British Journal of Nutrition (2022), 128, 793–801 doi:10.1017/S0007114521004931

Effects of dietary vitamin D_3 supplementation on the growth performance, tissue Ca and P concentrations, antioxidant capacity, immune response and lipid metabolism in *Litopenaeus vannamei* larvae†

Tianmeng Dai, Lefei Jiao*, Xinyue Tao, Jingjing Lu, Min Jin, Peng Sun and Qicun Zhou*

Laboratory of Fish and Shellfish Nutrition, School of Marine Sciences, Ningbo University, Ningbo 315211, People's Republic of China

(Submitted 10 September 2021 – Final revision received 14 November 2021 – Accepted 22 November 2021 – First published online 9 December 2021)

Abstract

An 8-week feeding trial was conducted to investigate the effects of dietary vitamin D_3 supplementation on the growth performance, tissue Ca and P concentrations, antioxidant capacity, immune response and lipid metabolism in *Litopenaeus vannamei* larvae. A total of 720 shrimp (initial weight 0·50 ± 0·01 g) were randomly distributed into six treatments, each of which had three duplicates of forty shrimp per duplicate. Six iso-nitrogenous and isolipidic diets were formulated to contain graded vitamin D_3 (0·18, 0·23, 0·27, 0·48, 0·57 and 0·98 mg/kg of vitamin D_3, measured) supplementation levels. The results revealed that *L. vannamei* fed diet containing 0·48 mg/kg of vitamin D_3 achieved the best growth performance. Compared with the control group, supplementing 0·48 mg/kg of vitamin D_3 significantly increased ($P < 0·05$) the activities of catalase, total antioxidative capacity, alkaline phosphatase and acid phosphatase in serum and hepatopancreas. Expression levels of antioxidant and immune-related genes were synchronously increased ($P < 0·05$). Carapace P and Ca concentrations were increased ($P < 0·05$) with the increased vitamin D_3 supplementation levels. Further analysis of lipid metabolism-related genes expression showed that shrimp fed 0·48 mg of vitamin D_3 per kg diet showed the highest value in the expression of lipid synthesis-related genes, while shrimp fed 0·98 mg of vitamin D_3 per kg diet showed the highest value in the expression of lipolysis-related genes. In conclusion, the results of present study indicated that dietary supplementation of 0·48 mg/kg of vitamin D_3 could increase Ca and P concentrations, improve antioxidant capacity and immune response, and influence lipid metabolism in *L. vannamei*.

Key words: **Vitamin D_3 requirements: Antioxidant capacity: Immune response: Lipid metabolism: *Litopenaeus vannamei***

The fat-soluble vitamin D, which comprises a group of secosterols found naturally in few foods, is an essential nutrient for animal growth and physiological metabolism[1]. Vitamin D consists of five different types: D_1, D_2, D_3, D_4 and D_5. Among these, the two major forms are vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamin D_2 is produced in plants and fungi, while vitamin D_3 is produced in animals. Generally, dietary vitamin D_3 supplementation in the diets can be used to maintain adequate health in aquatic animals. The classical action of vitamin D_3 is to maintain Ca and P homoeostasis by enhancing the absorption ability from the intestine[2,3]. In recent years, additional diverse physiological function of vitamin D_3, including its effects on immune response, antioxidant capacity and lipid metabolism, has gradually been explored[4–7].

Contrary to mammals, which are able to produce vitamin D_3 in the skin after exposure to solar UV-B radiation, fish and crustacean cannot synthesise vitamin D *de novo* and the diet is the only vitamin D source[8,9]. Currently, the research regarding the influence of vitamin D_3 on the growth and physiological metabolism in aquatic animals is limited. Previous studies mainly focus on the optimum dietary vitamin D_3 requirement for various fishes and found that vitamin D_3 requirement varies from different species[10,11]. A few researches have demonstrated the influence of vitamin D_3 on the antioxidant and immune capacity in pearl oyster *Pinctada fucata martensii*, Atlantic Salmon (*Salmo salar*) and yellow catfish (*Pelteobagrus fulvidraco*)[12–14]. It has been found that vitamin D_3 is also closely connected with regulating lipid metabolism in visceral adipose tissue of zebrafish[6]. In contrast, high dosage of vitamin D_3 could cause metabolic disorders and induce chronic stress effects in aquatic animals[12,15]. Therefore, optimum vitamin D_3 supplementation level in the formulated diets should be carefully evaluated and

Abbreviations: ACP, acid phosphatase; ALP, alkaline phosphatase; CAT, catalase; LZM, lysozyme; NOS, nitric oxide synthase; PWG, percent weight gain; SOD, superoxide dismutase.

* **Corresponding authors:** Lefei Jiao, email: jiaolefei@nbu.edu.cn; Qicun Zhou, email: zhouqicun@nbu.edu.cn

† The online version of this article has been updated since original publication. A notice detailing the change has been published at https://doi.org/10.1017/S000711452200188X.

Tianmeng Dai and Lefei Jiao were co-first authors.

determined, especially for unstudied aquaculture species. To the best of our knowledge, exploring vitamin D_3 optimal requirement and physiological function in crustaceans was currently only reported in *Penaeus monodon*[16], *Eriocheir sinensis*[17] and *Litopenaeus vannamei*[2].

Pacific white shrimp *L. vannamei* is one of the most important economic marine shrimp, which makes the shrimp aquaculture become the fastest-growing industry worldwide[18,19]. Currently, researches reporting the effects of dietary vitamin D_3 inclusion on growth and physiological metabolism in *L.vannamei* remain limited. Therefore, this study aims to evaluate the dietary vitamin D_3 requirement in *L.vannamei* based on growth performance, and further compare the specific effects of different vitamin D_3 supplementation levels on the tissue Ca/P concentrations, antioxidant capacity, non-specific immune response and lipid metabolism. This study would contribute to a in-depth understanding of vitamin D_3's physiological function in *L.vannamei*.

Materials and methods

Ethics statement

All experimental procedures complied with the Standard Operation Procedures (SOP) of the Guide for Use of Experimental Animals of Ningbo University.

Animals and experimental procedure

Our experiment was carried out in the Ningbo Marine Fishery Science and Technology Innovation Base. Healthy shrimp post-larvae were raised in aerated semi-intensive pond at room temperature (28 ± 2°C), salinity (21·6–23·5), pH (7·6–7·9) and dissolved oxygen (4·29–5·8 mg/l).

A total of 720 shrimp (initial weight 0·50 ± 0·01 g) were randomly distributed into six treatments, each of which had three tanks (300-litre cylindrical fibreglass tanks filled with 200 litre of water) of forty shrimp per tank. We firstly counted twenty shrimp and weighed, and then selected remaining twenty shrimp to make the final total weight of about 20 g. The six diets (45·71 % crude protein and 7·36 % crude lipid) were formulated to contain different vitamin D_3 levels: the control group was fed the basal diet without extra vitamin D_3 supplementation, and the other five treatment groups were supplemented with 0·05, 0·10, 0·30, 0·40, 0·80 mg/kg of vitamin D_3, respectively. Vitamin D_3 was purchased from Guangzhou Chengyi Aquatic Products Technology Co., Ltd. The vitamin D_3 content in each treatment group was measured in Guangzhou Analysis and Test Center in China (GB/T 17818-2010-1). The analysed vitamin D_3 supplementations levels in each diet were 0·18, 0·23, 0·27, 0·48, 0·57 and 0·98 mg/kg, respectively. The ingredient and composition of the basal diet are shown in Supplementary Table S1. Shrimp were fed manually three times a day (8–10 % of body weight) at 06.00 h (35 % of the diets), 12.00 h (30 % of the diets) and 18.00 h (35 % of the diets). Daily amount of formulated feed was adjusted every 2 weeks according to the weight of shrimp in each treatment. Dead shrimp were removed, weighed and recorded immediately, and above 60 % of seawater in each tank was exchanged before the first feeding every morning.

Sample collection

Following the 8-week feeding period, all the shrimp in each tank were taken out, counted and weighed to obtain the final body weight (FBW). The data were further used to determine the specific growth rate (SGR), percent weight gain (PWG), survival rate and feed efficiency (FE).

PWG, % = (final body weight (g) − initial body weight (g))/initial body weight (g) × 100;

SGR, % day^{-1} = (Ln (final body weight) − Ln (initial body weight)) × 100/days;

FE = weight gain (g, wet weight)/feed consumed (g, dry weight).

Ten shrimp per tank were sampled and pooled to make one sample per tank, and therefore three pooled samples per treatment. Haemolymph samples were collected from the pericardial cavity and placed into 1·5-ml centrifuge tubes overnight at 4°C before centrifugation (1811 g, 10 min). The supernatant was collected for anayzing Ca/P concentrations, antioxidant and immune enzyme activity. Muscle and carapace were collected for measuring Ca/P concentrations. Hepatopancreas of the remaining shrimp was harvest for measuring Ca/P concentrations, antioxidant and immune enzyme activity and related gene expression.

Experimental parameters measured

Determination of tissue calcium and phosphorus concentrations. Tissue samples were weighed, freeze-dried and then digested in 70 % HNO_3 solution at 80°C. Drops of HNO_3 solution were added until the liquid became clear and bright. Ca/P concentrations in serum, carapace, hepatopancreas and muscle of *L. vannamei* were determined by diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Analysis of antioxidant and non-specific immune enzymes activities. Hepatopancreas samples were homogenised on ice in 0·9 % NaCl solution, and the supernatant was removed after centrifugation (1811 g for 10 min at 4°C). The supernatant of haemolymph and hepatopancreas was stored at −80°C until further analysis.

The activities of catalase (CAT), superoxide dismutase (SOD) and total antioxidative capacity (T-AOC), alkaline phosphatase (ALP), acid phosphatase (ACP), nitric oxide synthase (NOS), malondialdehyde (MDA), phenoloxidase (PO) in serum and hepatopancreas were measured using diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute) according to manufacturer's instructions.

Gene expression analysis. Total RNA was extracted from samples using TRIzol reagent (Vazyme Biotech co., Ltd) and subsequently measured its quality using a Nano-Drop ND-2000 spectrophotometer (Nano-Drop Technologies). The cDNA was synthesised using the Primer-Script™ One Step RT-PCR Kit (TaKaRa Biotechnology). The primer sequences were designed

British Journal of Nutrition

using the Primer Premier 5.0 software and listed in the Supplemental Table S2. The target genes were as follows: glutathione (*gsh)*, *cat* and *sod* were antioxidant-related genes. Lysozyme (*lzm*), *alp*, *acp*, *tnf-α*, apoptosis-inducing factor (*aif*) and member RAS oncogene family (*rab6a*) were immune-related genes. Sterol-regulatory element binding protein (*srebp*), acetyl-CoA carboxylase 1 (*acc1*), fatty acid synthetase gene (*fas*) fatty acid transport proteins (*fatp*), fatty acid binding protein (*fabp*), carnitine palmitoyltransferase1 (*cpt*) and acyl-CoA oxidase (*aco*) were lipid metabolism-related genes.

Quantitative real-time PCR (qPCR) was carried out on a StepOne Plus Real-Time PCR system (Applied Biosystems) using a SYBR Premix Ex Taq II kit (Vazyme Biotech co., Ltd). The amplifications were carried out in a 20-μl reaction mixture, which consisted of 10 μl of 2 × SYBR Green I Master Mix, 1·0 μl each of the forward and reverse gene-specific primers (10 μM), 2 μl of cDNA, and 6 μl of DEPC water. The PCR program was conducted at an initial denaturation step at 95°C for 2 min, followed by forty-five cycles of 95°C for 10 s, 58°C for 10 s and 72°C for 20 s. The relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method. *β*-actin is used as an internal control. Three independent biological replicates were performed for each sample.

Statistical analysis

Statistical analysis was conducted using IBM SPSS statistics 23.0 (SPSS Inc.). Data presented are mean and standard error. The values of growth performance, tissue Ca/P concentrations, antioxidant enzyme activity, non-specific immune enzymes activity and gene expression among different vitamin D_3 levels were analysed with normality and homoscedasticity tests. Subsequently, one-way ANOVA was applied when the statistical assumptions were fulfilled. Duncan's multiple range test was conducted to compare the differences. Otherwise, Kruskal–Wallis test was applied. All tests were carried out at $\alpha = 0{\cdot}05$ confidence level. Taking PWG as the evaluation index, quadratic regression analysis was used to determine the optimal vitamin D_3 requirement. Effects were considered significant at $P < 0{\cdot}05$.

Results

Growth performance and feed utilisation

As shown in Table 1, FW, PWG and SGR showed a trend of increasing first and then decreasing. 0·48 mg/kg of vitamin D_3 supplementation group achieved the highest value ($P < 0{\cdot}05$) of FW, PWG, FE and SGR. Survival rate was not affected ($P > 0{\cdot}05$) by different vitamin D_3 supplementation levels in *L. vannamei*. Quadratic regression analysis showed that the optimum dietary vitamin D_3 requirement based on PWG was determined to be 0·49 mg/kg (Fig. 1).

Tissue calcium and phosphorus concentrations

As shown in Fig. 2, when dietary vitamin D_3 supplemental level exceeded 0·48 mg/kg, P concentration was significantly increased ($P < 0{\cdot}05$) in hepatopancreas and carapace of *L. vannamei*, while Ca concentration was significantly increased ($P < 0{\cdot}05$) in carapace of *L. vannamei*. Ca concentration was not affected ($P > 0{\cdot}05$) by different vitamin D_3 supplementation levels in serum, hepatopancreas and muscle of *L. vannamei*.

Antioxidant enzyme activity

As shown in Fig. 3, the activity of T-AOC and CAT in serum and hepatopancreas showed a trend of increasing first and then decreasing while hepatopancreas SOD activity had the same trend. The above enzyme activity reached the highest value ($P < 0{\cdot}05$) in the 0·48 mg/kg of vitamin D_3 supplementation group. MDA content was not affected ($P > 0{\cdot}05$) by different vitamin D_3 supplementation levels in serum and hepatopancreas of *L. vannamei*.

Non-specific immune enzymes activity

As shown in Fig. 4, the activity of ALP, ACP in serum and hepatopancreas, and NOS in hepatopancreas showed a trend of increasing first and then decreasing. The above enzyme activity reached the highest value ($P < 0{\cdot}05$) in the 0·48 mg/kg vitamin D_3 supplementation group. Serum NOS activity reached the highest value ($P < 0{\cdot}05$) in the 0·98 mg/kg vitamin D_3 supplementation group.

Expression of antioxidant- and immune-related genes

As shown in Fig. 5(a), antioxidant-related genes (*sod, gsh* and *cat*) showed a trend of increasing first and then decreasing. The above antioxidant-related gene expression reached the highest value ($P < 0{\cdot}05$) in the 0·48 mg/kg of vitamin D_3 supplementation group.

As shown in Fig. 5(b), expression levels of *alp*, *acp* and *lzm* genes showed a trend of increasing first and then decreasing. Expression of *aif* gene showed a trend of decreasing first and then increasing. The expression levels of *alp, acp* and *lzm* genes reached the highest value ($P < 0{\cdot}05$), while *aif* expression reached the lowest value ($P < 0{\cdot}05$) in the 0·48 mg/kg of vitamin D_3 supplementation group. Gene expression of *tnf-α* and *rab6a* was not affected ($P > 0{\cdot}05$) by different vitamin D_3 supplementation levels in hepatopancreas of *L. vannamei*.

Expression of lipid metabolism-related genes in hepatopancreas

As shown in Fig. 6, expression levels *of srebp, acc1* and *fas* genes showed a trend of increasing first and then decreasing. The above gene expression reached the highest value ($P < 0{\cdot}05$) in the 0·48 mg/kg of vitamin D_3 supplementation group. Compared with the control group, supplementing 0·98 mg/kg of vitamin D_3 significantly increased ($P < 0{\cdot}05$) the expression of *cpt1, aco, fabp and fatp* genes in the hepatopancreas of *L. vannamei*, while supplementing 0·23 mg/kg, 0·27 mg/kg, 0·48 mg/kg and 0·57 mg/kg of vitamin D_3 had no effects ($P > 0{\cdot}05$) on the gene expression of *srebp*, *acc1* and *fas* in hepatopancreas of *L. vannamei*.

Table 1. Effects of different vitamin D_3 supplementation levels on growth performance in *Litopenaeus vannamei* (Mean values and standard errors of three replications, *n* 3)

	D0·18		D0·23		D0·27		D0·48		D0·57		D0·98		
Parameters	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	*P*
IW (g)	0·50	0·003	0·50	0·001	0·50	0·005	0·50	0·002	0·50	0·002	0·50	0·004	0·30
FBW (g)	10·51	$0·12^{a}$	10·92	$0·24^{ab}$	11·34	$0·37^{ab}$	11·49	$0·19^{b}$	10·76	$0·01^{a}$	10·45	$0·09^{a}$	0·03
PWG (%)*	2060·65	$23·94^{ab}$	2094·2	$48·25^{ab}$	2177·49	$73·6^{ab}$	2208·98	$38·14^{b}$	2061·63	$28·46^{a}$	2025·2	$19·26^{a}$	0·02
SGR (%/d)†	18·2	$0·22^{a}$	18·95	$0·44^{ab}$	19·71	$0·67^{ab}$	19·99	$0·34^{b}$	18·2	$0·02^{a}$	18·1	$0·17^{a}$	0·02
FE‡	0·76	$0·02^{ab}$	0·81	$0·03^{bc}$	0·82	$0·18^{bc}$	0·86	$0·19^{c}$	0·83	$0·16^{bc}$	0·72	$0·11^{a}$	0·04
Survival rate (%)	70·33	5·78	72·67	4·37	66	5·77	73·56	4·48	73·33	2·4	64·67	10·35	0·22

IW, initial weight; FBW, final body weight; PWG, percent weight gain; SGR, special growth rate; FE, feed efficiency.
Mean in the same row with different superscripts are significantly different ($P < 0·05$).
*PWG, % = (final body weight (g) − initial body weight (g))/initial body weight (g) × 100.
† SGR, % day^{-1} = (Ln (final body weight) − Ln (initial body weight)) × 100/d.
‡ FE = weight gain (g, wet weight)/feed consumed (g, dry weight).

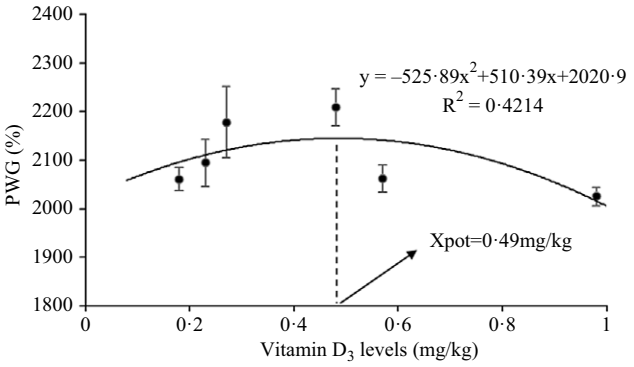

Fig. 1. Relationship between the percent weight gain and the vitamin D_3 levels. Quadratic regression analysis was used to determine the optimal vitamin D_3 requirement. Xpot represents the optimal dietary vitamin D_3 level for the maximum percent weight gain in *Litopenaeus vannamei*. PWG, percent weight gain.

Discussion

The present study clearly demonstrated that the growth performance of *L. vannamei* was significantly influenced by the dietary vitamin D_3 supplementation levels, and shrimp feeding 0·48 mg/kg of vitamin D_3 diet achieved the best growth performance. Further quadratic regression analysis showed that the optimum dietary vitamin D_3 requirement based on PWG was determined to be about 0·49 mg/kg. In a series of early work on vitamin D_3 nutrition showed that dietary essentiality of vitamin D_3 to maintain normal growth and a diet supplied with appropriate amount of vitamin D_3 could exert a positive effect on the growth of aquatic animals. In general, feed nutrition levels (purified diet or commercial feed), a consequence of a faulty managed feed, species and environmental condition might be partially responsible for the difference in optimal vitamin D_3 requirement in aquatic animals. Lock et al. (2010) summarised the minimum dietary requirement for vitamin D_3 in fish, including *Monopterus albus* (0·125 mg/kg), *Salmo gairdneri* (0·04 mg/kg), *Ictalurus punctatus* (0·006 mg/kg), *Salmo salar* (0·06 mg/kg) and *Oreochromis niloticus* × *O. aureus* (0·009 mg/kg)[10]. As for crustacean species, the dietary vitamin D_3 requirement of juvenile grass shrimp (*Penaeus monodon*) was 0·1 mg/kg (purified diets)[16]. The optimal vitamin D_3 requirement for larval crabs (*Eriocheir sinensis*) was 4825–5918 μg/kg (0·12 mg/kg–0·15 mg/kg), and crabs fed 9000 μg/kg (0·225 mg/kg) showed the highest survival rate after 120-h salinity stress[17]. Moreover, growth performance was not affected by the supplementation of vitamin D_3 (0·017 mg–0·081 mg/kg) for juvenile *L. vannamei* at low salinity rearing conditions (10–15 g/l)[2]. Interestingly, our result demonstrated that optimal vitamin D_3 requirement for *L. vannamei* was higher than other aquatic animals. The reason for the differences in vitamin D_3 requirements may be partially related to species, feed nutrition levels (protein level, lipid level, etc), initial weight and breeding density. However, the specific molecular mechanism is unknown at present, which needs to be further explored.

The primary biological function of vitamin D_3 is to maintain normal Ca and P homoeostasis by enhancing the absorption ability from the intestine[20,21]. Currently, the research regarding the influence of vitamin D_3 on the tissue Ca and P deposition in crustaceans is limited. The body Ca and P deposition was not affected by dietary vitamin D supplementation (0·1–62·5 mg/kg ergocalciferol or cholecalciferol) in juvenile grass shrimp (*Penaeus monodon*)[16]. In our experiment, carapace Ca and P concentrations increased with increasing vitamin D_3 supplementation levels (0·48–0·98 mg/kg), which confirmed vitamin D_3 function in Ca and P homoeostasis of *L. vannamei*. Cal and P absorption is mediated by both an active transcellular pathway and a passive paracellular pathway through tight junctions. $1,25(OH)_2D$, the hormonally active form of vitamin D, increases intestinal transcellular Ca and P absorption at least in part by enhancing expression of cotransporter[22,23]. Similar to our study, Coloso et al. (2001) reported that the dietary combination of low P (0·3 %) of 10 000 μg/kg (0·25 mg/kg) vitamin D_3 decreased soluble and faecal P levels in the effluent of rainbow trout *Oncorhynchus mykiss*[24]. Sundell *et al.* (1990) suggested that $25(OH)D_3$ and $24,25(OH)_2D_3$ that might be active regulators of Ca^{2+} transport across the intestinal mucosa were detected in *Atlantic cod*[25]. Miao et al. (2015) also found that whole body Ca content was increased with different dietary vitamin D_3 supplementation levels (0–0·2 mg/kg) in Wuchang bream (*Megalobrama amblycephala*)[15].

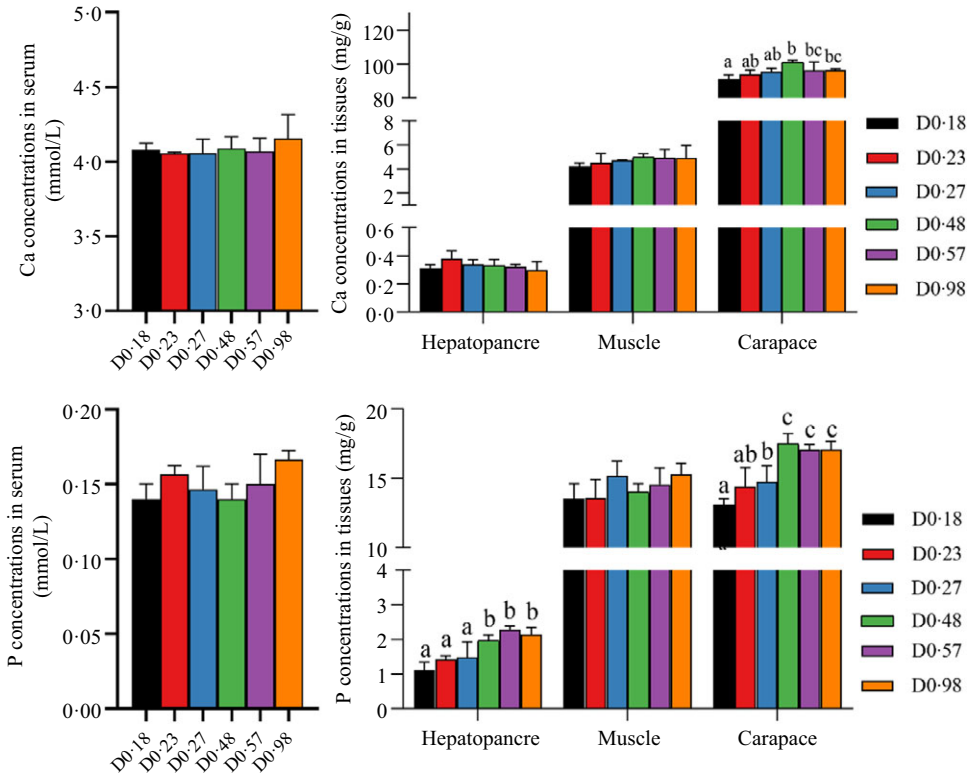

Fig. 2. Effects of different dietary vitamin D_3 supplementation levels on the calcium and phosphorus concentration in tissues of *Litopenaeus vannamei*. Data presented are mean ± SE (*n* 3), and different letters above bars represent significant differences between different treatments ($P < 0·05$).

It is now clear that vitamin D_3 has additional physiological function beyond its classic effect on Ca and P absorption. It has been suggested that the active form of vitamin D_3 may be shown as a membrane antioxidant. Our study showed that supplementing 0·48 mg/kg of vitamin D_3 significantly increased the activities of T-AOC and CAT in serum and hepatopancreas of *L. vannamei*. Meanwhile, the expression levels of antioxidant-related genes (*sod, gsh* and *cat*) were increased in hepatopancreas of *L. vannamei*. Antioxidant enzymes such as SOD, GSH and CAT participated in delaying or preventing the oxidation of cellular oxidisable substrates, and therefore protected cells against oxidative damage[26,27]. This finding suggested that dietary supplementing 0·48 mg/kg of vitamin D_3 could improve antioxidant capacity in *L. vannamei*. It has been reported that vitamin D_3 protected cell membranes against free radical-induced lipid peroxidation through interaction with phospholipid fatty acid side chains, then increased stabilisation of the membrane structure[28,29]. Mechanistic study demonstrated that the antioxidant role of vitamin D_3 was vitamin D receptor (VDR)-mediated transcriptional down-regulation of NOX2, a major isoform of NADPH oxidase, and up-regulation of Nrf2-keap1-mediated antioxidant pathways[30]. Similarly, some reports have demonstrated that dietary vitamin D_3 could improve antioxidant capacity in sea cucumber and pearl oyster[12,31]. Liu et al. (2021) reported that AOC and GSH activities were higher in the hepatopancreas of Chinese mitten crab (*Eriocheir sinensis*) fed with 6000 μg/kg (0·15 mg/kg) vitamin D_3 when compared with the control group (without extra vitamin D_3 supplementation)[17].

Increasing evidence suggested that vitamin D_3 could modulate the innate and adaptive immune responses and has been used to treat various infections before the advent of effective antibiotics[32]. It has been reported that vitamin D_3 could also affect the immune function in some fish species[33]. Like other invertebrates, non-specific immunity is shrimp's main defence against pathogens[34,35]. LZM, ALP, NOS, ACP and PO are identified as important immune indices of shrimp. NO, which is produced from L-arginine catalysed by the enzyme NOS, has be shown to be beneficial for defensing against pathogens[36]. LZM has the capacity to hydrolyse bacterial cell walls that simultaneously regulate the synthesis and secretion of other immune factors, while ACP and ALP are important parts of lysosomal enzymes of crustaceans[37]. The major enzyme produced during proPO system activation is phenoloxidase, which oxidises phenolic compounds to produce quinones and help to kill pathogens[38]. In the present study, we observed that both the activity of ALP, ACP, NOS and immune-related genes (*alp, acp* and *lzm*) expression in hepatopancreas increased and then decreased with increasing vitamin D_3 supplementation levels, while numeric value reached the highest in the 0·48 vitmain D_3 mg/kg supplementation level. This result seems to show that dietary vitmain D_3 at 0·48 mg/kg

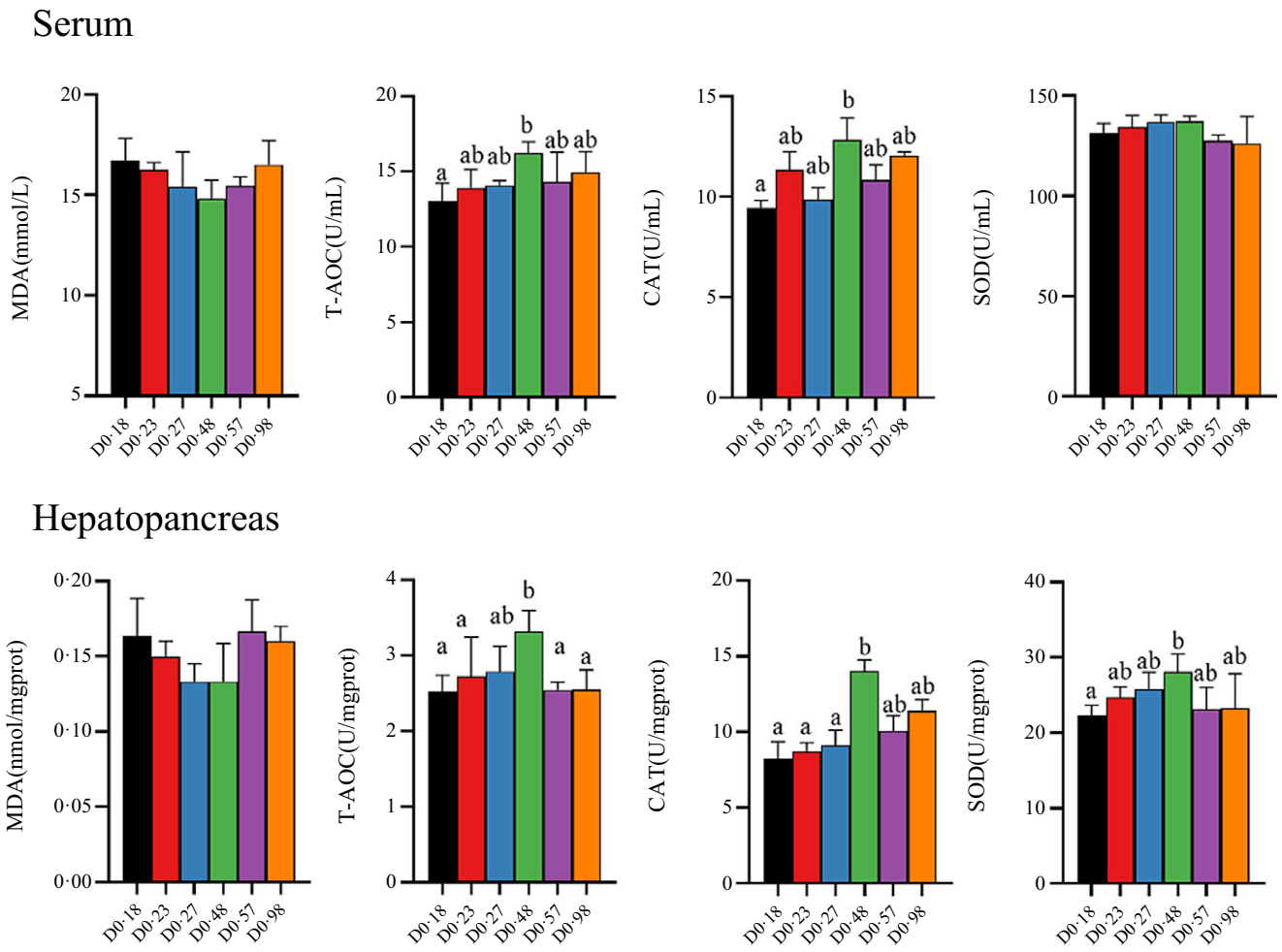

Fig. 3. Effects of different dietary vitamin D_3 supplementation levels on the antioxidant enzyme activities in serum and hepatopancreas of *Litopenaeus vannamei*. Data presented iare mean ± SE (n 3), and different letters above bars represent significant differences between different treatments ($P < 0{\cdot}05$). CAT, catalase; SOD, superoxide dismutase; T-AOC, total antioxidative capacity; MDA, malondialdehyde.

supplementation level exerted the best immune function in *L. vannamei*. Recent research has demonstrated that vitamin D_3 could exert its immunomodulatory actions via down-regulating inflammation-mediated signalling pathway (ROCK/NF-κB, JAK and STAT) and acitivating an anti-inflammatory action (up-regulation of the anti-inflammatory toll-like receptor)[30]. Similarly, Dioguardi et al. (2017) confirmed that a stimulation of phagocytosis and peroxidase activity of serum was observed in European sea bass (*Dicentrarchus labrax L.*) fed with vitamin D_3 (0·09 mg/kg–0·9 mg/kg) diets[39]. Liu et al. (2021) observed that the mRNA expression of LZM was upregulated with 9000 μg/kg (0·225 mg/kg) vitamin D_3 after 23-d feeding trial, suggesting that dietary vitamin D_3 could enhance immunity in *E. sinensis*[17]. Shiau and Hwang (1994) reported that adding 0·2 mg/kg of vitamin D_3 significantly increased ALP activity in juvenile grass shrimp (*Penaeus monodon*)[16].

Epidemiologically, the function of vitamin D is also related to lipid metabolism. Certain studies have suggested the vitamin D_3 negatively regulated the expression of various lipogenic genes in both the adipose tissue and liver in mammals[40,41]. Until now, the effects of vitamin D on lipid metabolism have not been studied much in aquatic animals. One recent paper demonstrated that vitamin D_3 deficiency induced retarded growth and excessive visceral adipose tissue in zebrafish using Cyp2r1 gene knockout model[6]. In our experiment, we found that supplementing different vitamin D_3 levels could influence hepatopancreas lipid metabolism. Expression of adipogenesis-related genes (*srebp, acc1* and *fas*) increased and then decreased in hepatopancreas of *L. vannamei*. Supplementing 0·48 mg/kg of vitamin D_3 achieved highest expression of lipid synthesis-related genes, which might benefit the improved growth performance. In addition, the expression of various lipolysis genes (*cpt1, fabp, fatp and aco*) showed an upward trend with the increased dietary vitamin D_3 supplemental levels, and supplementing 0·98 mg/kg of vitamin D_3 significantly increased the expression of lipolysis genes in hepatopancreas of *L. vannamei*. This result indicated that vitamin D_3 might modulate lipolysis with increased supplementation levels. Previous experimental studies in mammals might support our guess. Vitamin D can control energy metabolism in adipose tissue by affecting fatty acid

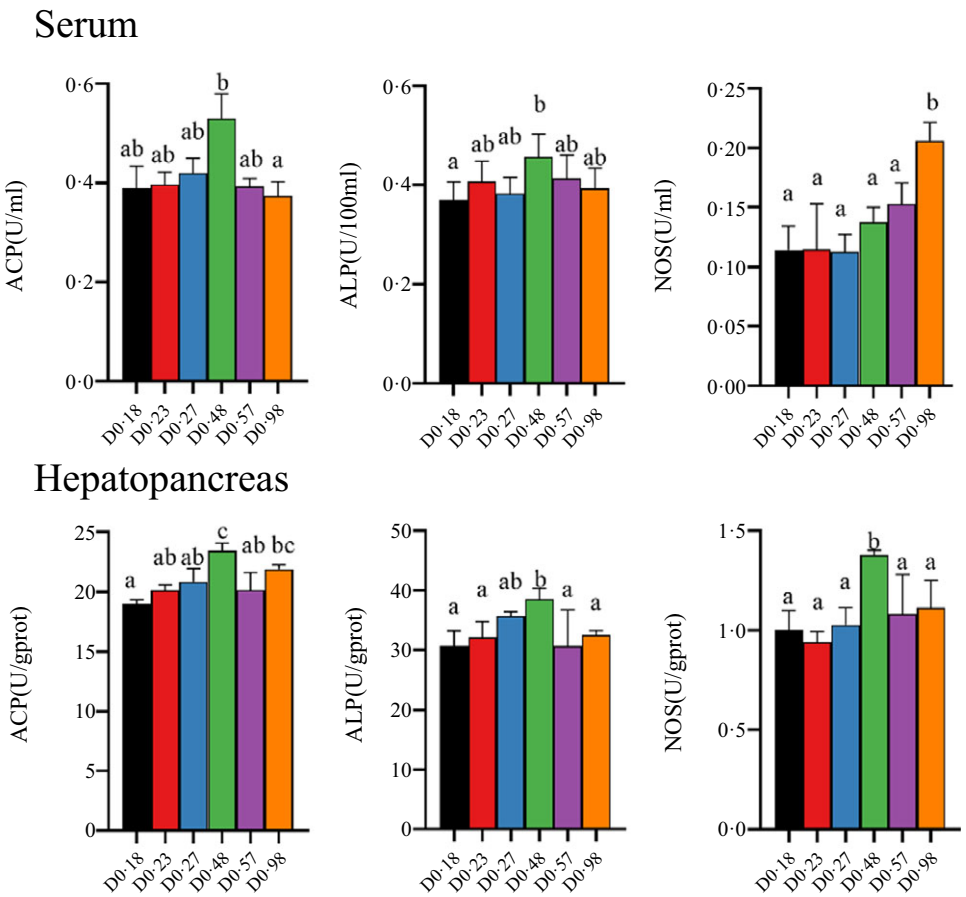

Fig. 4. Effects of different dietary vitamin D_3 supplementation levels on the immune enzyme activities in serum and hepatopancreas of *Litopenaeus vannamei*. Data presented are mean ± SE (n 3), and different letters above bars represent significant differences between different treatments ($P < 0{\cdot}05$). ALP, alkaline phosphatase; ACP, acid phosphatase; NOS, nitric oxide synthase; PO, phenoloxidase.

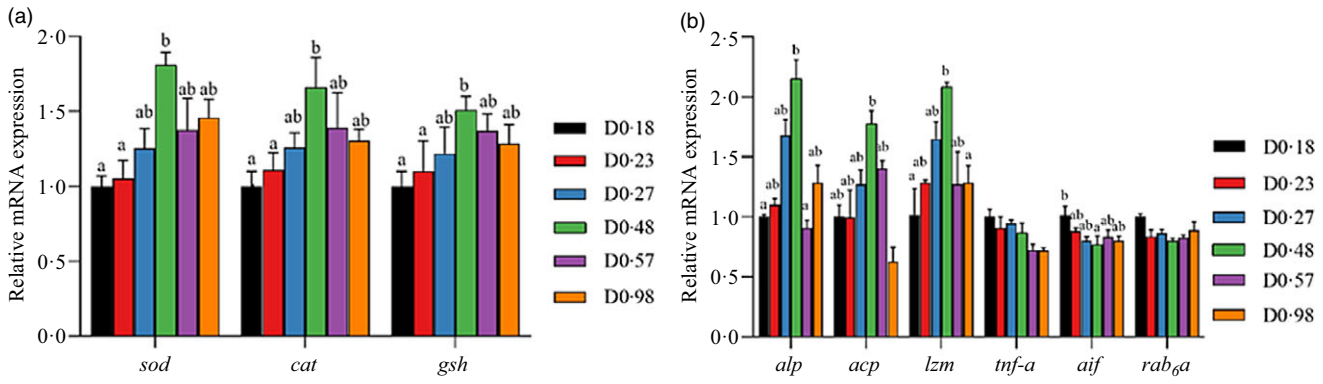

Fig. 5. Effects of different dietary vitamin D_3 supplementation levels on the expression of genes involved into antioxidant and immune status in hepatopancreas of *Litopenaeus vannamei*. (a) Relative mRNA expression of antioxidant genes. (b) Relative mRNA expression of immune-asscіated genes. Data presented are mean ± SE (n 3), and different letters above bars represent significant differences between different treatments ($P < 0{\cdot}05$). *gsh*, Glutathione; *cat*, catalase; *sod*, superoxide; *aif*, apoptosis-inducing factor; *rab6a*, member RAS oncogene family.

oxidation, expression of uncoupling proteins, insulin resistance and adipokine production[42]. This function of vitamin D_3 has be supported by previously published experiments on VDR knockout and overexpression models. It has been reported that 0·375 mg/kg of vitamin D_3 could protect against diet-induced obesity possibly by up-regulating genes involved in fatty acid oxidation and mitochondrial metabolism which led to increased energy expenditure[43]. Similarly, intraperitoneal injection of

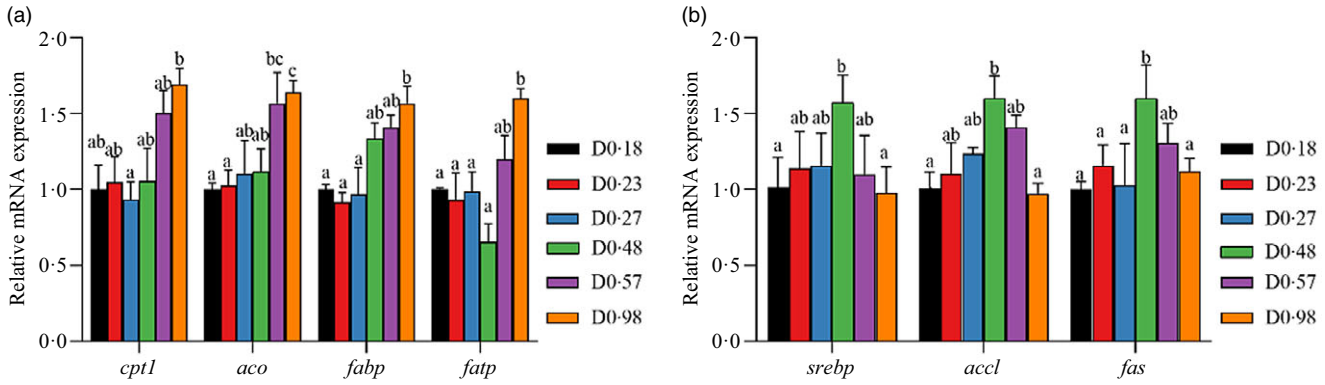

Fig. 6. Effects of different dietary vitamin D_3 supplementation levels on the expression levels of lipid metabolism-related genes in hepatopancreas of *Litopenaeus vannamei*. (a) Relative mRNA expression of lipolysis-related genes. (b) Relative mRNA expression of adipogenesis-related genes. Data presented are mean ± SE (n 3), and different letters above bars represent significant differences between different treatments ($P < 0{\cdot}05$). *srebp*, Sterol-regulatory element binding protein; *acc1*, acetyl-CoA carboxylase 1; *fas*, fatty acid synthetase gene; *fatp*, fatty acid transport proteins; *fabp*, atty acid binding protein; *cpt1*, arnitine palmitoyltransferase 1; *aco*, acyl-CoA oxidase.

vitamin D_3 (1–5 μg/kg) could prevent high-fat diet-induced hepatic steatosis through the inhibition of lipogenesis and the promotion of fat acid oxidation in liver[44].

Conclusion

The results of present study indicated that dietary supplementation of 0·48 mg/kg of vitamin D_3 could increase Ca and P concentraions, improve antioxidant capacity and immune response, and influence lipid metabolism in *L.vannamei*.

Acknowledgement

This research was supported by National Key R & D Program of China (2018YFD0900400), Ningbo Public Welfare Science and Technology Project (202002N3041), Industrial Chain Collaborative Innovation Project of the Demonstration Work on Innovative Development of the Marine Economy of the State Oceanic Administration (NBHY-2017-S2), K. C. Wong Magna Fund in Ningbo University.

TM. Dai conducted feeding experiments and drafted the article; LF. J designed this experiment, supervised project administration and revised the article; XY. T and JJ. L assisted in sampling and data analysis. M. J and P. S helped reviewing. QC. Z acquisited funding, supervised project administration, and gave constructive advice and valuable help during paper review.

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.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114521004931

References

1. Lu Z, Chen TC, Zhang A, *et al.* (2007) An evaluation of the vitamin D_3 content in fish: is the vitamin D content adequate to satisfy the dietary requirement for vitamin D? *J Steroid Biochem Mol Biol* **103**, 642–644.
2. Wen ML, Liu YJ, Tian LX, *et al.* (2015) Vitamin D_3 requirement in practical diet of white shrimp, *Litopenaeus vannamei*, at low salinity rearing conditions. *J World Aquacult Soc* **46**, 531–538.
3. DeLuca HF (1979) The vitamin D system in the regulation of calcium and phosphorus metabolism. *Nutr Rev* **37**, 161–193.
4. Bikle DD (2014) Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol* **21**, 319–329.
5. Prabhu AV, Luu W, Sharpe LJ, *et al.* (2016) Cholesterol-mediated degradation of 7-dehydrocholesterol reductase switches the balance from cholesterol to vitamin D synthesis. J Biol Chem 291, 8363–8373.
6. Peng X, Shang G, Wang W, *et al.* (2017) Fatty acid oxidation in zebrafish adipose tissue is promoted by 1α,25$(OH)_2D_3$. *Cell Rep* **19**, 1444–1455.
7. Zügel U, Steinmeyer A, May E, *et al.* (2009) Immunomodulation by a novel, dissociated Vitamin D3 analogue. Exp Dermato 18, 619–627.
8. Holick MF (1994) McCollum Award Lecture, 1994: vitamin D – new horizons for the 21st century. *Am J Clin Nutr* **60**, 619–630.
9. Lambert J (2007) Vitamin D deficiency. *Br J Gen Pract* **57**, 669.
10. Lock EJ, WaagbÃ˜ R, Wendelaar Bonga S, *et al.* (2010) The significance of vitamin D for fish: a review. Aquacult Nutr 16, 100–116.
11. Ling-Hong M, Xian-Ping G, Jun X, *et al.* (2015) Dietary vitamin D_3 requirement of Wuchang bream (*Megalobrama amblycephala*). *Aquaculture* **436**, 104–109.
12. Yang C, Du X, Hao R, *et al.* (2019) Effect of vitamin D_3 on immunity and antioxidant capacity of pearl oyster Pinctada fucata martensii after transplantation: insights from LC-MS-based metabolomics analysis. *Fish Shellfish Immunol* **94**, 271–279.
13. Cheng K, Ma C, Guo X, *et al.* (2020) Vitamin D_3 modulates yellow catfish (*Pelteobagrus fulvidraco*) immune function *in vivo* and *in vitro* and this involves the vitamin D_3/VDR-type I interferon axis. *Dev Comp Immunol* **107**, 103644.
14. Soto-Davila M, Valderrama K, Inkpen SM, *et al.* (2019) Effects of vitamin D_2 (Ergocalciferol) and D_3 (Cholecalciferol) on Atlantic Salmon (*Salmo salar*) primary macrophage immune response to aeromonas salmonicida subsp. salmonicida Infection. *Front Immunol* **10**, 3011.
15. Miao LH, Xie J, Ge XP, *et al.* (2015) Chronic stress effects of high doses of vitamin D_3 on *Megalobrama amblycephala*. *Fish Shellfish Immunol* **47**, 205–213.

16. Shiau SY & Wang H (1994) The dietary requirement of juvenile grass shrimp (*Penaeus monodon*) for vitamin D. *J Nutr* **124**, 2445–2450.
17. Liu S, Wang X, Bu X, *et al.* (2021) Influences of dietary vitamin D_3 on growth, antioxidant capacity, immunity and molting of chinese mitten crab (*Eriocheir sinensis*) larvae. *J Steroid Biochem* **210**, 105862.
18. Amaya EA, Davis DA & Rouse DB (2007) Replacement of fish meal in practical diets for the pacific white shrimp (*Litopenaeus vannamei*) reared under pond conditions. *Aquaculture* **262**, 393–401.
19. Alam SMM (2006) Safety in the Shrimp Supply Chain. *Regulating Safety of Traditional and Ethnic Foods,* 99–123.
20. Darias MJ, Mazurais D, Koumoundouros G, *et al.* (2011) Overview of vitamin D and C requirements in fish and their influence on the skeletal system. *Aquaculture* **315**, 49–60.
21. Darias MJ, Mazurais D, Koumoundouros G, *et al.* (2010) Dietary vitamin D_3 affects digestive system ontogenesis and ossification in european sea bass (*Dicentrachus labrax*, Linnaeus, 1758). *Aquaculture* **298**, 300–307.
22. Christakos S, Dhawan P, Porta A, *et al.* (2011) Vitamin D and intestinal calcium absorption. *Mol Cell Endocrinol* **347**, 25–29.
23. Fukumoto S (2014) Phosphate metabolism and vitamin D. *Bonekey Rep* **3**, 497.
24. Coloso RM, Basantes SP, King K, *et al.* (2001) Effect of dietary phosphorus and vitamin D_3 on phosphorus levels in effluent from the experimental culture of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **202**, 145–161.
25. Sundell K & Björnsson BT (1990) Effects of vitamin D3, 25 (OH) vitamin D3, 24, 25 (OH)2 vitamin D3, and 1, 25 (OH)2 vitamin D3 on the *in vitro* intestinal calcium absorption in the marine teleost. Atlantic Cod 78, 74–79.
26. Mates JM (2000) Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicol* **153**, 83–104.
27. Zhong W, Gu B, Gu Y, *et al.* (2014) Activation of vitamin D receptor promotes VEGF and CuZn-SOD expression in endothelial cells. *J Steroid Biochem Mol Biol* **140**, 56–62.
28. Boakye D, Jansen L, Schöttker B, *et al.* (2021) The association of vitamin D with survival in colorectal cancer patients depends on antioxidant capacity. *Am J Clin Nutr* **113**, 1458–1467.
29. Wolden-Kirk H, Gysemans C, Verstuyf A, *et al.* (2012) Extraskeletal effects of vitamin D. *Endocrin Metab Clin* **41**, 571–594.
30. Kim HA, Perrelli A, Ragni A, *et al.* (2020) Vitamin D deficiency and the risk of cerebrovascular disease. *Antioxidants* **9**, 327.
31. Wang L, Baoshan LI, Wang J, *et al.* (2019) Effects of dietary vitamin D3 on growth performance, body composition, and antioxidant capacity of the juvenile sea cucumber. Progr Fish Ences 40, 110–118.
32. White JH (2012) Vitamin D metabolism and signaling in the immune system. *Rev Endocr Metab Disord* **13**, 21–29.
33. Cerezuela R, Cuesta A, Meseguer J, *et al.* (2009) Effects of dietary vitamin D_3 administration on innate immune parameters of seabream (*Sparus aurata L.*). *Fish Shellfish Immunol* **26**, 243–248.
34. Li F & Xiang JJD (2013) Recent advances in researches on the innate immunity of shrimp in china. *Dev Comp Immunol* **39**, 11–26.
35. Jia XY, Wang F, Lu YL, *et al.* (2014) Immune responses of *Litopenaeus vannamei* to thermal stress: a comparative study of shrimp in freshwater and seawater conditions. *Mar Freshw Behav Phy* **47**, 79–92.
36. Chakravortty D & Hensel MJM (2003) Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes Infect* **5**, 621–627.
37. Liu T, Zhang G, Feng Y, *et al.* (2019) Dietary soybean antigen impairs growth and health through stress-induced non-specific immune responses in pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol* **84**, 124–129.
38. Cerenius L & Soderhall K (2004) The prophenoloxidase-activating system in invertebrates. *Immunol Rev* **198**, 116–126.
39. Dioguardi M, Guardiola FA, Vazzana M, *et al.* (2017) Vitamin D_3 affects innate immune status of european sea bass (*Dicentrarchus labrax L.*). *Fish Physiol Biochem* **43**, 1161–1174.
40. Kang EJ, Lee JE, An SM, *et al.* (2015) The effects of vitamin D_3 on lipogenesis in the liver and adipose tissue of pregnant rats. *Int J Mol Med* **36**, 1151–1158.
41. Lee H, Lee H & Lim Y (2020) Vitamin D_3 improves lipophagy-associated renal lipid metabolism and tissue damage in diabetic mice. *Nutr Res* **80**, 55–65.
42. Chan YP & Han SN (2012) The role of vitamin D in adipose tissue biology: adipocyte differentiation, energy metabolism, and inflammation. *J Lipid Atheroscler* **10**, 130.
43. Marcotorchino J, Tourniaire F, Astier J, *et al.* (2014) Vitamin D protects against diet-induced obesity by enhancing fatty acid oxidation. *J Nutr Biochem* **25**, 1077–1083.
44. Yin Y, Yu Z, Xia M, *et al.* (2012) Vitamin D attenuates high fat diet–induced hepatic steatosis in rats by modulating lipid metabolism. *Eur J Clin Invest* **42**, 1189–1196.

British Journal of Nutrition