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REVIEW

REVIEWS IN Aquaculture

Physiological function of vitamin D_3 in fish

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Abstract

Vitamin D (VD) is a steroid hormone, of which vitamin D_3 (VD_3) is the main form in fish. Fish obtain VD_3 majorly from diets, and VD_3 has been widely used in aquatic feed as feed additive. As one of the essential nutrients, VD_3 plays an indispensable role in the growth and health of fish. Due to the particularity of the fish living environment, the metabolic and physiological functions of VD_3 in fish have some differences from those of mammals. This paper reviews the metabolic process of VD_3 in fish, the main metabolites and vitamin D receptors, compares the differences in VD_3 metabolism between fish and mammals, summarises the recommended dietary requirements for VD_3 in several fish species, generalises the role of VD_3 in fish growth performance, bone development, lipid metabolism, immune regulation, antioxidant and other potential physiological functions, as well as its synergistic effects with other nutrients. Future perspectives of VD_3 application in aquaculture are proposed. Understanding the metabolic process and physiological function of VD_3 is of great significance for extending VD_3 application in the aquatic feed industry.

KEYWORDS
fish, immunoregulation, nutritional effect, recommended dosage, vitamin D_3

1 | INTRODUCTION

Vitamin D (VD) is a fat-soluble vitamin and a steroid hormone that can exist in two natural forms: one being ergocalciferol (vitamin D_2 [VD_2]) that is predominantly found in plants, the other is cholecalciferol (vitamin D_3 [VD_3]) coming from animal sources.[1] These two forms of VD can be hydroxylated in the liver to 25-hydroxy metabolites, which then undergo a second hydroxylation in both the liver and the kidney to produce active VD_3 (i.e., $1,25(OH)_2D_3$) in fish.[2,3] In mammals, 7-dehydrocholesterol undergoes photolysis to produce endogenous VD when ultraviolet rays irradiate the skin,[4,5] but fish in the natural environment can barely produce VD in this manner, and they can only meet their physiological VD needs through dietary sources, such as ingesting phytoplankton and zooplankton in water. In contrast, cultured fish species rely mostly on supplementation of VD via commercial diets. Previous studies have found that VD_3 is more bioavailable than VD_2 in fish and shrimps[2,6,7]; thus, VD_3 is usually added to aquatic feed in the form of a dry spray or enclosed beadlets to support the normal growth and metabolism of fish and shrimps.[1]

VD_3 was first recognised and is best known for the function of regulating calcium and phosphorus metabolism and bone mineralisation. In addition, VD_3 has hormone-like activity that widely contributes to the maintenance of animal health. Studies in mammals over the past decade have confirmed that beyond bone mineralisation, VD_3 can also regulate nutritional metabolism and immune function to optimise resistance and responsiveness to bacterial and viral infections and cancers.[8–16] The VD endocrine system in fish appears to function similarly as in mammals[17]; however, the function of VD_3 in fish and its mechanism of action are still relatively poorly studied compared with mammals. This review presents a general picture of the possible physiological function of VD_3 in fish, especially in terms of fish growth, bone development, antioxidant status and immunity as well as lipid metabolism, and compares the differences in VD_3 metabolism between fish and mammals.

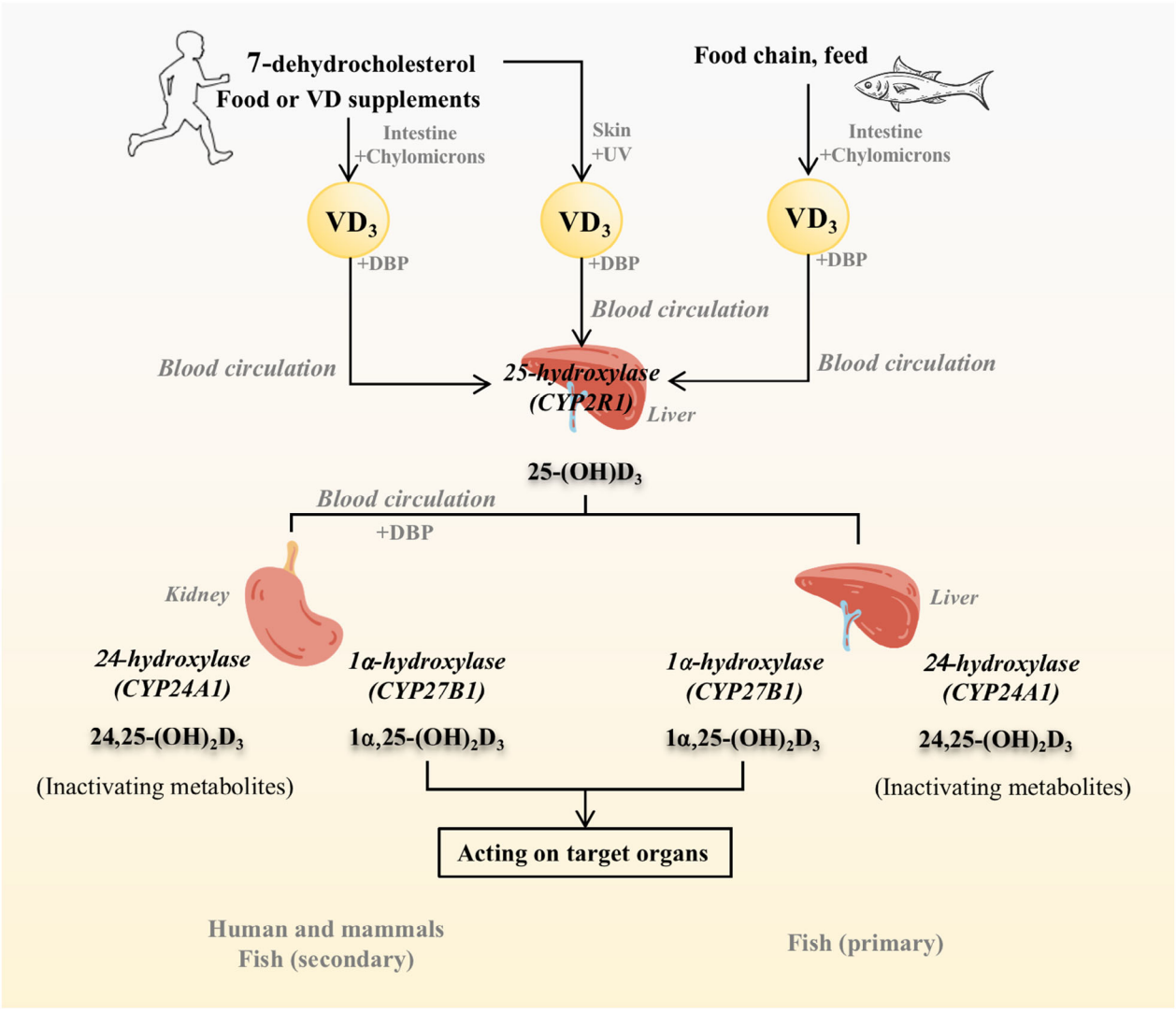

FIGURE 1 Main metabolic pathways and products of vitamin D_3 in higher animals and fish. Mammals and fish are able to absorb VD_3 from food, where fat-soluble VD_3 is absorbed and transported through chylomicrons in the intestine and then transported to the liver via plasma DBP. Terrestrial mammals can also synthesise VD_3 through the skin, and this part of VD_3 is transported directly to the liver by DBP. In fish and mammals, VD_3 is first hydroxylated to 25(OH)D_3 under the action of CYP27R1. In mammals, 25(OH)D_3 produced in the liver is again bound to DBP and transported to the kidney to be further hydroxylated to 1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ under the function of CYP27B1 and CYP24A1, respectively. The conversion of 25(OH)D_3 to biologically form-1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ in fish occurs primarily in the liver and possibly also in the kidney. The bioactive 1α,25(OH)$_2D_3$ subsequently acts on target organs. DBP, vitamin D binding protein; VD_3, vitamin D_3.

2 | METABOLISM AND BIOLOGICAL PROCESSES OF VD_3

2.1 | Comparison of VD_3 absorption and metabolism between mammals and fish

Previous studies have shown that VD_3 intake and utilisation is higher in fish compared to VD_2.[7] Throughout life, fish can bioaccumulate dietary VD_3, and although it is mainly deposited in the liver, it also accumulates in other organs such as kidney, intestine, spleen and gills.[18–21] Vitamin D binding protein (DBP), also abbreviated as Gc protein, was first reported by Hay and Watson as a lipoprotein that transports 25(OH)D_3 in cartilaginous and bony fish.[22] DBP is a trace hepatic plasma protein that transports VD and its metabolites in circulation, and can play an important role in maintaining the homeostasis of VD content and regulating the level of free VD.[17,23] As shown in Figure 1, both mammals and fish are able to absorb VD_3 from food, where fat-soluble VD_3 is absorbed and transported through chylomicrons in the intestine and then transported to the liver via plasma DBP.[24,25] The difference is that terrestrial mammals can also synthesise VD_3 through the skin, and this part of VD_3 is transported directly to the liver by DBP.[25] VD_3 is first hydroxylated to 25(OH)D_3 under the action of hepatic 25-hydroxylase (25-OHase), namely CYP27R1, both in fish and mammals. However, some class differences exist regarding organs for the second hydroxylation of 25(OH)D_3 into bioactive 1,25(OH)$_2D_3$ between fish and mammals.[26] In mammals, for example, 25(OH)D_3

produced in the liver is again bound to DBP and transported to the kidney to be further hydroxylated to $1,25(OH)_2D_3$ under the function of 1-α hydroxylase enzyme (1-OHase) (such as CYP27B1). In contrast, the conversion of $25(OH)D_3$ to biologically form-$1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ in fish occurs primarily in the liver and possibly also in the kidney due to the findings of two isotypes of 1-OHase, one is renal 1-OHase, both in seawater and freshwater fish,[27] and the other is hepatic 1-OHase.[2,28,29] An earlier study also pointed out that the second-step hydroxylation of VD_3 in fish can also occur in the intestines, muscle and gills of seawater-adapted European eel.[30]

Two other obvious differences in VD_3 metabolism were also found between fish and mammals. First, the content of VD_3 metabolites in body circulation is different. In humans, the level of $25(OH)D_3$ in serum is generally used to assess the human VD status due to the short half-life of $1,25(OH)_2D_3$ (4–6 h).[31,32] However, in fish, both 25(OH)D and $1,25(OH)_2D_3$ can be detected. Avioli et al. found that $1,25(OH)_2D_3$ was detected in fish plasma, with levels being 10 times higher than those detected in mammals.[33] $1,25(OH)_2D_3$ content in seawater-adapted Atlantic salmon (*Salmo salar*) was 55 times higher than detected in humans.[34] Another unique difference in VD_3 metabolism between fish and mammals is the storage amount of VD_3 in body; fish can store large amounts of VD_3 in the body, especially in liver and fat tissues, without being catabolised[17]; however, unused $1,25(OH)_2D_3$ and excess $25(OH)D_3$ are excreted from the body in mammals.[35] This makes fish an excellent source of VD_3 for human consumption.

2.2 | Vitamin D receptors

Vitamin D receptors (VDRs) can be divided into two categories: nuclear VDR (nVDR) and membrane VDR (mVDR). $1,25(OH)_2D_3$ can bind to these two specific VDRs to exert biological functions, which include regulating the absorption of calcium and phosphorus, increasing their concentrations in the blood, and maintaining their steady state.[36,37] Once $1,25(OH)_2D_3$ binds to nVDR, this complex forms a heterodimer with the retinol X receptor (RXR), which is then translocated into the nucleus where the heterodimer acts as a transcription factor after binding to the vitamin D response element (VDRE) within target genes; this can lead to activation or inhibition of target gene transcription.[38] The combination of $1,25(OH)_2D_3$ and mVDR/nVDR also regulates cell function through non-genomic pathways, which includes signals being transmitted through a pathway mediated by protein kinase C and the opening of L-shaped calcium channels on the cell membrane, which facilitates calcium influx and stimulates the release of calcium ions stored in the endoplasmic reticulum leading to a rapid increase in calcium ion concentration.[36,39,40] The sequences of two VDR subtypes of Japanese flounder (*Paralichthys olivaceus*) were identified,[41] which was the first reported sequence of bony fish nVDR mRNA. An mVDR in the enterocyte basal lateral membrane of carp was also identified in the same year.[37] The VDR has to date been sequenced in many species including the zebrafish, Japanese puffer (*Takifugu rubripes*), Atlantic salmon, common carp and yellow catfish (*Pelteobagrus fulvidraco*).[41–44] VDR has been found to commonly expressing in various fish tissues, which reflects the pleiotropic nature of the VD endocrine system.[34,41,43,45] Moreover, several studies have reported the presence of two isoforms of nVDR (nVDRα and nVDRβ) in fish.[41,46,47] In the zebrafish studies, $1,25(OH)_2D_3$ binding to nVDRα showed higher activation effect than binding to nVDRβ, and further studies found that nVDRα function is mainly associated with calcium and phosphorus metabolism,[48] while nVDRβ is mainly involved in regulation of organ development.[47,49] The ubiquitous expression of VDR in many different tissues indicates that the function of VD_3 is far beyond its actions on calcium homeostasis and bone development.[17]

3 | PHYSIOLOGICAL FUNCTION OF VD_3 IN FISH

In 1979, VD_3 was first recognised to be a very important requirement of rainbow trout diets.[50] Since then, VD_3 has become recognised as an essential nutrient in fish diets. A large number of studies have confirmed that VD_3 has a very wide range of physiological effects. The next section summarises the physiological effects of VD_3 on fish in terms of growth performance, skeleton health, lipid metabolism, immune and antioxidant function, and describes the different uses of VD_3 in aquaculture practice in the past several years (Table 1), while studies in the model organism zebrafish (*Danio rerio*) are presented in Table 2.

3.1 | Growth performance and feed utilisation

Studies have verified the positive effects of optimal range of dietary VD_3 on growth and feed utilisation. For example, Li et al. found that adding 250–2000 IU/kg of VD_3 to the diet could increase the weight gain (WG) and protein efficiency ratio (PER) of Asian swamp eel (*Monopterus albus*), while reducing the feed conversion rate (FCR).[51] Zhang et al. reported that WG, feed efficiency ratio (FER), body ash, and calcium and phosphorus levels of juvenile sea bass increased within the dietary VD_3 range of 34.2–393.8 IU/kg.[56] Xie et al. confirmed that the WG and FER of juvenile orange-spotted grouper (*Epinephelus coioides*) were the highest in the 2000 IU/kg dietary VD_3 group. Meanwhile, the juvenile orange-spotted grouper fed with high doses of VD_3 (8000 IU/kg) had lower crude protein content in whole fish and muscle than in the other treated groups.[60] This may mean that feeding on high doses of VD_3 is detrimental for protein accumulation in fish, but no more relevant studies have been reported yet to confirm this. Subsequently, He et al. conducted a study on mid-stage orange-spotted grouper and showed that, in the range of 0–4000 IU/kg dietary VD_3, WG increased with the increase in VD_3 concentration. FCR was the lowest, while body crude protein was the highest in the group containing 1000 IU/kg dietary VD_3.[62] Another study indicated that juvenile black carp (*Mylopharyngodon piceus*) had the highest WG when supplemented with 534.2 IU/kg VD_3, while 412, 840 and 1480 IU/kg dietary VD_3 could increase PER and decrease FCR.[61]

TABLE 1 Effect of dietary VD_3 on the physiological function of aquaculture fish species.

Species	Initial weight	VD/VD_3 level Test range-IU/kg (mg/kg)	Remarks		References
			Non-negative effects	Negative effects	
Asian swamp eel (*Monopterus albus*)	21.7 ± 2.1 g	VD_3: 0–4000 (0–0.1)	500–1000 IU/kg (0.013–0.025 mg/kg): WGR ↑; PER ↑; FCR ↓; immunologic function ↑	0 or > 1000 IU/kg (0.025 mg/kg): immunologic function ↓	51
Yellow catfish (*Tachysurus fulvidraco*)	3.0 ± 0.00 g	VD_3: 2000, 4000 (0.05, 0.1)	Whole body Na, K, P, Ca, Mg and Zn retention ↑ growth performance →	–	52
Wuchang bream (*Megalobrama amblycephala*)	17.71 ± 0.22 g	VD_3: 0–8000 (0–0.2)	① Crude protein, crude lipid, ash ↑; P → ② 0–1000 IU/kg (0–0.025 mg/kg): plasma calcium ↑; 2000, 8000 IU/kg (0.05, 0.2 mg/kg): plasma cholesterol, triglycerides, insulin ↑	–	53
White shrimp (*Litopenaeus vannamei*)	0.39 ± 0.01 g	VD_3: 685–7750 (0.017–0.194)	① Growth performance →; alkaline phosphatase activity in hepatosomatic → ② Moisture and Zn ↓; protein, ash content, Ca, P, Mg ↑	–	54
Jian carp (*Cyprinus carpio* var. Jian)	12.58 ± 0.23 g	VD: 0, 2400 (0, 0.06)	Anti-inflammatory function in intestine ↑	–	55
Japanese seabass (*Lateolabrax japonicas*)	2.26 ± 0.03 g	VD: 34.2–3091.2 (0.001–0.077)	① 34.2–393.8 IU/kg (0.001–0.01 mg/kg): WGR, SGR, FER, PER ↑; >393.8 IU/kg: WGR, SGR, FER, PER → ② Liver VD content ↑; liver lipid content ↓	–	56
European sea bass (*Dicentrarchus labrax* L.)	100 ± 4.0 g	VD_3: 0–37,500 (0–0.938)	Phagocytic ability ↑; serum peroxidase ↑; protease, anti-protease, natural haemolytic complement activities, total IgM →	–	57
Siberian sturgeon (*Acipenser baerii*)	3.47 ± 0.14 g	VD_3: 60–10,000 (0.002–0.25)	① 450–3300 IU/kg (0.011–0.083 mg/kg): SGR ↑; 1670–3300 IU/kg (0.042–0.083 mg/kg): crude lipid, ash ↑ ② 880–3300 IU/kg (0.022–0.083 mg/kg): liver and serum $25(OH)D_3/1,25(OH)_2D_3$ ↑; 880–10,000 IU/kg (0.022–0.25 mg/kg): osteocalcin ↑	–	58
Tilapia (*Oreochromis nilticus*)	78.58 ± 1.93 g	VD: 0–3200 (0–0.08)	① WGR ↑; crude protein, moisture → ② 200 IU/kg (0.005 mg/kg): crude lipid, serum total cholesterol, albumin, total protein (peak value); 200–1600 IU/kg (0.005–0.04 mg/kg): FER ↑; 200–800 IU/kg (0.005–0.024 mg/kg): serum alkaline phosphatase activity ↑	–	59
Orange-spotted grouper (*Epinephelus coioides*)	18.00 ± 0.15 g	VD_3: 0–100,000 (0–2.5)	2000 IU/kg (0.05 mg/kg): WGR, SGR, FER ↑; >2000 IU/kg (0.05 mg/kg): IPF, VSI ↓; <2000 IU/kg (0.05 mg/kg): Ca, P in bone ↑	–	60
Black carp (*Mylopharyngodon piceus*)	4.73 ± 0.13 g	VD_3: 96–3008 (0.002–0.075)	① 412–1480 IU/kg (0.01–0.037 mg/kg): PER, LPO5, SOD,	–	61

(Continues)

TABLE 1 (Continued)

Species	Initial weight	VD/VD_3 level Test range-IU/kg (mg/kg)	Remarks		References
			Non-negative effects	Negative effects	
			CAT, GPX, GR, GSH, T-AOC, C3, C9, LYZ, HEPC ↑ ② 534.2 IU/kg (0.013 mg/kg): WGR, SGR (peak value)		
Yellow catfish (*Tachysurus fulvidraco*)	5.0 ± 0.2 g	VD_3: 1120–16,600 (0.028–0.415)	WGR →; serum CAT, LYZ ↑; serum SOD, MDA →; anti-bacterial ability ↑	–	44
Orange-spotted grouper (*Epinephelus coioides*)	81.50 ± 0.05 g	VD: 0–10,000 (0–0.25)	① 1000 IU/kg: WGR, SGR ↑; FCR ↓; crude protein ↑ ②1000, 2000 IU/kg (0.025, 0.05 mg/kg): liver lipid metabolism ↑	–	62
Gilthead seabream (*Sparus aurata*)	20.50 ± 0.3 g	VD_3: 5800–26,000 (0.145–0.65)	① WG →; liver VD_3 ↑ ② 11,600 IU/kg (0.29 mg/kg): skeletal anomalies ↓	>11,600 IU/kg (0.29 mg/kg): skeletal anomalies ↑; > = 20,000 IU/kg (0.65 mg/kg): myocarditis ↑	63
Abalone (*Haliotis discus hannai*)	2.06 ± 0.01 g	VD_3: 0–5000 (0–0.125)	1000 IU/kg (0.025 mg/kg): serum glucose, crude lipid ↓; affect glucose metabolism	–	64
Chinese mitten crab (*Eriocheir sinensis*)	7.52 ± 0.10 mg	VD_3: 0–36,000 (0–0.9)	3000, 6000 IU/kg (0.075, 0.15 mg/kg): WGR, SGR, carapase growth ↑; 6000 IU/kg (0.15 mg/kg): survival, moulting frequency, SOD, GPX, T-AOC ↑; 12,000 IU/kg (0.3 mg/kg): whole-body VD_3 (peak value)	–	65
Turbot (*Scophthalmus maximus* L.)	13.00 ± 0.08 g	VD_3: 0–1600 (0–0.04)	400 IU/kg (0.01 mg/kg): WGR, SGR, serum $1,25(OH)_2D_3$ (peak value)	0 or > 400 IU/kg (0.01 mg/kg): intestinal inflammation ↑; diversity of gut microbiota, anti-bacterial ability ↓	66
Grass carp (*Ctenopharyngodon idella*)	257.24 ± 0.63 g	VD_3: 15.2–1980.1 (0–0.05)	WGR, SGR, amino acid absorption capacity ↑; intestinal injury ↓	–	67
White shrimp (*Litopenaeus vannamei*)	0.5 ± 0.01 g	VD_3: 7200–39,200 (0.18–0.98)	① Carapace P and Ca ↑ ② 19,200 IU/kg (0.48 mg/kg): WGR, SGR (peak value); antioxidant, immune response↑; affect lipid metabolism	–	68
Orange-spotted grouper (*Epinephelus coioides*)	7.4 ± 0.03 g	VD: 0–10,000 (0–0.25)	① Triglyceride, liver fatty acid synthase activity ↓; lipoprotein lipase, hepatic lipase activities ↑ ② 1000 IU/kg (0.025 mg/kg): SOD (peak value); 2000 IU/kg (0.05 mg/kg): liver VD ↑; 4000 IU/kg (0.1 mg/kg): AKP (peak value)	–	69

Note: Symbols indicate an increase (↑), decrease (↓), no effect (→) and not reported (–) on the parameters. The unit of VD is converted to: 1 mg = 40,000 IU.

Abbreviations: AKP, alkaline phosphatase; C, complement; CAT, catalase; FCR, feed conversion ratio; FER, feed efficiency ratio; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HEPC, hepcidin; IPF, intraperitoneal fat ratio; LPO5, lipid peroxide 5; LYZ, lysozyme; PER, protein efficiency ratio; SOD, superoxide dismutase; SGR, specific growth rate; T-AOC, total antioxidant capacity; VSI, viscerosomatic index; WGR, weight gain rate.

TABLE 2 Effect of VD_3 on the physiological function of zebrafish (*Danio rerio*).

Strain	Growth stage	Treatment method	Remarks	References
vdra MO	Adult	–	Ca^{2+} uptake ↓; ossification of vertebrae ↓	48
WT	Larva	Soak with CCF (2 and 4 μM)	Lipid accumulation ↓; adipogenic factors ↓	70
vdra/b MO	Embryo/larva	–	*vdrb* MO: heart rate ↓; *vdra/b* MO: cardiac laterality defects ↑; abnormal cardiac looping ↑; alters atrioventricular boundary formation	47
vdrb MO	Embryo/larva	–	Morphological ear defects ↑; balance and motor coordination ↓	49
cyp27b1 MO	Embryo/larva	–	*runx1* expression ↓; Flk1(+) cMyb(+) HSPC numbers ↓	71
cyp2r1 $^{-/-}$	Embryo/larva/adult	–	Growth ↓; obesity ↑; in VAT: mitochondrial biogenesis ↓ and free fatty acid oxidation ↑	72
WT	Adult	Fed with VDD or VDS diets	VDD: anxiety-like behaviour ↑; swimming activity ↓; startle response ↑	73
WT	Adult	Fed with VD null or VDS diets	VD null diet: growth ↓; central adiposity ↑; hepatic triglycerides ↑; plasma free fatty acids ↓; lipoprotein lipase activity ↑; insulin signalling dysregulation ↑	74
WT	Adult	Fed with VDD or VDS diets	VDD: bioactive lipids accumulation ↑; anandamide synthesis and metabolism ↑; EC tone ↑	75
cyp2r1 $^{-/-}$	Larva	–	Granulopoiesis ↓	76
WT	Larva	① Tail clip ② Soak with VD_3 (10 nM)	*il-8* expression, neutrophil recruitment to caudal fin ↑	
csf3r $^{-/-}$	Larva	① Pretreated with VD_3 (50 nM) ② Microinjected with *E. tarda*	Mortality, bacterial colonization ↓	
ZFL	–	① Pretreated with 1,25 $(OH)_2D_3$ (200 pM) ② Treated with RSL3	Ferroptosis ↓; ROS, LPO, MDA ↓; GPx4 activity ↑	77
cyp24a1 ^{mGFP}, dn-*vdra*	Embryo/larva	–	*cyp24a1* ^{mGFP}, dn-*vdra*: regeneration of amputated fin ↓ VD remedies: regeneration of amputated fin ↑	78
ZFL	–	① Pretreated with 1,25$(OH)_2D_3$ (1 nM) ② Glucose (20 mM)	Glucose metabolism ↑	79
WT	Adult	Fed with VD null or VDS diets	VD null: one-hour postprandial glucose ↑ and postprandial insulin ↓ VDS diets: one-hour postprandial glucose ↓ and postprandial insulin ↑	
cyp2r1 $^{-/-}$	Adult	Injected glucose	One-hour postprandial glucose ↑ and postprandial insulin ↓; glucose metabolism ↓	

Note: Symbols indicate an increase (↑) or decrease (↓) on the parameters.
Abbreviations: CCF, cholecalciferol, a precursor of active vitamin D; dn-*vdra*, dominant-negative zebrafish *vdra* gene; EC, endocannabinoid; GPx4, glutathione peroxidase 4; HSPC, haematopoietic stem progenitor cell; LPO, lipid peroxide; MDA, Malonic dialdehyde; MO, morpholino; ROS, reactive oxygen species; RSL3, a kind of ferroptosis activator; VAT, visceral adipose tissue; VDD, vitamin D deficiency; VDR, vitamin D receptor; VDS, vitamin D sufficient; WT, wild type; ZFL, zebrafish liver cell line.

These existing studies clarify the positive effect of the appropriate amount of dietary VD_3 in increasing the WG rate of fish, improving FER and reducing FCR, and illustrate the potential role of VD_3 in feed saving. However, in terms of fish composition, such as crude protein, the effect of dietary VD_3 may not be determined due to different fish species, growth stage, metabolic capacity and even body size. In addition, there is a lack of research on the mechanism of VD_3 affecting fish growth and feed utilisation. This part of research is of great significance for understanding the regulation of fish growth by VD_3. But overall, VD_3 as a commonly used feed additive is also beneficial to improve the economic benefits of aquaculture besides the aspect of nutritional supplement.

3.2 | Effects of VD_3 on skeletal development and bone mineralisation

VD_3 maintains fish bone development through regulating body mineral homeostasis.[80] This has been confirmed by the fact that increasing dietary VD_3 concentrations improved the retention of phosphorus (P), sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and zinc (Zn) in yellow catfish[52]; Ca, P and Mg deposition in juvenile white shrimp (*Litopenaeus vannamei*)[54]; and P, Ca, Mg, Na, K, copper (Cu), Zn and manganese (Mn) contents in the bones of mrigal carp (*Cirrhinus mrigala*) fingerlings.[81] Research in zebrafish elucidated the possible mechanism for Ca metabolism regulated by VD_3 in which 1,25

$(OH)_2D_3$ stimulated epithelial calcium channel (ECaC) gene expression by VDR leading to increased Ca^{2+} uptake and circulating Ca^{2+} content.[48] In addition, both VD_3 deficiency and high dietary VD_3 reduced levels of a calcium transporter TRPV6, resulting in inhibited Ca^{2+} absorption, coupled with reduced osteocalcin levels, eventually leading to poor bone mineralisation and even deformity in juvenile European sea bass, and 19,200 IU/kg VD_3 is the necessary concentration for complete bone development of juvenile European sea bass.[82] The addition of 11,600 IU/kg VD_3 to a basal diet with a high plant content reduced the rate of bone deformities in juvenile gilthead seabream (*Sparus aurata*); however, higher VD_3 content than 11,600 IU/kg increased the rate of deformity.[63] Beyond dietary VD_3, intraperitoneal injection of VD_3 (5 ng/g fish weight), and the addition of VD_3/1,25$(OH)_2D_3$ to embryo medium, also effectively enhanced the bone mineralisation of a marine Antarctic teleost (*Pagothenia bernacchii*)[83] and zebrafish,[45] respectively. These findings directly identify the key role of VD_3 in regulating skeletal development and bone mineralisation in fish.

3.3 | Effects of VD_3 on lipid metabolism

In aquaculture, fatty liver is one of the common nutritional diseases of fish, and diseased fish have symptoms such as slow growth, poor resistance to stress, and reduced disease resistance. In terms of physiological indicators, the content of muscle fat is significantly increased, and this results in a decline in product quality, which seriously hinders the sustainable and healthy development of the aquaculture industry.[84] As early as 1979, VD_3 was first found to regulate lipid metabolism in rainbow trout.[50] In recent years, with the development of molecular biology, researchers have begun to pay attention to the effect of VD_3 on fish lipid metabolism. Miao et al. discovered that dietary VD_3 was associated with dyslipidemia and metabolic syndrome in bluntnose black bream (*Megalobrama amblycephala*), with plasma triglyceride and cholesterol levels decreased in their low dietary VD_3 group (500 and 1000 IU/kg) and increased in their high dietary VD_3 group (2000 and 8000 IU/kg), but the mechanism of lipid metabolism disorder was unclear.[53] Dietary VD_3 was later negatively correlated with liver fat content in juvenile sea bass.[56] Similar results were also found in turbot (*Scophthalmus maximus* L.), and according to transcriptome analysis, specific expression of key genes involved in fatty acid biosynthesis, peroxisome proliferators-activated receptors (PPARs) and other lipid metabolism-related signal pathways was observed in the VD_3 diet deficient group, indicating that VD_3 deficiency would cause abnormal fatty acid metabolism,[66] thus confirming the critical role of VD_3 in regulating fatty acid metabolism in fish. Appropriate amounts of dietary VD_3 (1000–2000 IU/kg) improved hepatic lipid metabolism in grouper, inhibited *fatty acid synthase* (*FAS*) and upregulate *hepatic lipase* (*HL*) expression,[62] indicating VD_3 can simultaneously reduce lipid accumulation in fish liver by inhibiting lipid synthesis and promoting lipid metabolism. Craig et al. treated zebrafish (7-day post fertilisation [dpf]) with 1,25$(OH)_2D_3$ for transcriptomic analysis and enriched to signalling pathways associated with lipid metabolism, in which the expression of *leptin*, *ppars* and adipocyte differentiation genes was affected.[85] Kim et al. treated zebrafish larvae with cholecalciferol and found that lipid accumulation was inhibited, and expression of *adipogenesis factor* (*pparγ*, *c/EBPα*) and *lipid-binding protein aP2* was down-regulated, indicating that VD_3 inhibition of lipid accumulation in zebrafish larvae was associated with negative regulation of adipogenic factors.[70] Peng et al. constructed a 1,25$(OH)_2D_3$-deficient zebrafish model (CYP2R1$^{-/-}$) and observed that 1,25$(OH)_2D_3$-deficient zebrafish showed growth retardation, obese size and substantial visceral fat accumulation, but exogenous supplementation of 25$(OH)D_3$ relieved the above symptoms.[72] This study also found that the effect of 1,25$(OH)_2D_3$ on lipid metabolism was accomplished by regulating mitochondrial biogenesis and oxidative metabolism in zebrafish visceral adipose tissue through PGC-1a signalling pathway.[72] VD_3 deficiency promoted the accumulation of bioactive lipids such as fatty acids, fatty amines in zebrafish liver, accompanied by increasing liver triglycerides and decreasing plasma-free fatty acids.[74,75] The above-mentioned studies have clearly verified that VD_3/1,25$(OH)_2D_3$ can effectively regulate fish lipid metabolism. However, most studies on the regulation of lipid metabolism by VD_3 are limited to the exploration of model organisms, and whether or not VD_3 can effectively solve the annoying lipid metabolism problems as fatty liver in aquaculture, remains to be determined.

3.4 | Effects of VD_3 on immunoregulation and antioxidant function

Due to the particularity of the living environment of fish, the spread of diseases in aquaculture mainly relates to the large and dense populations in tanks or net pens. Diseases in aquaculture spread rapidly, resulting in heavy economic losses. One way to avoid such problems in intensive culture is to improve the disease resistance of cultured fish through nutrition. Nutritional immunology focusses on supplying nutrients to optimise the immune response.[86] VD is one of the essential nutrients required to regulate the immune response of animals.[87] Research in humans and other mammals over the past decade has demonstrated that the cellular expression of VDR provides significant levels of VD_3 to leukocytes such as macrophages, dendritic cells, T and B lymphocytes for functional outcomes,[88,89] and it is evident from studies that almost all leukocytes respond to 1,25$(OH)_2D_3$.[90] As previously mentioned, researchers have realised that the VD endocrine system in fish may function similarly to mammals,[17] so whether VD_3 has the same immunomodulatory function in fish has gradually become a research hotspot. Below are studies that have confirmed that VD_3 is indeed able to modulate the immune function in fish.

3.4.1 | Effects of VD_3 on effector molecules of the humoral immune system

VD_3 can modulate various effector molecules of the humoral immune system including complement (C) proteins, antimicrobial peptides

(AMPs), immunoglobulins (Ig), enzymes and so on. Cerezuela et al. showed that 37,500 IU/kg dietary VD_3 significantly increased serum peroxidase and phagocytic cell activity in sea bream, while there was no significant change in C protein activity.[91] Similar results were confirmed in European sea bass.[57] In contrast, 1000 and 2000 IU/kg dietary VD_3 increased the concentration of serum C4 in bluntnose black bream, while 2000 and 4000 IU/kg dietary VD_3 increased the levels of C3 and C4 after stimulation by *Aeromonas hydrophila*.[92] Likewise, 500–1000 IU/kg dietary VD_3 reduced Asian swamp eel mortality, increased serum content of C3 and C4, enhanced lysozyme (LYZ) activity, increased the number of whole blood leukocytes, including neutrophils and lymphocytes as well as increased the number of CD3, CD8 molecules and the ratio of CD4 to CD8, reflecting the increase in the total number of T cells and the activation of cellular immunity, indicating the role of dietary VD_3 in innate and adaptive immunity in Asian swamp eel.[51] In addition, Wu et al. reported that in juvenile black carp, 421–1480 IU/kg dietary VD_3 increased the content of C3 and C4 in serum and upregulated the *C3* and *C9* expression in the haemocytes and liver compared with the VD_3 deficiency group.[61]

Possible reasons for the discrepancy in C proteins across studies may be as follows: First, the settings of the control groups in each study are not uniform (generally, the control group is VD_3 deficiency or the minimum demand of VD_3 in this species). If the controls are VD_3 deficient, it may cause more serious inflammation in the fish body.[51,66] Second, fish C protein gene is not only expressed in the liver, but also in brain, intestine, kidney, gill, skin, gonad and other organs, and participates in the body fluid, lymph and blood circulation.[93] Thus, differences between environmental factors (temperature, breeding density and stress), biological factors (growth stage and maturity), and nutritional conditions (i.e., dietary VD_3 concentration) will also lead to different changes in C protein. Another reason may be that C proteins can mediate inflammatory and immune responses, so the function of VD_3 to enhance C proteins concentration is amplified in immunostimulated fish, that is, the change of immune stimulus source. According to the above data, VD_3 had no inhibitory effect on the concentration of C3 and C4 in fish serum.

AMP is a kind of small molecule polypeptide with antibacterial effect, which has broad-spectrum antibacterial property. The direct regulation of VD on AMP transcription has been confirmed in human studies.[94,95] $1,25(OH)_2D_3$ can also induce autophagy of human monocytes by activating autophagy-related genes through cathelicidin.[96] The effects of VD_3 on AMPs have been documented in fish. Li et al. found that 500 IU/kg dietary VD_3 could increase the content of the antibacterial peptide hepcidin (hepc) in the hepatopancreas and head kidney of Asian swamp eel.[51] The expression of *hepc* was also up regulated by 18,750–37,500 IU/kg dietary VD_3 in the gut of European sea bass[57] and 412–1480 IU/kg dietary VD_3 in the liver of black carp juveniles.[61] Additionally, after incubation of a chinook salmon (*Oncorhynchus tshawytscha*) embryonic cell line with VD_3 for 72 h, the expression of *cathelicidin-2* in the cells was increased, and the antibacterial ability of the cells was enhanced.[97]

Igs are effectors of vertebrate adaptive immunity. At present, three categories of Igs, IgM, IgD and IgT, have been found in teleost fish, among which IgT is a specific type of Ig in teleost fish.[98] In fish, 500 and 1000 IU/kg dietary VD_3 increased the level of IgM in the serum of Asian swamp eel,[51] 400 IU/kg dietary VD_3 upregulated the expression of IgM mRNA in the intestine of juvenile turbots,[65] while 0–37,500 IU/kg dietary VD_3 had no significant effect on the level of IgM in the serum of European sea bass.[57] No more evidence about the effect of VD_3 on other types of Ig was found in fish. In fact, both IgM and IgT are related to mucosal immunity of fish, but the relationship between VD_3 and Igs in fish has not been well studied. Further exploration of the relationship between VD_3 and Igs will help to understand the regulatory mechanism of Ig in fish and promote the development of VD_3-related immune functional aquatic feeds.

3.4.2 | Effects of VD_3 on cells of the immune system

VD_3 can modulate the function of various effector cells of the innate and acquired immune system. $1,25(OH)_2D_3$ inhibited the inflammatory response of Jian carp (*Cyprinus carpio* var. jian) primary intestinal cells induced by bacterial lipopolysaccharide (LPS).[55] $1,25(OH)_2D_3$ also reduced macrophage reactive oxygen species (ROS) production and apoptosis after stimulation with (LPS) or the viral mimic Poly(I:C), and enhanced the phagocytic activity in the yellow catfish, indicating that $1,25(OH)_2D_3$ can help resist oxidative damage and apoptosis in fish.[44] Soto-Davila et al. isolated primary macrophages from healthy Atlantic salmon (without extra dietary VD_3) and found that the inactive form of VD_3 (10,000 ng/mL) could reduce Salmonella's ability to attach to primary macrophages and reduce bacterial invasion through the activation of leukocyte cell-derived chemotaxin 2 (LECT-2) in vitro.[99] In addition, 400 IU/kg dietary VD_3 upregulated *T-bet* ($CD4^+$ T cell transcription factor) and *gata3* (Th2 cell-specific transcription factor) in intestine of juvenile turbots after *Edwardsiella tarda* infection, while 10 nM VD_3 increased the level of LYZ and downregulated the inflammatory factors *tnf-α* and *il-1β* in healthy turbot (without extra dietary VD_3) head kidney macrophages in vitro,[65] suggesting the important role of VD_3 in both innate and adaptive immunity. Finally, Liao et al. observed accelerated aggregation of neutrophils into the injured site after soaking fin-cut zebrafish larvae with 10 nM $1,25(OH)_2D_3$, and neutrophil chemokine *il-8* was upregulated, implying that VD_3 may promote neutrophil aggregation by regulating IL-8.[76] A study in mice showed that DBP-G-actin complex was a necessary condition for neutrophil recruitment in inflammatory reaction,[100] which may mean that VD_3 promote the healing of injured sites.

3.4.3 | Antioxidant properties of VD_3

Antioxidant capacity is closely linked to immune function and VD_3 has potent antioxidant properties. Catalase (CAT) is a kind of oxidase that catalyses the decomposition of hydrogen peroxide in cells and prevents oxidation.[101] High concentration of dietary VD_3 (8030 and 16,600 IU/kg) significantly increased serum CAT in yellow catfish

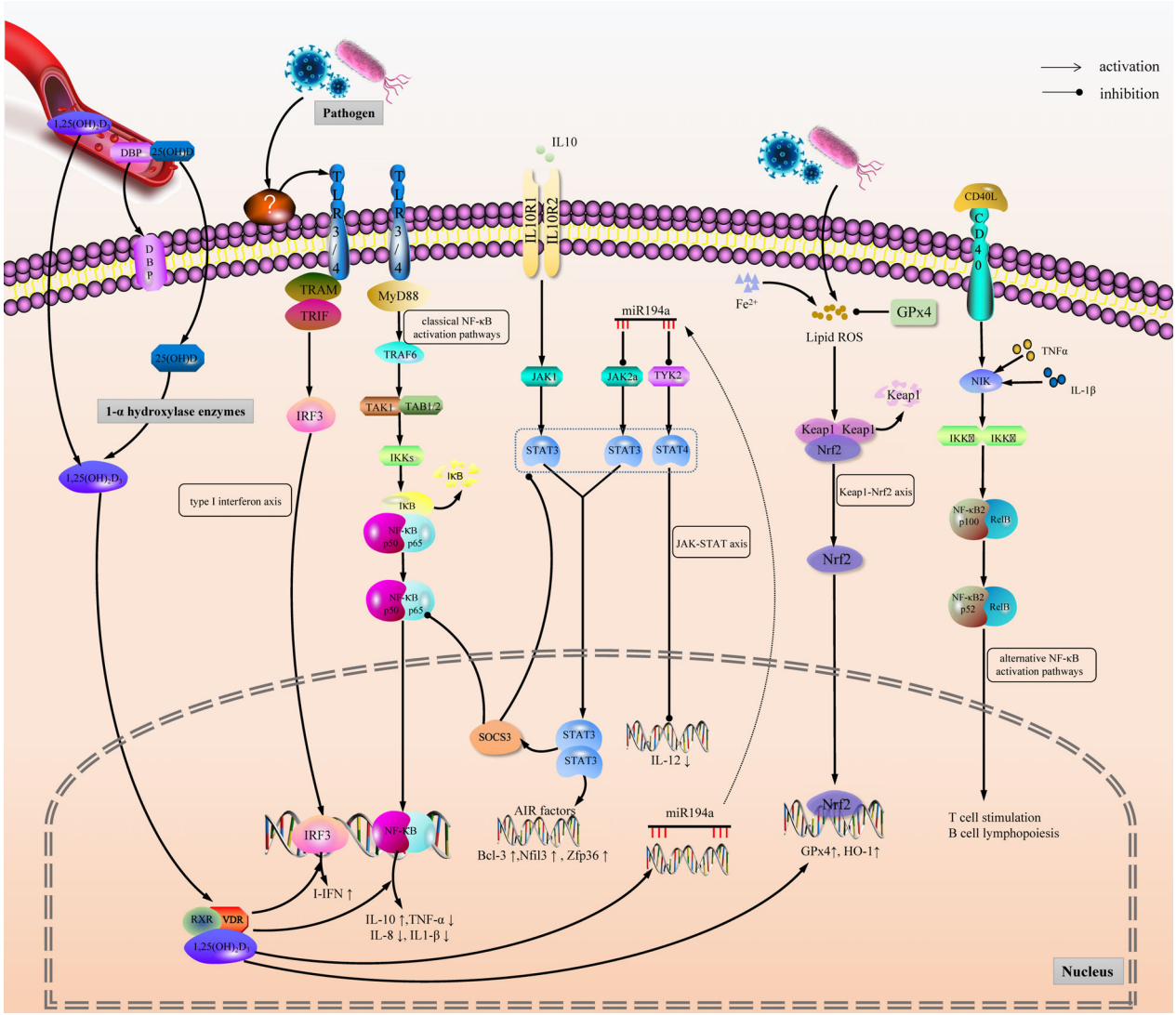

FIGURE 2 Regulation mechanism of vitamin D_3 on partial innate immunity of fish. The previously reported possible immunomodulatory and antioxidant pathways of VD_3 in fish include type I interferon axis, classical and alternative NF-κB activation signalling pathways, JAK–STAT axis, Keap1-Nrf2-ARE axis. Bcl, B-cell lymphoma; DBP, vitamin D binding protein; GPx4, Recombinant Glutathione Peroxidase 4; HO-1, Heme Oxygenase-1; I-IFN, type I interferon; IL, interleukin; JAK, janus kinase; Keap1, Kelch-like ECH-associated protein-1; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B; Nfil3, nuclear factor interleukin 3; NIK, NF-κB-inducing kinase; Nrf2, Nuclear factor erythroid 2-related factor 2; RXR, retinoid X receptor; SOCS3, suppressors of cytokine signalling-3; STAT, signal transducer and activator of transcription; TAB1/2, TGF-beta activated kinase 1 (MAP3K7) binding protein 1/2; TAK1, TGF β-activated kinase 1; IKK, I kappa B kinase; TLR3/4, toll-like receptor 3/4; TNF-α, tumour necrosis factor-α; TRAF6, TNF receptor associated factor 6; TRAM, TRIF-related adaptor molecule; IRF3, interferon regulatory factor 3; TRIF, TIR-domain-containing adaptor inducing interferon-β; TYK2, tyrosine kinase 2; VDR, vitamin D receptor; Zfp36, zinc finger protein 36.

after being infected with *Edwardsiella ictaluri*.[44] Wu et al. studied the effects of dietary VD_3 on the immune and antioxidant functions of black carp juveniles. They found that dietary VD_3 (412–1480 IU/kg) could upregulate the expression level of lipid peroxide 5 gene (*lpo5*) in the liver, and enhance the activity of superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx), glutathione reductase (GR), reduce the activity of glutathione S-transferase (GST) and malondialdehyde (MDA) content.[61] Similarly, VD_3 (1370–1430 IU/kg) improved the ability of largemouth bass (*Micropterus salmoides*) to resist infection by *A. hydrophila*, and enhanced the activities of SOD and CAT in the liver, and reduce the activity of aspartate aminotransferase in serum, thereby increasing the total antioxidant capacity.[102]

3.4.4 | Mechanism of VD_3 regulating immune function and antioxidant function

Studies at the cellular and molecular levels have confirmed the innate anti-inflammatory properties of VD_3 and identified some of the immunomodulatory pathways. We have summarised the possible immune regulation and antioxidant pathways of VD_3 that have been reported in fish in Figure 2.

The type I interferon axis is modulated by VD_3 and is typically activated during inflammation. Supplementation of 3964 IU/kg VD_3 in low-phosphorus diet was reported to significantly affect the expression of *interferon-induced protein 35* (*IFI35*) in the small intestine,

kidney and liver of yellow catfish.[103] VD_3 supplementation also played an anti-inflammatory role through VD_3/VDR-type I interferon axis and upregulated the expression of *ifn-β*, *ifi56*, *ifp35* to achieve the anti-inflammatory state in both in vivo (3950–8030 IU/kg dietary VD_3) and in vitro (10–200 pM $1,25(OH)_2D_3$) experiments of yellow catfish involving next-generation sequencing of head kidney tissue.[44,104]

The classical and alternative NF-κB activation signalling pathways are also modulated by VD_3. Jiang et al., for example, found evidence that 2400 IU/kg dietary VD_3 can reduce the expression of *nf-κb p65*, and downstream inflammatory factors, by influencing the regulation of the TLR4-MyD88 signalling pathway in Jian carp.[55] However, TLR4 in fish does not have the ability to recognise LPS,[105] so the outcome of VD_3 interacting with the TLR4 signalling pathway in fish remains unclear; perhaps it responds to danger signals released during inflammation.[54] Nevertheless, dietary VD_3 was found to regulate the innate immune response within yellow catfish splenic tissue after *E. ictaluri* infection via the NF-κB classical and alternative activation signalling pathways using next-generation sequencing analysis.[106] Previous studies have pointed out that inflammatory factors (TNF-α, IL-1β) produced downstream of the classical NF-κB activation pathway can act as activators of the alternative NF-κB activation pathway.[107] Similarly, Huang et al. also confirmed that VD_3/VDR inhibited the ubiquitination and degradation of IκB in abalone (*Haliotis discus* hannai), thereby negatively regulating the NF-κB signalling pathway, and VD_3/VDR affected the biological process of autophagy and apoptosis.[108]

VD_3 also modulated the JAK–STAT axis in fish. Cheng et al. reported that 3950 IU/kg dietary VD_3 upregulated the expressions of *jak1* and *stat1* in the head kidney and liver of yellow catfish after immune stimulation, and 10 pM $1,25(OH)_2D_3$ also activated the expressions of *jak1* and *stat1* in the head kidney macrophages of healthy yellow catfish (without extra dietary VD_3) after LPS and Poly(I:C) stimulation in vitro.[44] Analysis and validation of next-generation sequencing data revealed that dietary VD_3 exerts immunoregulatory function in yellow catfish through the regulation of JAK–STAT signalling pathway by microRNA-194a (our work in press). This immunomodulatory pathway is conserved across vertebrate species.[109,110]

The Keap1-Nrf2-ARE axis also modulated by VD_3. Keap1-Nrf2-ARE is the core antioxidant signalling pathway that can regulate the transcription and expression of antioxidant enzyme genes including *GPx4* and *HO-1*.[111] Disruption of this pathway can lead to an iron ion-dependent programmed cell death referred to as ferroptosis, and its essence is lethal lipid peroxidation.[112] Ferroptosis is associated with many diseases and antioxidant capacity. Recent studies from our laboratory confirmed that $1,25(OH)_2D_3$ could inhibit ferroptosis in zebrafish liver cells through the Keap1-Nrf2-GPx4 signalling pathway and the NFκB-hepcidin axis,[77] which is the first report of a relationship between VD_3 and ferroptosis in fish, and more in-depth studies are still in progress.

In addition to the aforementioned signalling pathways, major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells, which present antigens to T lymphocytes and consequently stimulate an adaptive immune response, are also affected by VD_3. Dietary supplementation of 3964 IU/kg VD_3 intensified the expression of major histocompatibility complex class II gene (*MHC class II*) in the small intestine and kidney of yellow catfish.[103] Collectively, the mechanisms by which VD affects the immune response and antioxidant capacity of fish have not been well studied. Studies on the immunomodulatory effect of VD_3 on fish have mainly focused on the innate immune response; thus, the specific immunoregulatory mechanism of VD_3 still needs further investigation, especially in terms of adaptive immunity.

3.5 | Other physiological functions of VD_3

Apart from the above-mentioned effects on fish growth performance, bone development, lipid metabolism and immune function, VD_3 also has extensive and important roles in other aspects, with only a few relevant studies. Taking glucose metabolism as an example, Knuth et al. detected a dysregulation of genes associated with insulin signalling in zebrafish in the VD-deficient group.[75] Also, 1000 IU/kg dietary VD_3 was shown to promote glycolysis and inhibit the pentose phosphate pathway in abalone,[64] which confirmed that the regulation of serum glucose homeostasis by VD_3 was completed through two ways: enhancing glycolytic capacity, and by promoting glucose transport.

Besides, the VD_3 analogue alfacalcidol can promote the proliferation of cardiomyocytes and regeneration of damaged hearts in zebrafish because activation of VDR leads to cardiomegaly, and alfacalcidol can upregulate the expression of ErbB2 signal-related genes in the heart and promotes mitosis of cardiomyocytes.[113] Chen et al. carried out a tail amputation experiment with transgenic fluorescent-labelled CYP24A1 (negative regulator of VD_3 metabolism) and dominant-negative *vdra* zebrafish; they found that CYP24A1 expression and *vdra* deletion inhibited tail fin regeneration, and exogenous VD treatment accelerated its regeneration.[78] These research results may bring enlightenment to human medicine, especially in terms of the enhancement of regeneration signal of injured parts and organ regeneration.

A few other interesting physiological functions appear to be affected by VD_3. A study by Oliveri et al., for example, observed that VD_3 deficiency reduced swimming frequency in zebrafish and increased startle responses, suggesting that VD_3 may play a certain role in fish neurodevelopment.[73] VD_3 also appears to be of great significance to fish gut health. According to Darias et al., dietary VD_3 can promote the maturation of the intestinal digestive function of European sea bass and enhance the activity of digestive enzymes.[82] A new study showed that dietary VD_3 deficiency decreased the gut microbial population of turbot, while the abundance of beneficial bacteria such as *Lactobacilli* increased in the VD_3-sufficient group.[66]

3.6 | Combination effect of VD_3 with other substances

In addition to the separate role of VD_3, there are several documented effects of VD_3 in combination with other nutrients. Formic acid (FA) is an organic acid commonly used as acidifying agent for aquatic feed;

this FA appears to interact with VD_3. Luqman et al., for example, found that mrigal (*C. mrigala*) had higher WG, SGR, content of protease and muscle crude fat when fed a diet supplemented with both 5000 IU/kg VD_3 and 2% FA when compared with any single component.[81] Similarly, in the grass carp, higher WG, SGR and contents of skeletal P, Ca, Mg, Na, K, Cu, Fe and Mn were also observed in the group fed combined 5000 IU/kg VD_3 and 2% FA diet,[114,115] indicating that FA combined with VD_3 have positive impacts on fish growth performance and mineral deposition, but whether they are synergistic or additive is still unclear.

Another study claimed that dietary supplementation of 1.5% Ca and 9000 IU/kg VD_3 could improve the growth performance, serum $1,25(OH)_2D_3$ and Ca level, enhance liver P content and antioxidant capacity of Chinese mitten crab (*Eriocheir sinensis*).[116] Also, Li et al. studied the effect of carbohydrate combined with VD_3 on glucose metabolism of abalone[64]; Farmed fish and shellfish naturally lack the utilisation of carbohydrates.[117] The results showed that 36.5% carbohydrate and 1000 IU/kg VD_3 in diet could increase the WGR of abalone juvenile, affect the expression of genes related to phosphoenolpyruvate carboxykinase (PEPCK), 6-phosphate glucose metabolism and glycogen degradation, activate insulin signal pathway and improve the utilisation of dietary carbohydrate of abalone. Up to now, there are few reports on the combined effect of VD_3 and other nutrients, despite aquatic feed being a compound feed with multiple nutrients; this suggests that researchers should consider exploring the interactive effects of VD_3 and other substances in promoting fish growth performance.

3.7 | Negative effects of VD_3 deficiency or excess on fish

It is likely that many of the beneficial effects of VD_3 supplementation to fish reflect the negative effects of deficiency, as is the case with other vertebrates.[118] The challenge with fish is that sufficient levels of VD_3 are unknown for many species; however, analysis of fish VD_3 tissue levels in their natural environment may help to better understand their nutrient requirement. Although the positive role of VD_3 in aquaculture fish species was described previously in terms of growth performance, skeletal health, lipid metabolism, immune and antioxidant function, it has been reported that insufficient dietary VD_3 concentration can have a negative effect on growth in some fish species. Very low levels of VD_3 resulted in poor mineralisation and skeletal deformities in larval stages of European sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata L.*)[82]; VD_3 deficiency is also responsible for human skeletal disorders.[119] Taveekijakarn et al. found that salmon (*Oncorhynchus keta*) fed with the diet free of VD_3 developed epidermis thinning and extensive necrotisation of the underlying musculature and hypocalcaemia[120]; however, the pathological changes were reversed when the same fish were fed a VD_3 supplemented diet after the deficiency period. Dietary VD_3 deficiency has also been associated with decreased immune function in Asian swamp eel,[51] as well as high incidence of intestinal inflammation, decreased abundance of intestinal flora and weakened antibacterial ability of turbot.[66] On the other hand, fish appear to be very tolerant to high concentrations of VD_3 in their diet; most studies have not reported negative outcomes associated with high dietary concentrations of VD_3. The Norwegian Food Safety Authority even proposed that fish, especially salmonids, have a safe upper limit of 60,000 IU/kg of dietary VD_3.[121]

A few negative effects on growth and physiological endpoints have been reported, and these outcomes appear to be variable across fish species. High dose of dietary VD_3 (38,400–140,000 IU/kg) caused skeletal malformations in European sea bass larvae.[82] Also, excessive dietary VD_3 negatively affected intestinal phosphate absorption in fish.[122] Similarly, Miao et al. observed that high-dose dietary VD_3 (200,000 IU/kg) led to liver and intestinal damage in the bluntnose black bream (*M. amblycephala*), negatively affecting liver metabolism and intestinal absorption.[123] In addition, 10,000 IU/kg of dietary VD_3 inhibited the growth of orange-spotted grouper.[62] Dietary VD_3 content greater than 20,000 IU/kg also increased the risk of myocarditis of gilthead seabream (*Sparus aurata*),[63] and greater than 400 IU/kg decreased the intestinal microflora diversity and the body antibacterial capacity of turbot (*Scophthalmus maximus L.*).[66]

3.8 | Safety and recommended dosage of VD_3

There is still a great controversy in the restriction of fish dietary VD_3. In fact, as the examples listed in Table 1, many high-dose dietary VD_3 did not show negative effects on fish growth, such as European sea bass (37,500 IU/kg), Orange spotted grouper (100,000 IU/kg), Chinese mitten crab (36,000 IU/kg) and so on, but this is only within the experimental concentration range, and the indicators evaluated have limitations. Therefore, the current evidence is unable to draw a conclusion on the safety of VD_3 in fish and should be considered for each cultured species. As we all know, some fish and fish products are good sources of VD_3 for human beings. In the assessment of safety, researchers should not only consider the impact of dietary VD_3 on fish growth, but also consider the deposition of dietary VD_3 in fish, that is, feed-fillet transfer ratio for VD_3, to assess the indirect impact of VD_3 on human nutrition through fish. As far as we know, there are few studies on feed-fillet transfer ratio for VD_3 in fish. When Atlantic salmon was fed 80,000 IU/kg (2 mg/kg) dietary VD_3, the transfer ratio was 0.1–0.13[124,125]; however, when Atlantic salmon were fed 8000 IU/kg (0.2 mg/kg) dietary VD_3, the transfer ratio increased to 0.4.[125] In the unpublished data from the Denmark and Portugal governments, the transfer rate of 56,000 IU/kg (1.4 mg/kg) dietary VD_3 in salmon is 0.07, and 30,000 IU/kg (0.75 mg/kg) dietary VD_3 in rainbow trout is 0.16.[121] Data show that the upper limit of VD intake per day for adults and children is 100 and 50 μg, respectively.[126] Rychen et al. investigated the dietary structure for adults and children to obtain VD_3. After excluding VD_3 from other animal sources (such as milk) and uncertain factors, it was concluded that even if the content of VD_3 in salmon feed reached 60,000 IU/kg (1.5 mg/kg), it would not exceed the upper limit of human VD_3 intake.[121] At present, the upper

TABLE 3 Recommended addition of dietary VD/VD_3 for several aquaculture fish species.

Species	Initial weight	Culture conditions	Diet type	Feeding rhythm	Evaluation criteria	Recommended concentration-IU/kg (mg/kg)	References
White shrimp (*Litopenaeus vannamei*)	0.39 ± 0.01 g	Indoor sea water recirculating system, salinity: 10–15	Isonitrogenous, isolipidic	50 g/kg body weight, twice/day, 10 weeks	Whole-body ash	VD_3: 6366 (0.159)	54
Wuchang bream (*Megalobrama amblycephala*)	17.71 ± 0.22 g	Indoor freshwater recirculating system	Isonitrogenous, isolipidic, semipurified	Satiation, 3 times/day, 90 days	SGR FCR	VD_3: 5430 (0.136) VD_3: 4970 (0.124)	53
Japanese seabass (*Lateolabrax japonicas*)	2.26 ± 0.03 g	Indoor sea water recirculating system	Isonitrogenous, isoenergentic	Satiation, twice/day, 9 weeks	WGR Liver VD content	VD: 431 (0.011) VD: 2444.4 (0.061)	56
Siberian sturgeon (*Acipenser baerii*)	3.47 ± 0.14 g	Flowing-water ponds	Isonitrogenous, isoenergetic, practical	3 times/day, 90 days	WGR Osteocalcin	VD_3: 1683.3 (0.042) VD_3: 1403.27 (0.035)	58
Tilapia (*Oreochromis nilticus*)	78.58 ± 1.93 g	Indoor freshwater recirculating system	Isonitrogenous, isolipidic	3 times/day, 12 weeks	WGR	VD: 259.8 (0.006)	59
Orange-spotted grouper (*Epinephelus coioides*)	18.00 ± 0.15 g	Indoor sea water recirculating system, salinity:30	Isonitrogenous, isoenergetic	Satiation, twice/day, 8 weeks	WGR and Ca/P contents in bones	VD_3: 2000 (0.05)	56
Black carp (*Mylopharyngodon piceus*)	4.73 ± 0.13 g	Flowing-water tanks	Isonitrogenous, isolipidic, purified	Twice/day, 9 weeks	WGR and SGR	VD_3: 534.2 (0.013)	61
Orange-spotted grouper (*Epinephelus coioides*)	81.50 ± 0.05 g	Indoor sea water recirculating system, salinity: 26–28	Isonitrogenous, isoenergetic	Twice/day, 10 weeks	WGR	VD: 750.19 (0.019)	62
Chinese mitten crab (*Eriocheir sinensis*)	7.52 ± 0.10 mg	Stagnant water	Isonitrogenous isolipidic	4% body weight, 3 times/day, 23 days	Moulting frequency WGR SGR	VD_3: 5918 (0.148) VD_3: 4825 (0.121) VD_3: 5428 (0.136)	65
Gilthead seabream (*Sparus aurata*)	20.50 ± 0.3 g	Not described	Isonitrogen, isoenergy, plant-based	Satiation, 3 times/day, 10 weeks	Skeletal health	VD_3: 11,600 (0.29)	63
White shrimp (*Litopenaeus vannamei*)	0.5 ± 0.01 g	Aerated semi-intensive pond, salinity: 21.6–23.5	Isonitrogenous, isolipidic	8%–10% body weight, 3 times/day, 8 weeks	WGR and SGR	VD_3: 19,200 (0.48)	68
Grass carp (*Ctenopharyngodon idella*)	257.24 ± 0.63 g	Outdoor freshwater ponds	Isonitrogenous, isolipidic	4 times/day, 10 weeks	WGR FER Trypsin activity	VD_3: 968.33 (0.024) VD_3: 1005 (0.025) VD_3: 1166.67 (0.029)	67
Orange-spotted grouper (*Epinephelus coioides*)	7.4 ± 0.03 g	Not described	Isonitrogenous, isolipidic	Twice/day, 8 weeks	WGR	VD: 930 (0.023)	69
Turbot (*Scophthalmus maximus* L.)	13.00 ± 0.08 g	Flow-through system	Isonitrogenous, isolipidic	Twice/day, 8 weeks	WGR	VD_3: 400 (0.01)	66

Note: The unit of VD is converted to: 1 mg = 40,000 IU.
Abbreviations: FCR, feed conversion ratio; SGR, specific growth rate; WGR, weight gain rate.

limit of VD content in fish feed is 3000 IU/kg according to international regulations (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52004XC0225(03)&from=EN), but the rationale is unclear. The requirement of VD_3 varies greatly due to different living habits, feeding properties and growth stages. Thus, it is difficult to draw firm conclusions about the safe range of VD_3 use in aquaculture. It is advised, therefore, that researchers develop a multi-factor evaluation criterion to provide complete data for the safe use of VD_3 in aquaculture. In addition, tolerance and bioaccumulation are highly variable across fish species, so this should be taken into consideration when formulating diets. It would be meaningful to learn more about the genetics involved in this species-to-species variation. Lock et al. and Darias et al. reviewed the fish VD/VD_3 requirements that had been reported.[17,80] Here, we list the recommended additions of VD/VD_3 in some fish diets reported in the past several years for reference (Table 3).

4 | CONCLUSION AND FUTURE PERSPECTIVES

Compared with humans and other mammals, the study of VD_3 in fish is still in its infancy. As a commonly used fish feed additive, the function of VD_3 and mechanisms of action need to be studied more extensively and deeply. Existing evidence has testified that VD_3 has positive effects on fish growth performance, bone development, immune function, nutritional metabolism, neurodevelopment, heart regeneration and intestinal maturation. In aquaculture, nutritional regulation can combine nutritional supplementation with functional regulation. For example, how to simultaneously achieve yield enhancement and disease resistance is one of the topics of concern for researchers, and VD_3 has shown great potential in this regard. Therefore, exploring the potential physiological regulation mechanism of VD_3 in fish will become a future research focus, especially in the aspects of adaptive immunity, antibacterial and antiviral, nutritional metabolism and intestinal health, which are relatively lacking at present. Furthermore, there are differences in the demand and metabolic level of VD_3 for different fish species. The threshold of VD_3 addition in fish feed is also controversial. Researchers may be able to develop a set of scoring criteria to evaluate the amount of VD_3 addition according to the different requirements of practical production. At the same time, it is necessary to fully understand the nutritional needs of each stage of development and make dynamic adjustments to the feed formula to ensure the scientific rationality of culture. Another area of concern is the interaction among vitamins, as lipid-soluble vitamins such as vitamin A (VA) can interact with VD through steroid hormone receptors, $1,25(OH)_2D_3$ and retinoic acid (RA), the active metabolite of VA, and have common targets such as bone.[127] Vitamin C and vitamin E also have strong antioxidant function, but interactions between VD and these two vitamins have not been reported in fish. A scientific rationale for establishing the ratio of various vitamins in aquatic feed deserves attention. Research enriching this field can provide theoretical guidance for the development of precise guidelines for the application of VD_3 in the aquatic feed industry so as to promote healthy, efficient and sustainable development of aquaculture.

AUTHOR CONTRIBUTIONS

Ke Cheng: Writing – original draft; investigation; data curation; software. **Yanqing Huang:** Investigation; resources. **Chunfang Wang:** Writing – review and editing; project administration; supervision; funding acquisition. **Wajeeha Ali:** Investigation; writing – original draft. **Niel A. Karrow:** Writing – review and editing; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no data sets were generated or analysed during the current study.

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