and others) and non-enzymatic antioxidants (Viarengo et al., 1991). Among non-enzymatic antioxidants, VE as a potent biological antioxidant can efficiently scavenge superoxide anion (Traber and Packer, 1995). In the present study, the groups fed VE-deficient diets showed significantly lower T-AOC, SOD, and CAT activities in the liver in comparison with the treatment group, which agrees with the results obtained in female shrimp (*Macrobrachium nipponense*) (Li et al., 2018b), largemouth bass (Li et al., 2018a), and sea cucumber (Li et al., 2020). The reduction in antioxidant enzymes activities might be ascribed to scavenging superoxide anion (Li et al., 2014). Similarly, the antioxidant enzymes activities of liver decreased in fish fed VE-excess diet, probably because VE possesses the property of resisting ROS (Hamre, 2011), thereby reducing lipid peroxide production. This result may also be related to a mild oxidative stress caused by excess VE. Thus, proper supplementation of VE in diet is conducive to enhancing the antioxidant enzyme activities of fish. MDA is a principal product the reaction between polyunsaturated fatty acids and ROS, used as a biomarker of oxidative stress (Del Rio et al., 2005) and an important indicator to evaluate the degree of lipid peroxidation (Trenzado et al., 2006). The current study found the MDA content of liver decreased with increasing VE levels in diet, suggesting that VE can suppress the degree of lipid peroxidation and reduce oxidative damage in the liver. The results obtained agree with other studies on coho salmon (*Oncorhynchus kisutch*) (Huang et al., 2004), Nile tilapia (Tang et al., 2013), and meagre (Lozano et al., 2017). Tocher et al. (2002) discovered that VE deficiency in diet promotes fatty acid peroxidation because of the formation of large amounts of oxidized free radicals in tissues. However, further exploration is still needed.

The signaling molecule Nrf2 plays a critical role in regulating the antioxidant system (Jaiswal, 2004). It can translocate into the nucleus and then activate the transcription of antioxidant genes through antioxidant response element (Wang and Gallagher, 2013; Ma, 2013), thereby maintaining cellular defenses (Jaiswal, 2004). Huang et al. (2020) reported that VE positively affects antioxidant gene expression by upregulating the mRNA level of Nrf2 in *Sillago sihama* Forskál. The present study found that the CAT, CuZnSOD, and Nrf2 mRNA levels of liver increased in the groups fed the diet with VE. Similar phenomena were also found in grass carp (Pan et al., 2017, 2018). These results imply that the upregulation of antioxidant gene expression may be partially correlated with the activation of the Nrf2 gene. Thus, VE not only acts as a non-enzymatic antioxidant by itself but also strengthens the enzymatic antioxidant defense system by regulating antioxidant gene and signaling molecule Nrf2 to promote antioxidant capacity.

The regulation of lipid metabolism involves lipogenesis, lipolysis, and lipid transport, which depend on many critical enzyme activities and transcription factors (Zhuo et al., 2018). FAS, a key enzyme in fatty acid synthesis, plays an crucial role in lipid deposition in animals (Semenkovich, 1997). HSL is key rate-limiting enzyme that catalyzes the hydrolysis of triacylglycerol, diacylglycerol, and monoacylglycerol in adipose tissue and then releases free fatty acid used for oxidative energy supply in the organism (Lafontan and Langin, 2009). LPL and HL are collectively known as total lipase and are recognized as essential factors in lipid degradation. LPL can function in lipoprotein metabolism and energy metabolism; it is responsible for the conversion of TG-rich lipoproteins and the supply of free fatty acids (Auwerx et al., 1992). Similar to LPL, HL can hydrolyze TGs and phospholipids present in circulating lipoproteins (Kobayashi et al., 2006). The current results in this study showed that the diets supplemented with VE reduced the activity and mRNA level of FAS and increased the activity and mRNA level of HSL in the liver. In addition, the mRNA level of HL increased in the treatment group. These results indicate that the VE inhibits lipid deposition in the liver by slowing down the synthesis of fatty acids and promoting the hydrolysis of TGs. Nevertheless, the mRNA level of LPL decreased in the groups fed the diets with 98 and 193 mg/kg diet, which may be partly relevant to the downregulation of PPAR-γmRNA level in this study. Schoonjans et al. (1996) reported that the downregulation of LPL mRNA level can be attributed to the depression of PPAR-γmRNA level and that a positive regulatory feedback loop possibly exists between LPL and PPAR-γ, which support our assumption.

As a member of the nuclear receptor superfamily, PPAR-γinvolved in differentiation of adipose cells and adipogenesis (Tontonoz et al., 1998; Lehmann et al., 1995), is rather related to the improvement of insulin sensitivity in rodents and humans (Tontonoz and Spiegelman, 2008). The beta-oxidation of fatty acid is regulated in the mitochondrial matrix by CPT-1 (Gutières et al., 2003), which is mediated by ligand-activated PPARs (Mascaró et al., 1998). The present study showed that the PPAR-γand CPT-1 mRNA levels in the liver were downregulated by the diet with VE supplementation. A similar research found that the significantly lower expression level of hepatic PPAR-γin blunt snout bream is caused by the supplementation of dietary VE (Zhang et al., 2017). It may be responsible for the antioxidant function of VE, which can suppress fatty acid oxidation and thereby decrease PPAR-γand CPT-1 expression. In addition, n-3 highly unsaturated fatty acids can enhance lipid catabolism and decrease deposition (An et al., 2020), which may also be a critical reason for the reduction of hepatic lipid droplets in the treatment group. Hence, another important reason for reduced lipid deposition in the liver is that vitamin E hinders the

activation of CPT-1 by downregulating PPAR-γ expression, thereby inhibiting the oxidation of highly unsaturated fatty acids.

5. Conclusion

The result of present study indicated that dietary VE could improve growth performance, enhance antioxidant capacity, and reduce hepatic lipid deposition of hybrid grouper. These positive and favorable effects resulted from the increased enzyme activities and mRNA levels related to antioxidation and lipid metabolism in liver. Finally, based on the WGR, the requirement of dietary VE for hybrid grouper was evaluated to be 133.45 mg/kg diet.

CRediT authorship contribution statement

Dazhi Liang: Responsible for breeding and analytical experiments, data analysis and article writing. **Qingzhi Zheng**: Responsible for analytical experiments. **Qihui Yang**: Responsible for directing experiments, paper revisions, and Funding acquisition. **Beiping Tan**: Responsible for Funding acquisition. **Xiaohui Dong**: Responsible for the guidance of breeding experiments. **Shuyan Chi**: Responsible for the guidance of breeding experiments. **Hongyu Liu**: Responsible for the maintenance of laboratory equipment. **Shuang Zhang**: Responsible for the purchase of experimental consumables.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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