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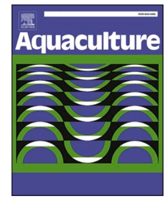

Calcifediol is a safe and effective metabolite for raising vitamin D status and improving growth and feed conversion in rainbow trout

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A B S T R A C T

The vitamin D endocrine system is required for the transcriptional regulation of a myriad of vertebrate genes including those involved in bone health, growth, nutrient metabolism and immunity. The requirements of salmonids for vitamin D are amongst the highest for any aquaculture species. With nuances, the metabolism of the pre-vitamin cholecalciferol (D_3) via calcifediol (25-OH-D_3), required to produce the physiologically active hormone calcitriol (1,25-OH-D_3) is conserved in fish. The composition of modern aquafeeds, growth in seawater and production challenges, such as disease, may result in the suboptimal biochemical activation of vitamin D hormone in fish.

A 91-day experimental feeding trial was used to test the safety and efficacy of calcifediol for the supply of vitamin D to rainbow trout with an initial body weight of 57.6 g. A practical diet containing cholecalciferol within recommended levels (5240 IU) was supplemented with either 69.8, 687 or 6854 μg/kg calcifediol. The efficacy of calcifediol was determined by the assessment of zootechnical performance and the appearance of vitamin D metabolites in the blood. The safety of the dietary interventions was assessed from generic health indices, examination of gross pathologies, hematology, and blood chemistry.

Test fish increased body weight at least 5.6-fold to 323.5 g over the experimental feeding period. The supplementation of 687 or 6854 μg/kg calcifediol resulted in significant improvements in growth rate and feed conversion (FCR). Whilst not detectable in control fish, calcifediol increased linearly according to dietary levels in the blood and to a lesser extent in the white muscle. The increases of calcifediol in the blood were accompanied by saturable increases of circulating active vitamin D. At the end of the 91-day feeding period, survival was 100%, no gross pathologies relating to the diets were observed, and health indices, hematology, and blood chemistry, including calcium and phosphorus, were not significantly altered.

The supplementation of calcifediol to practical diets containing recommended levels of cholecalciferol improves zootechnical performance and ensures that maximal levels of active vitamin D are present in the blood to meet physiological demands. With a lack of significant effects on health indices, hematology, and blood chemistry, including calcium and phosphorus, the tested high doses of calcifediol are concluded to be safe for salmonids.

1. Introduction

With evolutionary origins in bony fish, the vitamin D endocrine system plays a well-known role in calcium and bone homeostasis (Vielma et al., 1999; Darias et al., 2010; Bouillon and Suda, 2014; Fraser, 2018; Sivagurunathan et al., 2022). Calcitriol (1,25-OH-D_3), the physiologically active form of vitamin D, is a secosteroid hormone with major roles as a signaling molecule. It is now recognized, including in fish, that calcitriol is not only essential for bone health, but it also regulates a myriad of physiological processes including immune function, nutrient

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metabolism, growth, and cell differentiation (Lock et al., 2010; Pierens and Fraser, 2015; Fraser, 2018; Han et al., 2019; Knuth et al., 2020; Liu et al., 2022; Shao et al., 2022). Salmonids deficient in vitamin D exhibit reduced growth, fin tip damage, thinner skin, changes to gill epithelium, liver damage and hypocalcemia (Taveekijakarn et al., 1996). Across vertebrate species, calcitriol regulates hundreds of genes via the nuclear vitamin D receptor (VDR), which is expressed in a wide variety of cell types indicating the numerous physiological roles of the vitamin D system (Carlberg, 2014). Photosynthesis of vitamin D is documented in the skin of fish, but this de novo supply of the pre-vitamin, cholecalciferol (D_3), does not meet physiological demands, therefore farmed fish rely on a dietary source (Lock et al., 2010).

In both terrestrial vertebrates and fish, two hydroxylation steps by hydroxylase enzymes are necessary to convert dietary cholecalciferol, via calcifediol (25-OH-D_3), into calcitriol (Norman, 2008; Lock et al., 2010; Fraser, 2018). In humans, the first hydroxylation of circulating vitamin D_3 principally takes place in the liver, after which 25-OH-D_3 is transported in the blood to the kidney for the second hydroxylation to 1,25-OH-D_3 (Norman, 2008). 25-OH-D_3 is the main circulating vitamin D metabolite in humans and livestock and is consequently routinely used as a biomarker of vitamin D status (Holick, 2009). Salmonids are particularly reliant on the liver for the activation of circulating vitamin D, as both hydroxylation steps take place in this organ. In contrast to humans, the principal circulating metabolite in salmonids is 1,25-OH-D_3 and not 25-OH-D_3, the latter which can be below limits of detection (Pierens and Fraser, 2015).

In both terrestrial and aquatic species, a variety of factors can cause a suboptimal production of circulating 1,25-OH-D_3. In fish and shrimp, these may include metabolic disorders, disease and increased demands arising from rapid growth rates. The importance of vitamin D for optimal immune function has been demonstrated in several aquaculture species (Soto-Dávila et al., 2020; Yang et al., 2021; Shao et al., 2022). Moreover, disease may significantly increase the metabolism of vitamin D metabolites from the diet (Yang et al., 2021). The dietary supplementation of the intermediate vitamin D metabolite 25-OH-D_3 allows a bypass of the first step in the metabolic activation of vitamin D_3 in vivo. Consequently, dietary 25-OH-D_3 is considerably more potent than the pre-vitamin D_3 in humans, poultry and swine, yet very few studies have assessed the nutritional utility of this vitamin D metabolite in fish (Yarger et al., 1995; Burild et al., 2016; Quesada-Gomez and Bouillon, 2018). 25-OH-D_3 may be particularly beneficial to salmonids, which have higher requirements and tolerances to vitamin D than reported in terrestrial species (Woodward, 1994; Liu et al., 2022). Indeed, prior to their lives at sea, Atlantic salmon parr residing in freshwater increase concentrations of vitamin D metabolites in their blood plasma in preparation for their transformation to smolts (Graff et al., 2004). Production stressors, such as elevated stocking density may also modulate the metabolism of vitamin D metabolites in salmonids (Rebl et al., 2017). Furthermore, modern diets with reduced levels of marine-derived feedstuffs that are rich sources of vitamin D, can increase the requirement for supplemented cholecalciferol in salmonids (Sissener et al., 2013; Antony Jesu Prabhu et al., 2019).

A dose response approach was undertaken to establish the efficacy of supplemented dietary 25-OH-D_3 for improving vitamin D status, growth and feed utilization in rainbow trout fed a basal practical diet containing recommended levels of cholecalciferol. The bioavailability of dietary 25-OH-D_3 was tested after a 91-day feeding period by measurement of the metabolites present in the blood plasma and muscle, and by quantification of the levels of circulating 1,25-OH-D_3. The efficacy of 25-OH-D_3 was determined by the assessment of growth performance and feed utilization. The safety of supplemented 25-OH-D_3 was also assessed by the determination of a variety of health indices and blood parameters.

2. Materials and methods

2.1. Rearing of test fish

Rainbow trout eyed-eggs were obtained from Aqualor, Fénétrange, France. From first-feeding, fish were raised on site in recirculating aquaculture systems (RAS) and fed commercial fish feeds until the required starting size. Before any handling, fish were anesthetized in 0.08 g/l buffered tricaine methane sulfonate; MS222 (PharmaQ Ltd., Overhalla, Norway). At the beginning of the trial, six hundred and forty test fish were individually weighed and randomly allocated to sixteen 500-l tanks of an indoor RAS system. Experimental groups consisted of four randomly assigned replicate tanks ($n = 4$) each containing 40 fish. At the start of the experimental feeding period, initial body weight (IBW) was 57.6 g. Thereafter, mean body weight in individual tanks was determined by bulk weighing every two to three weeks. The RAS was supplied with ground water and water temperature and oxygen maintained within 15.2 ± 1.3 °C and 8.9 ± 1.2 mg/l, respectively. Water quality was kept within the following ranges: pH 8.1 ± 0.2, NH_3 0.004 ± 0.002 mg/l, nitrites 0.05 ± 0.09 mg/l and nitrates 27.4 ± 5.7 mg/l. Indoor lighting was used with a constant photoperiod of 12 h light: 12 h dark.

2.2. Experimental diets and feeding

A basal practical experimental diet was formulated to contain macro ingredients and a proximate composition that was representative of current commercial rainbow trout feeds, for details see Table 1. The basal feed contained a total of 131 μg/kg (5240 IU) cholecalciferol (D_3), originating from both the feed ingredients and the vitamin premix supplying 37.5 μg/kg (1500 IU) D_3, see Table 1. The D_3 content of the basal feed exceeded the minimum of 40 μg/kg (1600 IU) dietary D_3 requirement for rainbow trout by 91 μg/kg (NRC, 2011) and was within recommended optimal levels for this vitamin (Liu et al., 2022). Experimental diets were manufactured under standard conditions using a Bühler twin-screw extruder (Uzwil, Switzerland) and a Forberg vacuum oil coater (Stoltz, Paris, France) at the DSM Nutritional Products feed

Table 1
Composition of the basal experimental diet.

Ingredients	(%)
[1]Fishmeal (Norvik LT) (29.11.19)	15
Soybean meal (Soja 50)	9.0
[2]Soy protein concentrate (X-SOY 600)	20
Rapeseed meal	7.0
Wheat	11
Wheat gluten (Viten)	14
Mono calcium phosphate (MCP)	1.5
[3]Soy lecithin (Emulpur IP)	0.5
Choline chloride (60%)	0.5
[4]Vitamin and Mineral premix – VMP1A	0.5
L-Lysine	0.2
DL-Methionine FG	0.5
[5]Winterised fish oil	5.0
Rapeseed oil	15.3

[1] Sopropêche, Wimille, France.
[2] Koster Marine Proteins GmbH, Germany.
[3] Emulpur IP, Arles, Marseille, France.
[4] Low-range OVN vitamin and mineral premix added to supply the following (per kg basal diet): retinol acetate, 3000 IU; cholecalciferol, 1500 IU; all-race-a-tocopheryl acetate, 200 IU; menadione sodium bisulfite, 30 mg; thiamine. HCl, 17 mg; riboflavin, 25 mg; calcium d-pantothenate, 40 mg; biotin, 1 mg; folic acid, 10 mg; cyanocobalamine, 0.05 mg; niacin, 150 mg; pyridoxine. HCl, 20 mg; ascorbate phosphate (Stay-C) 150 mg ascorbic acid equivalents; iron, 30 mg; copper, 5 mg; zinc, 100 mg; manganese, 20 mg; selenium, 0.2 mg; iodine, 2 mg. DSM, Heanor, England.
[5] Winterised fish oil (HPW), Olvea Fish oils, Saint-Léonard, France.

mill, Village-Neuf, France. Four experimental diets were produced in both 3- and 4-mm pellet sizes. The control diet contained no test additive, and three test diets were supplemented with various levels of calcifediol from ROVIMIX® Hy-D® 1.25%, DSM Nutritional Products, Kaiseraugst, Switzerland. Nominal supplementation levels of 25-OH-D_3 were 80, 800 and 8000 μg/kg - as fed. For the actual recovered 25-OH-D_3 in test diets see Table 2.

Fish were fed the 3 mm diets for 44 days followed by the 4 mm diets for the remaining 47 days. Feeding was carried out by hand twice per day during weekdays and by automatic belt feeders during weekends. Feed rations were pre-determined based on recommendations for a water temperature of 15 °C by feed ration tables for a similar commercial diet. At each feeding, the complete consumption of feed was confirmed by checking the outflow of each experimental tank for any uneaten feed. The specific feeding rate throughout the experimental feeding period ranged from 1.65% to 1.40% body weight per day. Fish were adapted to the control diet for 36 days prior to the experimental feeding period.

2.3. Proximate and vitamin D_3 metabolite analysis of diets

Analyses of the nutrient content in feed samples were performed in duplicate according to standard methods. Dry matter was determined after drying at 105 °C for 24 h. Ash was measured after combustion at 500–600 °C for 48 h. Crude protein was determined by a nitrogen analyzer (FP 528, LECO, St. Joseph, USA) using the Dumas (1826) method (CP = N x 6.25), as cited by (Buckee, 1994). Gross energy measurements were performed using an adiabatic bomb calorimeter (C 2000 basic, IKA, Staufen, Germany). Crude lipid content was determined following petroleum ether extraction (Soxtherm, Gerhardt, Germany).

The concentrations of D_3 and 25-OH-D_3 in experimental diets were determined in duplicate using HPLC (Agilent Infinity LC System) coupled with mass spectrometry (MS) detection (API4000 SCIEX). Briefly, for 25-OH-D_3, after addition of the internal standard, the sample was saponified and 25-OH-D_3 extracted with methyl tert-butyl ether (TBME). The extract was dried by evaporation and then analyzed with reverse-phase HPLC with MSMS detection. For D_3, after saponification with a KOH alkaline ethanol solution and extraction with cyclohexane solvent, D_3 was quantified by a reverse-phase HPLC-MSMS method using 6,19,19- trideuterovitamin D_3 as a stable isotope internal standard. Data acquisition, integration and quantification were performed by Analyst® software (Sciex). The measured proximate composition and vitamin D metabolites of test diets are shown in Table 2. Diet treatment groups are named hereafter according to the recovered 25-OH-D_3.

2.4. Growth performance and feed conversion

Growth performance and feed conversion during the 91-day experimental feeding period were determined from measures of feed intake, tank biomass and mean water temperature. The following parameters were calculated:

$$\text{Weight gain (WG), from : final body weight (FBW)–initial body weight (IBW) (g)}$$

$$\text{Specific growth rate (SGR) from : } (100 \times [ln\ (\text{FBW/IBW})]) \times \text{days of growth (unit : \%BW/day)}$$

Thermal growth coefficient (TGC) from:

$$1000 \times \left[\text{FBW}^{(1/3)}–\text{IBW}^{(1/3)}\ (\text{g})\right] / \left[\text{days of growth} \times \text{mean temperature}^{0}\text{C}\right]$$

$$\text{Feed conversion ratio, as} - \text{fed basis (FCR) from : feed intake} \times \text{biomass gain}$$

Table 2
Diet proximate composition and vitamin D_3 metabolite content.

Diet (μg/kg 25-OH-D_3)	0	69.8	687	6854	0	69.8	687	6854
Pellet size	3 mm				4 mm			
Dry Matter (%)	95.2	95.3	95.2	95.3	94.4	94.1	94.0	94.1
Ash (%)	6.70	6.75	6.76	6.69	6.54	6.67	6.65	6.66
Protein (%)	44.0	43.4	43.6	42.7	42.7	42.3	42.3	42.9
Energy (MJ/kg)	22.7	22.6	23.0	22.4	22.1	22.4	22.2	22.2
Lipid (%)	24.5	24.2	24.0	23.1	23.5	23.8	23.3	23.7
D_3 metabolites								
†Total D_3 (μg/kg)	131	131	133	134	137	137	144	135
Nominal 25-OH-D_3 (μg/kg)	0	80	800	8000	0	80	800	8000
Measured 25-OH-D_3 (μg/kg)	*< LOQ	60.0	680	7190	2.55	78.9	693	6540
Mean (weighted average) dietary 25-OH-D_3 over entire 91-day feeding period								
25-OH-D_3(μg/kg)	1.3	69.8	686.7	6854				

† 37.5 μg/kg D_3 from vitamin premix and remaining endogenous contributions from feed ingredients.
* <LOQ: below 10 μg/kg limit of quantification.

2.5. Fish sampling and determination of health indices

At the end of the 91-day experimental feeding, veterinarians from the Institute for Fish and Wildlife Health, Vetsuisse Faculty Bern, University of Bern, examined and sampled ten randomly selected fish from each tank. The fish sampled represented 25% of the individuals in each tank. Feeding was stopped one day prior to sampling. Fish were externally examined for any malformations or external lesions and internally the viscera, stomach, intestine, liver, gall bladder, spleen and kidney assessed for any gross pathologies. Body weight (g), length (cm) and liver weight (g) were measured for the determination of condition factor (K) according to Fulton (100*weight / length^3) and liver somatic index (LSI) using the formula, 100*weight of liver / body weight. Immediately after anesthesia, blood was sampled from the caudal vein with Li-Heparin-treated syringes (Laboklin GmbH & Co.KG, Bad Kissingen, Germany) for blood smears and the determination of hematocrit. Remaining blood was kept on ice prior to the recovery of plasma by centrifugation at 2657 G for four minutes. Following blood sampling, fish were euthanized by a blow to the head and a gill cut. Five grams of

white muscle from the Norwegian Quality Cut (NQC) was sampled from the first five fish sampled. All samples were stored at −20 °C prior to the analysis of vitamin D metabolites and blood chemistry parameters.

2.6. Analysis of vitamin D metabolites in plasma and muscle

Vitamin D_3 and the metabolites 24(R),25-dihydroxy vitamin D_3, 25-OH-D_3, 3-epi-25 OH-D_3, 25-OH-D_2, and 1,25-OH-D_3 in the plasma, and 25-OH-D_3 in the muscle, were determined using UHPLC (1290 Agilent Infinity II LC System) coupled with MS detection (SCIEX API4000 or Agilent 6495QQQ) by the Bio-analytics lab of DSM Nutritional products, Kaiseraugst, Switzerland. Briefly, for plasma samples, after addition of a deuterated internal standard to an aliquot of plasma, proteins were precipitated with acetonitrile. After centrifugation and a concentration step, vitamin D metabolites (except 1.25-dihydroxyvitamin D_3) were analyzed using a LC-MS/MS system. 1,25-OH-D_3 was extracted from an aliquot of plasma by immunoaffinity using a commercial kit (Immundiagnostik AG, Germany), followed by a derivatization step with AmplifexTM diene reagent (Sciex, Germany) to enhance the detection sensitivity. The resulting fraction was analyzed by LC-MS/MS. The muscle samples were first homogenized by milling. After addition of a deuterated internal standard, 25-OH-D_3 was extracted by mechanical shaking with acidified methanol, followed by a solid phase extraction. The eluted fraction was concentrated and analyzed using a LC-MS/MS system. Data acquisition integration and quantification were performed by Analyst® software (Sciex) or MassHunter software (Agilent). Quantification was done by applying dedicated external calibrations (using deuterated internal standard). To assess the daily and long-term laboratory performance (accuracy and precision) of the method, dedicated standard and quality-control samples were analyzed daily with unknown samples.

3-epi-25-OH-D3 (LOQ <0.5 ng/ml), 24(R),25-dihydroxy vitamin D_3 (LOQ <0.5 ng/ml) and 25-OH-D_2 (LOQ <0.5 ng/ml) were measured, but none of these vitamin D metabolites were detectable in the plasma of rainbow trout.

2.7. Hematology and blood chemistry

Blood smears were stained using a modified Wright-Giemsa stain on an automated staining instrument (HemaTek, Siemens, Zurich, Switzerland). Blood smears were used for the microscopic determination of total leukocyte counts determined per 1000 x optic field and leukocyte proportions in percentages. Hematocrit was measured using a Haematokrit 200 centrifuge (Hettich, Bäch, Switzerland). Analysis of blood chemistry was carried out at the Clinical Laboratory of the Vet-Suisse faculty, Zürich. Plasma samples were measured for blood chemistry on a Cobas C (Roche Diagnostics, Rotkreuz, Switzerland) according to IFCC-conditions (International Federation of Clinical Chemistry and Laboratory Medicine). Routine daily internal controls were performed at two levels using human control material. Alanine aminotransferase (ALT) was measured enzymatically according IFCC, while for protein the Biuret method was applied. Uric Acid was measured with a kinetic test with urease and GLDH (glutamate dehydrogenase). The following blood parameters were measured in recovered plasma: albumin, urea, gamma-glutamyltransferase (GGT), creatine kinase (CK), amylase, lactate dehydrogenase (LDH), total protein, alkaline phosphatase (ALP), ALT, aspartate aminotransferase (AST), creatinine, calcium, uric acid, magnesium, bilirubin, phosphate, glucose, cholesterol, sodium, potassium and chloride. The following parameters were below the limit of detection (<LOD) in all examined plasma samples and no further analysis was possible: creatinine (<15 μmol/l), bilirubin (<2.5 μmol/l) and GGT (<3 U/l). For the remaining parameters where only a portion of samples were < LOD, those were replaced with LOD/2 and included in the analysis. All test results that were flagged to be out of the technical range of the test were not included in further data analysis. The distribution of flagged values across treatment groups was tested and not conspicuous, see supplement.

2.8. Statistical analysis

The experimental unit for zootechnical performance endpoints was the tank. Differences in group means were investigated with a one-way ANOVA followed by post-hoc pairwise comparisons. The experimental unit for all other endpoints was the individual fish. Point estimates of the respective mean values per dose group were calculated together with their 95% confidence intervals based on a linear mixed model on the respective endpoint, including dose group and location within the room (block) as fixed effects and tank as random effect. The same model was used to perform an overall ANOVA followed by multiplicity-unadjusted pairwise comparisons (*t*-tests) and multiplicity-adjusted pairwise comparisons (Tukey). If a parametric approach was not possible due to skewed data, even after log-transformation of the data, non-parametric Kruskal-Wallis tests for overall comparisons of treatments followed by Dunn-tests for pairwise comparisons were performed. To account for the hierarchical data structure (fish in tank) with the non-parametric approach, data were first summarized by tank using the median of the data as robust measure of location before the statistical methods were applied across treatments. Vitamin D metabolites were modelled by linear or non-linear regression with a line of best fit using Graphpad Prism 9, San Diego, CA, USA.

2.9. Ethical statement

All animal experimentation was conducted at the Research Centre for Animal Nutrition and Health (CRNA) of DSM Nutritional Products located in Village-Neuf, France in accordance with French legal requirements adopted from European Directive 86/609/CEE (26th November 1986) on live animal experimentation. The study was designed based on the guidance published by the European Food Safety Authority (EFSA) in the publication; Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel (EFSA Panel on additives and products or substances used in animal feed) et al., 2017).

3. Results

3.1. Fish growth performance and feed utilization

During the experimental feeding period, survival was 100% and good zootechnical performance was achieved by the test fish. In fish sampled at the end of the experimental feeding period, irrespective of the test group, no obvious gross pathologies were detected, either externally or internally. The body weight of test fish increased at least 5.6-fold from 57.6 to 323.5 g over the 91-day period. Dietary 25-OH-D_3 at the higher doses of 687 and 6854 μg/kg significantly improved all performance and feed utilization parameters compared to the control group. The lower 69.8 μg/kg dose of 25-OH-D_3 only resulted in marginal insignificant improvements in performance. The greatest improvements in performance were found in fish fed diets containing 687 μg/kg 25-OH-D_3. Weight gain, SGR and FCR in fish fed 687 μg/kg 25-OH-D_3 improved by 6.6, 3.0 and 3.3%, respectively, see Table 3. Dietary 25-OH-D_3 had no significant effect on the health indices of sampled test fish. Both condition factor (K) and liver somatic index (LSI) remained constant across all dietary 25-OH-D_3 treatments, see Table 4.

3.2. Hematology

The tested doses of dietary 25-OH-D_3 had no significant effects on either hematocrit or hemoglobin. The highest mean hematocrit was 44.2 in fish supplemented 69.8 μg/kg 25-OH-D_3 and the lowest mean hematocrit was 42.2 in the highest dose group. Similarly, the highest mean hemoglobin was 7.48 g/dl in fish fed the 69.8 μg/kg 25-OH-D_3 diet and

Table 3
Mean growth performance of fish fed various levels of dietary 25-OH-D_3 over 91 days.

Diet (µg/kg 25-OH-D_3)	0	69.8	687	6854	ANOVA
Survival (%)	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	NA
IBW (g)	57.6 ± 0.0	57.6 ± 0.0	57.6 ± 0.0	57.6 ± 0.0	0.698
FBW (g)	323.5 ± 3.6 [a]	326.5 ± 2.5 [a]	341.0 ± 2.9 [b]	336.0 ± 3.8 [b]	*<0.001*
*Diff. to control (%)		0.96	5.39	3.80	
WG (g/animal)	266.0 ± 3.5 [a]	268.9 ± 2.5 [a]	283.4 ± 2.9 [b]	278.4 ± 3.9 [b]	*<0.001*
*Diff. to control (%)		1.16	6.56	4.63	
Feed Intake (g/animal)	197 ± 1 [a]	199 ± 2 [ab]	203 ± 2 [b]	201 ± 2 [ab]	*0.005*
*Diff. to control (%)		1.06	3.05	1.75	
SGR (% BW / day)	1.90 ± 0.01 [a]	1.91 ± 0.01 [a]	1.95 ± 0.01 [b]	1.94 ± 0.01 [b]	*<0.001*
*Diff. to control (%)		0.54	3.04	2.18	
FCR (as-fed basis)	0.74 ± 0.01 [b]	0.74 ± 0.01 [b]	0.72 ± 0.01 [a]	0.72 ± 0.01 [a]	*<0.001*
*Diff. to control (%)		−0.33	−3.3	−2.72	
TGC	2.17 ± 0.02 [a]	2.19 ± 0.01 [a]	2.26 ± 0.01 [b]	2.24 ± 0.02 [b]	*<0.001*
*Diff. to control (%)		0.72	4.04	2.87	

Initial body weight (IBW); final body weight (FBW); weight gain (WG); specific growth rate (SGR); thermal growth coefficient (TGC); feed conversion ratio (FCR).
Data presented are means ($n = 4$ tanks) ± standard deviation (SD).
Groups sharing the same superscript are not significantly different from the control (after adjusting for multiple testing).
Percent differences to the control were calculated prior to data rounding.

the lowest mean hemoglobin was 7.09 g/dl in the highest dose group, see Fig. 1. White blood cell counts (WBC) were not significantly modulated by dietary 25-OH-D_3. The lowest mean WBC counts were in the control group (1.99 cells/1000× optic field (OF)) and the highest WBC counts were found at the highest 25-OH-D_3 dose (2.26 cells/1000× OF). Within the WBC populations, the proportions of lymphocytes, neutrophils and monocytes were also not significantly modulated by dietary 25-OH-D_3, see Fig. 1.

3.3. Blood chemistry

There were no significant effects of dietary 25-OH-D_3 on the blood chemistry parameters measured. Calcium, phosphorus, magnesium, and a variety of blood chemistry parameters were tested as a holistic approach to assess normal physiological function. At all dietary doses of 25-OH-D_3, calcium, phosphorus, magnesium, sodium, potassium, chloride, total protein, albumin, glucose, urea, uric acid, cholesterol, amylase, creatinine kinase, ALT, AST, LDH and ALP were not statistically different to the control group. For urea, there was a significant difference between the group fed 69.8 µg/kg 25-OH-D_3 and the highest dose group using unadjusted *p* values, which turned insignificant using p values adjusted for multiplicity testing. Similarly, creatine kinase was significantly increased in the fish fed the highest dietary 25-OH-D_3 compared to control fish. However, when adjusted in consideration of multiplicity testing, no significant group differences were observed, see Table 5. In summary, up to 687 µg/kg dietary 25-OH-D_3 there were no significant effects on blood chemistry compared to control fish, even when using unadjusted p values. In fish fed the highest 6854 µg/kg dose, significant differences in urea and creatinine kinase were observed, but if accounting for multiplicity testing, these differences were not significant.

3.4. Vitamin D metabolites in the plasma and muscle

The supplementation of dietary 25-OH-D_3 for 91 days resulted in increased vitamin D_3 metabolites in both the plasma and muscle. Increased dietary 25-OH-D_3 resulted in respective linear increases in the plasma. 25-OH-D_3 was not detectable in the plasma of control fish (LOD <1.0 ng/ml). In fish fed diets with 69.8, 687 and 6854.3 µg/kg 25-OH-D_3, mean (± SD) plasma 25-OH-D_3 increased from 0.38 ± 0.2 to 8.5 ± 2.3 and 95.5 ± 2.3 ng/ml, respectively, see Fig. 2, A. The response of the muscle to dietary 25-OH-D_3 up to the highest tested dose of 687 µg/kg was also linear, but the concentration of 25-OH-D_3 in the muscle was much lower than found in the plasma. 25-OH-D_3 was not detectable in the muscle of control fish (LOD <1.0 ng/g). The mean (± SD) concentration of 25-OH-D_3 in the muscle of fish supplemented 69.8 and 687 µg/kg 25-OH-D_3 was 0.38 ± 0.1 and 3.1 ± 1.0 ng/g, respectively, see Fig. 2, B.

The higher doses of dietary 25-OH-D_3 resulted in modest increases of D_3 in the plasma of test fish. In control fish fed no 25-OH-D_3 and fish fed 69.8 µg/kg, the mean (± SD) plasma D_3 was 4.4 ± 1.7 and 4.0 ± 2.3 ng/ml, respectively. In fish fed 687 and 6854.3 µg/kg 25-OH-D_3, plasma D_3 increased slightly to 6.5 ± 1.9 and 6.3 ± 2.1 ng/ml, respectively, and indicates that increases in plasma D_3 are slight and reach a plateau, see Fig. 2, C. Levels of plasma 25-OH-D_3 exceed levels of D_3 in plasma when dietary 25-OH-D_3 was supplemented at >69.8 µg/kg.

Supplementing 25-OH-D_3 results in a 68.6% increase in plasma levels of the active metabolite 1,25-OH-D_3. In control fish fed no 25-OH-D_3 the mean (± SD) plasma 1,25-OH-D_3 was 740.3 ± 80.7 pg/ml. Supplementing 69.8, 687 and 6854 µg/kg 25-OH-D_3 increased 1,25-OH-D_3 in the plasma to 847.5 ± 80.7, 1123.8 ± 192.6 and 1248.0 ± 223.4 ng/ml, respectively. These data show that although dietary 25-OH-D_3 increased respective plasma levels of this metabolite in a liner manner, levels of 1,25-OH-D_3 reach a plateau, see Fig. 2, D.

4. Discussion

Whilst the efficacy of the supplementation 25-OH-D_3 has been well reported in humans and terrestrial livestock (Yarger et al., 1995; Burild et al., 2016; Quesada-Gomez and Bouillon, 2018), very few studies have focused on this vitamin D metabolite in cultivated fish and shrimp. This is surprising given the increasingly realized diversity of the physiological functions of the vitamin D endocrine system in vertebrates, including fish (Lock et al., 2010; Fraser, 2018; Liu et al., 2022). The

Table 4
Mean health indices of fish fed various levels of dietary 25-OH-D_3 over 91 days.

Diet (µg/kg 25-OH-D_3)	0	69.8	687	6854	ANOVA
K	1.39 ± 0.18	1.40 ± 0.11	1.39 ± 0.08	1.39 ± 0.09	0.992
*Diff. to control in % (90% CI)		−0.18 (−7.31;6.95)	−0.55 (−7.4;6.3)	0.33 (−6.8;7.46)	
LSI	1.13.5 ± 0.37	1.11.5 ± 0.18	1.13 ± 0.17	1.15 ± 0.15	0.998
*Diff. to control in % (90% CI)		0.29 (−13.54;14.13)	0.21 (−13.08;13.5)	0.97 (−12.87;14.8)	

Fulton's condition factor (K); Liver somatic index (LSI).
Data presented are means ($n = 4$ tanks) ± standard deviation (SD).

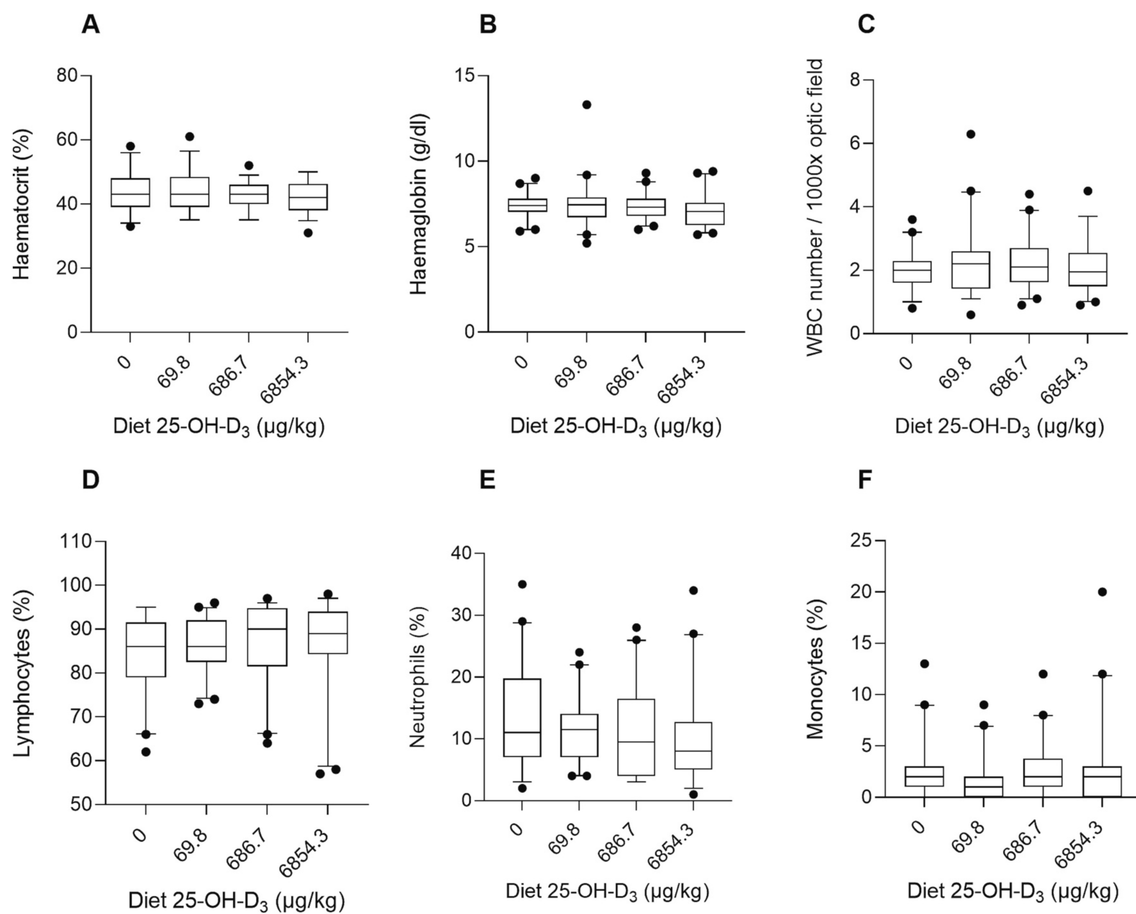

Fig. 1. Hematological response to dietary 25-OH-D_3. Box and whisker plots showing 5–95% confidence intervals and outliers. (A) Hematocrit (ANOVA p 0.902) and (B) hemoglobin (ANOVA p 0.568) are not significantly different to the control. C) White blood cell (WBC) counts are not significantly affected by dietary 25-OH-D_3, ANOVA p 0.779. D-F) Proportions of lymphocytes (Kruskal Wallis p 0.164), neutrophils (ANOVA p 0.167) and monocytes (Kruskal Wallis p 0.114) are not significantly different to the control.

Table 5
Ranges and mean blood chemistry values of test fish fed various dietary levels of 25-OH-D_3.

Diet (μg/kg 25-OH-D_3)	† Range	0	69.8	687	6854	Adjusted *P*-value
Means ± standard deviation (normal distributions)						
Albumin (g/l)	15.4–23.7	18.9 ± 1.5	19.0 ± 2.0	18.4 ± 1.3	19.0 ± 1.0	0.658 [1]
Chloride (mmol/l)	117–140	128 ± 4	130 ± 5	128 ± 4	126 ± 4	0.573 [1]
Cholesterol (mmol/l)	5.0–12.6	8.16 ± 1.18	8.48 ± 1.75	8.19 ± 1.23	8.77 ± 0.74	0.436 [1]
Phosphate (mmol/l)	3.46–9.18	5.82 ± 1.05	5.68 ± 1.43	5.84 ± 1.13	5.92 ± 0.91	0.941 [1]
Protein (g/l)	31.0–51.6	39.9 ± 4	40 ± 4.6	39.1 ± 3.1	40.4 ± 2.1	0.872 [1]
Sodium (mmol/l)	140–170	155 ± 7	156 ± 7	155 ± 6	154 ± 6	0.941 [1]
Medians with lower and upper quartiles in parenthesis (skewed distributions)						
ALP (U/l)	40–130	78 (62–97)	73 (59–86)	69 (60–82)	83 (74–102)	0.287 [2]
ALT (U/l)	5–35	5 (5–7)	5.5 (5–7.5)	6.5 (5–9)	5 (5–8.5)	0.937 [3]
Amylase (U/l)	216–1780	623 (395–876)	644 (470–942)	711 (520–978)	672 (528–927)	0.232 [2]
AST (U/l)	155–1806	333 (310–373)	315 (250–395)	362 (292–471)	339 (285–426)	0.912 [3]
Calcium (mmol/l)	2.63–4.32	3.12 (2.93–3.38)	3.15 (2.84–3.5)	3.08 (2.96–3.28)	3.1 (2.99–3.23)	0.936 [2]
CK (U/l)	705–144,800	2780 (1880–5136)	3213 (2176–5505)	4515 (2135–9509)	5474 (2772–7927)	0.317 [3]
Glucose (mmol/l)	0.11–7	1.8 (1–3.1)	2.6 (1.5–3.9)	1.8 (1–2.7)	1.4 (0.8–2.5)	0.533 [3]
LDH (U/l)	466–9496	874 (718–1069)	939 (802–1561)	967 (810–1672)	1003 (849–1809)	0.326 [3]
Magnesium (mmol/l)	1.1–1.98	1.46 (1.38–1.57)	1.46 (1.26–1.52)	1.48 (1.39–1.54)	1.38 (1.31–1.52)	0.927 [2]
Potassium (mmol/l)	1.5–17.2	2.2 (1.5–11)	2.6 (2–10.8)	2.2 (1.6–9.3)	2.4 (1.8–9.3)	0.927 [3]
Urea (mmol/l)	0.7–1.5	0.9 (0.9–1)	0.9 (0.9–1)	0.9 (0.9–1)	1 (1–1.1)	0.126 [3]
Uric acid (μmol/l)	12–42	15 (12–21)	15 (12–23)	15 (12–22)	16 (13–19)	0.998 [3]

Alanine aminotransferase (ALT); aspartate aminotransferase (AST); lactate dehydrogenase (LDH); alkaline phosphatase (ALP); creatine kinase (CK).
† Range between individual fish. * Groups not sharing subscripts are significantly different to the control, P values unadjusted for multiplicity testing. [1] ANOVA [2] ANOVA on log-transformed data [3] Non-parametric Kruskal-Wallis test.

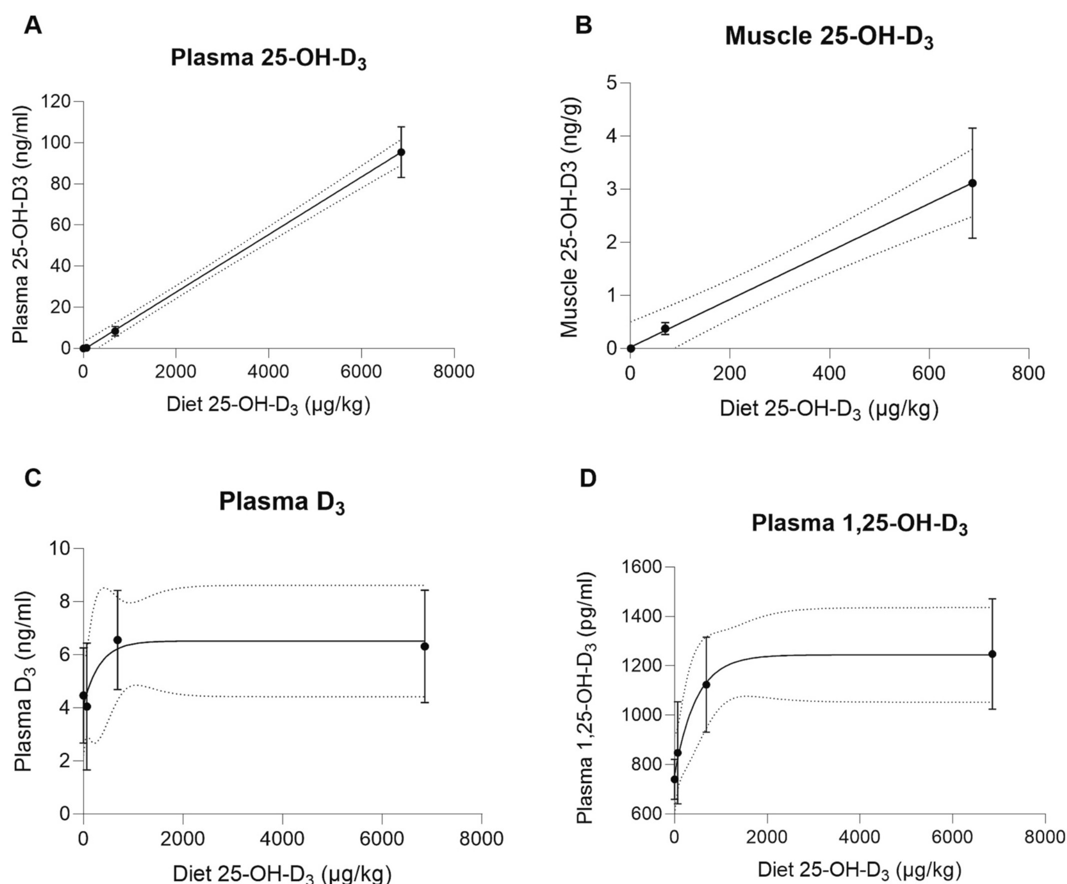

Fig. 2. The response of vitamin D_3 metabolites in the plasma and muscle to dietary 25-OH-D_3. All graphs show mean values per group ± SD. Linear or non-linear lines of best fit are shown with 95% confidence intervals. A) Increased 25-OH-D_3 in the feed linearly increases respective levels in the plasma; linear regression, $p <$ 0.0001, R^2 0.9820 and slope 0.01400. B) Increased 25-OH-D_3 in feed results in linear increases of 25-OH-D_3 in the muscle; linear regression, $p < 0.0001$, R^2 0.8760 and slope 0.004510. C) Increases of dietary 25-OH-D_3 results in a slight increase of plasma D_3 up to a plateau; non-linear regression, p 0.001 and R^2 0.2464. D) Increased dietary 25-OH-D_3 results in a 68.6% increase of plasma 1,25-OH-D_3 up to modelled plateau of 1245 pg/ml compared to control fish; non-linear regression 0.0001 and R^2 0.6166.

results from this study supplementing 25-OH-D_3 show that combined levels of dietary vitamin D metabolites that are approximately 10-fold higher than current recommendations for D_3 alone improve growth performance and feed utilization with at least a 10-fold margin of safety. This study focused on D_3 metabolites as opposed to ergocalciferol (D_2), as the latter is widely reported to have a much-reduced physiological activity and potency in fish, including salmonids (Barnett et al., 1982a; Lock et al., 2010; Soto-Dávila et al., 2020).

The current findings of improved performance with the supplementation of 25-OH-D_3 suggest that in some instances, optimal vitamin D_3 status may not be met with current recommendations of dietary D_3. This study was based on a basal diet not only apparently replete for vitamin D_3, but one containing 5240 IU (131 μg)/ kg diet D_3, which well covers the established recommended ranges for salmonids (NRC, 2011). The latest recommendations for vitamin D_3 in salmonids suggest, that based on the saturation of whole body D_3, 2400 to 3600 IU (60–90 μg)/ kg diet are required for parr and post-smolts fed diets with a low inclusion (~10%) of fish meal and krill meal (Antony Jesu Prabhu et al., 2019; Liu et al., 2022). It is frequently reported that salmonids require higher levels of vitamin D than other fish species (Lock et al., 2010). A low inclusion of marine-derived ingredients reduces endogenous D_3 contributions to aquafeeds, as fishmeal and fish oil are rich sources of D_3 (Sissener et al., 2013). The improved growth and feed utilization observed in the present study indicates that increasing dietary vitamin D_3 above recent recommendations by use of supplemental 25-OH-D_3 can benefit the zootechnical performance of salmonids fed practical diets. In contrast to plasma calcium concentration and bone ash content, weight gain is currently regarded as the most appropriate criterion for assessing the vitamin D_3 requirements of fish (Woodward, 1994; Lock et al., 2010).

Our data indicate that trout are not only well tolerant to 25-OH-D_3, but growth performance and feed conversion are improved in fish fed diets containing this metabolite, particularly at higher dietary doses. Both vitamin D deficiency and toxicity are known to reduce growth and feed conversion in fish (Brown and Robinson, 1992; Vielma et al., 1998; Graff et al., 2002; Knuth et al., 2020; Shao et al., 2022). Vitamin D_3 deficiency in salmonids has been reported to cause reduced growth in several studies (Barnett et al., 1982a; Barnett et al., 1982b; Taveekijakarn et al., 1996). Salmonids are known to be well tolerant to high dietary doses of vitamin D_3 and reports of reduced growth due to high vitamin D_3 in salmonids are rare, even in the presence of hypercalcemia (Hilton and Ferguson, 1982; Graff et al., 2002; Lock et al., 2010). In contrast to our findings with 25-OH-D_3, high doses of dietary D_3 (up to 1,004,000 IU /25.1 mg/kg) do not significantly improve growth (Hilton and Ferguson, 1982; Hayes et al., 1986; Graff et al., 2002). The improved growth of fish supplemented with the two higher doses of 25-OH-D_3 in the current study can be, in part, attributed to the concurrent improvements in feed conversion. Similar enhancements in feed utilization have been reported in other fish species fed diets containing optimal dietary levels of dietary D_3 (Brown and Robinson, 1992). Another physiological factor that may have contributed to the improved growth may the potent mitogenic properties of vitamin D_3 in fish, as demonstrated in vivo in proliferating zebrafish cardiomyocytes with alfacalcidol, an analogue of vitamin D (Han et al., 2019). In the current

study, the maximum growth was found in the 687 μg/kg 25-OH-D_3 group indicating that the optimal dietary dose of 25-OH-D_3 lies within the dose range tested.

Supplementation of 25-OH-D_3 to fish diets results in corresponding increases of the molecule in the blood, confirming that this metabolite is readily absorbed by fish. In humans a serum 25-OH-D_3 of >20 ng/ml is regarded as sufficient (Munns et al., 2016). In contrast, several studies have shown that in fish, circulating plasma levels of 25-OH-D_3 are lower than in terrestrial species. In farm raised rainbow trout fed commercial diets, 25-OH-D_3 is not detectable (<1.2 ng/ml) and similar findings have been reported for other fish species (Lock et al., 2010; Pierens and Fraser, 2015). In experimental salmonids fed diets containing D_3, reported plasma 25-OH-D_3 ranges from 1.63 to 6.37 to ng/ml (Nahm et al., 1979; Lock et al., 2007; Fjelldal et al., 2009). Even in salmon fed diets containing extremely high levels of D_3 (28.7 mg/kg), plasma 25-OH-D_3 only reached 10.4 ng/ml (Horvli and Aksnes, 1998). In the present study, 25-OH-D_3 was below limits of quantification (<0.5 ng/ml) in most individual fish fed the control diet. By contrast, in fish fed the highest 6854 μg/kg 25-OH-D_3 dose, corresponding plasma levels increased significantly to 95.5 ng/ml. This may indicate a limitation in the conversion of D_3 to 25-OH-D_3 in fish. A greater bioavailability of 25-OH-D_3 compared to D_3 has been well documented in humans and terrestrial livestock (Yarger et al., 1995; Burild et al., 2016; Quesada-Gomez and Bouillon, 2018). In rainbow trout, over 80% of (injected) circulating 25-OH-D_3 is rapidly metabolized within two hours to four more polar metabolites, the majority of which includes 1,25-OH-D_3 and 25,26-OH-D_3 (Hayes et al., 1986). In goldfish and trout, 25-OH-D_3 is rapidly metabolized and excreted via the bile compared to excess D_3, some of which esterified in the liver, possibly for metabolic inactivation by sequestration (Oizumi and Monder, 1972; Pierens and Fraser, 2015). In the current study, neither the 3C epimer 3-epi-25-OH-D_3 nor 24(R), 25-dihydroxy vitamin D_3 (24,25-OH-D_3) were detected in any of the test fish (LOQ <0.5 ng/ml). In terrestrial species, 24,25-OH-D_3 results from hydroxylation of 25-OH-D_3 by 25-hydroxycholecalciferol-24-hydroxylase, but may not constitute an important catabolic pathway for 25-OH-D_3 in trout residing in freshwater (Lock et al., 2010). 24,25-OH-D_3 has, however, been detected salmonids residing in seawater, where this metabolite maybe required for the increased uptake of calcium (Lock et al., 2007; Lock et al., 2010).

Supplementing 25-OH-D_3 to trout diets results in a regulated increase of the physiologically active form of vitamin D. In vertebrates, including fish, 1,25-OH-D_3 regulates hundreds of genes via the vitamin D receptor (VDR), which imparts its regulation on the genome by forming a heterodimer with the retinoid-X receptor (Lock et al., 2007; Craig et al., 2008; Carlberg, 2014; Carlberg, 2019). Given the widespread expression of the VDR accross the majority of fish tissues, active vitamin D can be expected to be implicated in a wide number of physiologocal processes in fish, some of which are yet to be fully elucidated. (Lock et al., 2007; Craig et al., 2008; Peng et al., 2017). The essentiality 1,25-OH-D_3 in teleosts has been demonstrated in zebrafish by the knockout of the cyp2r1 gene, which encodes cytochrome P450 2r1, the principal vitamin D 25-hydroxylase, which converts D_3 to 25-OH_2-D_3 in the liver. The knockout of cyp2r1 reduces plasma 1,25-OH-D_3 by two thirds causing significant reductions in growth and the impairment of lipid metabolism. Both these effects are rescued, and even improved, by the supplementation of 25-OH-D_3 (Peng et al., 2017). Similarly, in zebrafish, dietary vitamin D_3 deficiency causes growth retardation, dyslipidemia, and impacts growth hormone and cytokine signaling (Knuth et al., 2020). Our study in rainbow trout and previous studies in Atlantic salmon show that the concentrations of circulating 1,25-OH-D_3 in salmonids are in orders of magnitude greater than in mammalian blood plasma (Lock et al., 2007; Lock et al., 2010). In contrast to mammals where 25-OH-D_3 is the metabolite used to assess vitamin D status, our study shows that in salmonids, 1,25-OH-D_3 is a more relevant marker of vitamin D status. In the current study, the 687 μg/kg 25-OH-D_3 dose at which the plateau of circulating 1,25-OH-D_3 was reached was also the dose that achieved the maximal growth. In trout, the second hydroxylation of 25-OH-D_3 to 1,25-OH-D_3 principally takes place in the liver prior to its secretion into the circulation; a much lesser conversion occurs in the kidney (Hayes et al., 1986; Pierens and Fraser, 2015). This contrasts with mammals where the second hydroxylation of 25-OH-D_3 to 1,25-OH-D_3 principally takes place in the kidney (Hayes et al., 1986; Lock et al., 2010). The supplementation of D_3 versus 25-OH-D_3 may have different consequences on levels of circulating active vitamin D_3. In contrast to our study supplementing 25-OH-D_3, previous studies in salmonids replete for D_3 show that excess supplemented D_3 does not increase circulating 1,25-OH-D_3 or results in decreases (Horvli and Aksnes, 1998; Avila et al., 1999).

The plateau of circulating 1,25-OH-D_3 in the present study suggests that active vitamin D derived from dietary 25-OH-D_3 is well regulated by fish, with no effects on blood chemistry or hepatic health. A panel of blood chemistry and hematology markers were measured and interpreted as performed for veterinary purposes (Bush, 1991). These common blood tests have also previously been successfully applied in rainbow trout (Manera and Britti, 2006). Levels of blood electrolytes, including calcium and phosphorus, were used as a measure of homeostasis in acid-base, water balance and ion transport. Plasma proteins including albumin are produced in the liver and retained by the kidney, thus providing a measure of liver function and kidney health. Total plasma cholesterol indicates any changes in lipid metabolism. Blood urea and uric acid are waste metabolites from protein and purine metabolism, respectively, and reflect renal function. Amylase breaks down carbohydrates and is used as an indicator of pancreatic function. Creatinine kinase is involved in energy metabolism and changes in blood levels can signify muscle and kidney damage. ALT, AST, ALP and LDH are largely, but not exclusively, hepatic enzymes involved protein and energy metabolism. Changes in these enzymes can be associated with impaired liver function and tissue damage (Bush, 1991). A potential toxic action of excess 25-OH-D_3 could be the induction of hypercalcemia and nephrocalcinosis by the endocrine actions of 1,25-OH-D_3 secreted by the liver (Pierens and Fraser, 2015). Several studies have shown hypercalcemic effects of vitamin D in fish, but these depend on the metabolite fed, its concentration, the species and waterborne calcium levels (Lock et al., 2010). In the present study, circulating levels of calcium, phosphorus and magnesium were all stable and no gross observations of nephrocalcinosis were observed at any of the doses tested in this study. Similar findings have been reported in trout fed diets with high levels of D_3 (25,100 μg/kg), which had no effects on plasma glucose, calcium, protein or hemoglobin (Hilton and Ferguson, 1982). Above sufficiency (2500 IU/kg), increasing dietary D_3 does not modulate plasma phosphorus (Avila et al., 1999). The tolerance of trout to 25-OH-D_3 is shown in the current study by the lack of changes in hepatic enzyme activity and liver somatic index (LSI). Similarly, a wide range of dietary D_3 doses (40 to 28,000 μg/kg) does not affect the LSI of Atlantic salmon (Horvli and Aksnes, 1998).

The supplementation of dietary 25-OH-D_3 up to 687 μg/kg 25-OH-D_3 does not significantly increase corresponding levels of this metabolite in the fish fillet. Salmon fillets are regarded as a rich source of vitamin D for humans (Graff et al., 2016). Whilst the vitamin D content of wild fish can vary between location and season, Atlantic salmon from the Baltic and North Seas are reported to contain between 94 and 180 ng/g D_3. The same study reports that farmed salmon contain between 29 and 95 ng/g D_3 (Jakobsen et al., 2019). The 25-OH-D_3 content of muscle in the same wild salmon is much lower, ranging from 2.0 to 3.0 ng/g. Feeding dietary D_3 at 230, 2600 and 2900 μg/kg improves the D_3 content from 90 to 350 and 380 ng/g D_3 in the fillet of Atlantic salmon, respectively (Graff et al., 2016). In another study, in salmon fed 2210 μg/kg cholecalciferol, fillet D_3 was 210 ng/g, and this increased to 2100 ng/g in fish fed a high dose of 28.7 mg/kg. In the current study, fish fed 69.8 and 686.7 μg/kg dietary 25-OH-D_3 only deposited 0.38 ± 0.1 and 3.1 ± 1.0 ng/g 25-OH-D_3 in the fillet, respectively. This is orders of magnitude less than the D_3 levels reported to be deposited in Atlantic salmon.

Collectively these data indicate that the muscle of salmonids has a high capacity to store vitamin D_3, but not 25-OH-D_3. The storage capacity of the liver for 25-OH-D_3 was not tested in this study. Similarly, in livestock tissue vitamin D_3 levels were higher in the pigs fed D_3 compared with those fed 25-OH-D_3 (Burild et al., 2016). As with the results for blood metabolites, these data for the muscle also suggest that D_3 may be more readily stored in the body than 25-OH-D_3, the latter which is more readily metabolized to produce active vitamin D or for excretion.

This study has revealed the beneficial properties and safety of 25-OH-D_3 for the supplementation of vitamin D to salmonids. Salmonids have a particularly high requirement for vitamin D, which further increase with the use of modern diets containing low inclusions of marine-derived feed ingredients, which are rich sources of vitamin D. 25-OH-D_3 is effectively absorbed into the blood plasma and increases active 1,25-OH-D_3 according to the needs of the fish. Due to the bypass of the first hydroxylation step, 25-OH-D_3 may prove particularly effective during periods of increased 1,25-OH-D_3 demand, such as during smoltification or disease. The low accumulation of 25-OH-D_3 by the muscle suggests that rather than being stored in the body of fish, 25-OH-D_3 is more readily metabolized for the production of active vitamin D, or for excretion when in excess. Optimal growth, lipid metabolism and immunity all depend on an optimal status of active vitamin D, which may be ensured using 25-OH-D_3. Crucially, the supplementation of 25-OH-D_3 allows the fish to determine the optimal levels of this potent active hormone without risks of impaired liver function or hypercalcemia.

CRediT authorship contribution statement

S. Rider: Conceptualization, Methodology, Visualization, Investigation, Formal analysis, Writing – original draft, Project administration. **V. Verlhac-Trichet:** Conceptualization, Methodology. **D. Constant:** Methodology, Investigation. **E. Chenal:** Methodology, Investigation, Writing – original draft, Writing – review & editing. **S. Etheve:** Methodology, Investigation. **B. Riond:** Investigation, Writing – original draft, Writing – review & editing. **H. Schmidt-Posthaus:** Investigation, Visualization, Writing – original draft, Writing – review & editing. **R. Schoop:** Formal analysis, Visualization, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Sebastien Rider reports financial support was provided by DSM Nutritional Products AG. Sebastien Rider reports a relationship with DSM Nutritional Products AG that includes: employment. Sebastien Rider has patent pending to DSM. S. Rider, R. Schoop, E. Chenal, and S. Etheve are all employees of DSM nutritional products AG, Wurmisweg 576, Kaiseraugst, Switzerland.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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