Aquaculture 543 (2021) 736991

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Effects of vitamin D_3 supplementation in gilthead seabream (*Sparus aurata*) juveniles fed diets high in plant based feedstuffs

David Dominguez [a,*], Daniel Montero [a], Maria Jesus Zamorano [a], Pedro Castro [a], Ramon Fontanillas [b], Philip Antony Jesu Prabhu [c], Marisol Izquierdo [a]

[a] Aquaculture Research Group (IU-ECOAQUA), University of Las Palmas de Gran Canaria, Carretera de Taliarte, s/n, 35200 Telde, Gran Canaria, Spain
[b] Skretting Aquaculture Research Centre AS, PO Box 48, N-4001 Stavanger, Norway
[c] Fish nutrition program, Institute of Marine Research, P.O. Box 1870, 5817 Bergen, Norway

ARTICLE INFO

Keywords:
Vitamin D
Gilthead seabream
Requirements
Optimum levels
Skeletal anomalies
Nutrition

ABSTRACT

Modern aquaculture feeds tend to contain lower levels of fish based ingredients, while increasing the content of plant ingredients. However, this may alter the vitamin profile of the feeds, leading to unbalanced vitamin supply. Requirements for several vitamins have been established for species such as carps and salmonids, but adequate levels for gilthead sea bream are yet unknown.

Vitamin D is mainly involved in Ca homeostasis by regulating Ca uptake and liberation from bone intervening in bone remodeling. Fish are unable to synthesize vitamin D and so require absorbing it directly from the diet, thus, it is considered essential for fish. A practical plant-based diet containing 10% fish meal and 6% fish oil containing five levels of vitamin D_3 (0.15, 0.43, 0.50, 0.55 and 0.65 mg kg^{-1} or 5.8, 17.0, 20.0, 22.0 and 26.0 IU g^{-1}) were formulated to identify the optimum levels for gilthead seabream juveniles. Feeding juveniles of gilthead seabream with a range of vitamin D_3 levels between 5.8 and 26.0 IU g^{-1} for 70 days did not markedly alter growth. Increase dietary vitamin D_3 significantly raised the liver contents in vitamin D_3 in a dose-dependent manner following a potential regression. Increased dietary vitamin D_3 levels up to 11.6 IU g^{-1} may reduce the incidence of skeletal anomalies, particularly caudal and maxillary anomalies, whereas further elevation of dietary vitamin D_3 levels increased the concentration of vitamin D_3 in liver as well as skeletal anomalies in association to the up-regulation of *alp* and *bmp2* gene expression. The occurrence of myocarditis signs in fish fed vitamin D_3 levels of 20.0 IU g^{-1} or more denote the toxic effects of these dietary levels. These results, together with the increased occurrence of skeletal anomalies in seabream fed the highest dietary vitamin D_3 levels, suggest initial signs of hypervitaminosis D. Thus, the recommended level for vitamin D_3 for gilthead seabream juveniles fed diets containing high levels of plant ingredients was suggested to be 11.6 IU g^{-1}.

1. Introduction

The current trend in substituting marine based ingredients for alternative ingredients in aquaculture feeds translates in changes in their nutritional profile, and can cause an unbalanced vitamin supply (Hansen et al., 2015). In this sense, several studies have been conducted in order to elucidate the vitamin requirements in species of major interest for aquaculture (NRC, 2011). Despite its importance in the Mediterranean aquaculture, little attention has been paid to the vitamin requirements of gilthead seabream.

Vitamin D is mainly involved in Ca homeostasis, acting in synergy with calcitonin and parathyroid hormone. Together they regulate Ca uptake and liberation from bone intervening in bone remodeling (Halver, 2002; NRC, 2011; Boglione et al., 2013). Fish are unable to synthesize vitamin D, unlike humans (Lock et al., 2010), probably because UV radiation is absorbed by the water before it reaches the fish (Boglione et al., 2001), and so require absorbing it directly from the diet (Hamre et al., 2010; Lock et al., 2010). Once it is absorbed, deposition takes place in liver, intestine, kidney, spleen, gills, skin and muscle (Lock et al., 2010). Vitamin D_3 is known to modulate bone and trace mineral metabolism in fish (Vielma et al., 1999). Thus, vitamin D_3 supplementation increases bone mineralization in a dose-dependent manner, but may also have a bone catabolic effect in fish (Fleming et al., 2005; Wendelaar Bonga et al., 1983). Consequently, bone mineralization has

* Corresponding author.
E-mail address: david.dominguez103@alu.ulpgc.es (D. Dominguez).

https://doi.org/10.1016/j.aquaculture.2021.736991
Received 22 March 2021; Received in revised form 26 May 2021; Accepted 27 May 2021
Available online 29 May 2021

been used as a biomarker for vitamin D status (Fleming et al., 2005). Bone formation is tightly regulated by a series of genes and proteins that affect cell differentiation and mineralization, especially at early developmental stages. These markers include *runx2,* bone morphogenic proteins (*bmp*), alkaline phosphatase (*alp*) or osteocalcin (*oc*) (Fleming et al., 2005; Darias et al., 2010; Saleh et al., 2014). Vitamin D_3 essentiality on preventing the onset of skeletal anomalies was proved in European seabass (*Dicentrarchus labrax*), where diets containing low (11.2 IU VD_3/g diet) and high vitamin D_3 levels (42 IU VD_3/g) induced several skeletal anomalies in larvae (Darias et al., 2010). Besides its relevance for bone formation, vitamin D also plays important roles in muscle function and cardiovascular physiology (Lock et al., 2010).

Vitamin D requirements markedly vary among species. Optimum dietary levels have been reported to be as low as 0.00004, 0.005 or 0.00625 and 0.00935 mg vitamin D kg^{-1} diet, for Atlantic salmon (*Salmo salar*;Horvli et al., 1998), Wuchang bream (*Megalobrama amblycephala*; Ling-Hong et al., 2015*)*, channel catfish (*Ictalurus punctatus*; Brown, 1988) and hybrid tilapia (*Oreochromis niloticus* x *O. aureus*; Shiau and Hwang, 1993), respectively. On the opposite end, recommended levels for Siberian sturgeon (*Acipenser baerii*) and rice field eel (*Monopterus albus*) are as high as 1683–1403 and 5000 IU vitamin D kg^{-1} diet, respectively (Wang et al., 2017; Tan et al., 2007). Intermediate values are obtained for channel catfish (500–2000 IU vitamin D kg^{-1} diet Lovell and Li, 1978; Andrews et al., 1980) or salmonids, such as rainbow trout 1600 IU vitamin D_3 kg^{-1}; Barnett et al., 1982) or Atlantic salmon (0.06 or < 0.2 mg vitamin D_3 kg^{-1} diet; Woodward, 1994; Graff et al., 2002). Recent studies define the optimum levels for Atlantic salmon to be in the range of 0.06–0.09 mg vitamin D kg^{-1} as part of a practical approach using a multi-nutrient package with reduced levels of marine ingredients (Antony Jesu Prabhu et al., 2019), suggesting that slightly higher levels of supplementation are needed when feeds are based on ingredients alternative to fish meal and fish oil.

Inadequate doses of vitamin D in fish may reduce growth, tetany, alteration of thyroid hormone levels, thin epidermis, muscle necrosis, hypocalcaemia, erosion of fins, and increased liver and muscle lipid deposition (Halver, 2002; Taveekijakarn et al., 1996; Lock et al., 2010). On the other hand, toxicity symptoms can reduce growth, hypercalcaemia, and elevated haematocrit levels (Fleming et al., 2005; Lock et al., 2010). However, these are rare and even vitamin D levels as high as 1,004,000 IU kg^{-1} dry diet did not cause toxic effects in rainbow trout (*Oncorhynchus mykiss*Hilton and Ferguson, 1982).

As for gilthead seabream, studies demonstrated that dietary vitamin D_3 stimulated some cellular innate immune parameters, such as phagocytosis and serum peroxidase, after only 2 weeks in 150 g fish (Cerezuela et al., 2009). However, little knowledge is available at the moment regarding the essentiality of this vitamin in gilthead seabream. Thus, the aim of this study was to investigate on the effect of dietary vitamin D levels in practical diets on growth performance, proximate composition, and morphology of bone, liver and heart of gilthead seabream juveniles.

2. Material and methods

All the experimental conditions and sampling protocols have been approved by the Animal Welfare and Bioethical Committee from the University of Las Palmas de Gran Canaria.

2.1. Feeding trial and growth performance

A practical low fish meal (containing 68.8% crude protein and 10.5% crude lipid), plant-based diet (FM 10% and FO 6%) containing five increasing supplementation levels for vitamin D (0.15, 0.43, 0.50, 0.55 and 0.65 mg kg^{-1} or 5.8, 17.0, 20.0, 22.0 and 26.0 IU g^{-1} vitamin D_3), supplied by CV. China Vitamins, LLC (New Jersey, U.S.A.) was formulated (Table 1). The same basal diet was used, thus the energy (Gross Energy = 22 mJ/kg) and nitrogen composition were equal, and were designed to cover all known nutritional requirements for this species. Feeds were manufactured by extrusion process by Skretting Aquaculture Research Centre AS (Stavanger, Norway).

Table 1
Ingredients of the experimental diets supplemented with increasing levels of D_3 fed to gilthead seabream juveniles for 70 days.

Ingredient	g/kg
Corn gluten (Cargill B.V., Amsterdam, The Netherlands)	149.0
Wheat gluten (Skretting, Stavanger, Norway)	216.5
Soya bean concentrate (Skretting, Stavanger, Norway)	230.0
Faba beans (Skretting, Stavanger, Norway)	49.5
Fish meal, Scandinavian (Norsildmel, Bergen, Norway)	100.0
Wheat (Skretting, Stavanger, Norway)	115.0
Rapeseed oil (Skretting, Stavanger, Norway)	21.1
Linseed oil (Linagro, Lichtervelde, Belgium)	8.2
Fish oil, S.American (Skretting, Stavanger, Norway)	60.0
Palm oil (AAK AB, Karlshamn, Sweden)	16.3
Premixes (Trouw Nutrition, Boxmeer, The Netherlands*)	34.4

* Proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according NRC (2011) except for vitamin D that was added separately.

Four hundred and fifty gilthead seabream (*Sparus aurata*) juveniles, weighing 20.5 ± 0.3 g body weight, were distributed into 15 tanks in triplicate groups per diet and fed until apparent satiation thrice daily for 70 days under a natural photoperiod (12 h light). Water temperature (21.9 ± 0.2 °C), oxygen (>5.8 mg kg^{-1}) and feed intake were monitored daily. Growth and productive parameters were monitored along the trial. And on the end of the 10-week trial all the fish were sampled for weight and length, and euthanized using ice. Before sampling, fish were previously fasted for 24 h and, then, anesthetized with clove oil (Guinama S.L.U., Valencia, Spain). Tissues from 10 fish per tank were frozen (−20 °C) as samples for proximal composition and vitamin concentration; vertebrae from 5 fish per tank were frozen in liquid nitrogen and later kept at −80 °C for further gene expression analyses of bone molecular markers; samples from 5 fish per tank were submitted to 10% buffered paraformaldehyde for histological evaluation; the remaining 10 fish were frozen at −20 °C and X-ray were taken for osteological assessment of skeletal anomalies.

2.2. Vitamin D contents and proximate composition

Vitamin D_3 (cholecalciferol) content was evaluated in liver by Eurofins Mas Control S.L. (Santa Cruz de Tenerife, Spain) according to the European Standard UNE-EN 12821:2009. Homogenised and pooled liver samples were submitted to saponification through ethanolic solution of potassium hydroxide, and a double extraction with ethyl di-ester. A reverse-phase HPLC was used to quantify vitamin D_3 using UV/DAD detector at 265 nm.

Standard procedures were employed to evaluate the biochemical composition of diets and muscle (Association of Official Analytical Chemists (AOAC, 2000). Crude lipid was extracted according to the method of Folch et al. (1957) and ash by combustion in a muffle furnace at 600 °C for 12 h. Protein content (N × 6.25) was determined by using the Kjeldahl method (AOAC, 2000) and dry matter content was determined after drying the sample in an oven at 105 °C until reaching constant weight.

2.3. Skeletal anomalies

X-Ray analyses were conducted using a fixed X-ray apparatus (Bennett B-OTC, Bennett X-Ray Corp., Chicago, IL, USA) and a 35 × 43 cm digital film (Fujifilm FDR D-EVO (Fujifilm Corporation, Tokyo, Japan). Radiographs were treated digitally (Onis 2.4, DigitalCore, Co.Ltd., Tokyo, Japan) and skeletal anomalies classified according to Boglione et al. (2001).

2.4. Gene expression

2.4.1. RNA extraction

Total RNA was extracted from 60 mg of vertebrae using TRI Reagent Solution (Life Technologies, Carlsbad, CA, USA) and purified on RNeasy Mini Spin Columns (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.4.2. Reverse transcription

Reverse transcription of 1 μg total RNA from each experimental sample was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, 1 μg total RNA and nuclease-free water to a final volume of 15 μl were heated at 65 °C for 10 min and cooled in ice. Afterwards 1 μl of iScript reverse transcriptase and 4 μl of 5 × iScript reaction mix were added, reaching a final reaction volume of 20 μl. The complete reaction mix was incubated for 5 min at 25 °C, 30 min at 42 °C, and then 5 min at 85 °C to inactivate reverse transcriptase. For gene quantification, the reverse transcription reactions were diluted 1:10.

2.4.3. Quantitative PCR

The nucleotide sequences of primers used in this study are reported in Table 2. A total of 2 μl of diluted cDNA was used in real-time PCR for gene expression quantification using IQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Duplicate analyses were performed for each sample for both the housekeeping and the target gene in a final reaction volume of 20 μl. Beta actin (*bact*) and Elongation Factor 1- alpha (*ef1α*) were used as housekeeping genes to normalize the expression of the target genes in vertebrae. Real-time quantitative PCR was performed using the iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR conditions were as follows: 95 °C for 3 min and 30 s, followed by 40 cycles of 95 °C for 15 s, 58.1 °C for 30 s, and 72 °C for 30 s; 95 °C for 1 min, and a final denaturation step from 58 to 95 °C for 10 s. The $2^{-\Delta\Delta Ct}$ method was applied to analyse the relative changes in gene expression.

2.5. Histological studies

Liver and heart samples were further segmented to allow a better penetration of the alcohol and introduced in histology cassettes. Dehydration of the samples was carried out using a Histokinette 2000 (Leica, Nussloch, Germany) with gradually increasing alcohol grades beginning with 70° and ending with 100°, being the last two steps xylene and paraffin. Once the paraffin block was obtained it was sliced at a thickness of 3 μm using a Leica RM 2135 microtome (Leica, Nussloch, Germany) and fixed to a slide including as much parts of the tissue as possible. Samples were then stained with haematoxylin – eosin staining (Martoja and Martoja-Pierson, 1970) for optical evaluation. Once the preparations were ready they were subjected to optical analysis in search for signs of liver and pancreas damage such as fat accumulation, signs of inflammation and presence of eosinophils, bile duct obstruction, etc.; as well as for symptoms of heart damage including cardiac congestion, swollen cardiac muscle and presence of eosinophils in cardiac tissue, and analyzed by pair evaluators in a 0–3 scale, where 0 was absence of observation and 3 presence in most of the tissue.

Table 2
Sequences of primers used for gene expression analysis.

Gene	Nucleotide sequence (5′-3′)	Accession number
Beta-actin (*bact*)	F: TCTGTCTGGATCGGAGGCTC R: AAGCATTTGCGGTGGACG	X89920
Elongation factor 1a (*ef1a*)	F: CATGGTTGTGGAGCCCTTCT R: TCCTGCACGACCATTCATTTC	AF184170
Alkaline phosphatase (*alp*)	F: AGAACGCCCTGACGCTGCAA R: TTCAGTATACGAGCAGCCGTCAC	AY266359
Runt-related transcription factor 2 (*runx2*)	F: GCCTGTCGCCTTTAAGGTGGTTGC R: TCGTCGTTGCCCGCCATAGCTG	AJ619023
Osteocalcin (*oc*)	F: GGCAGCCATCTGTCTGACTT R: GGTCCGTAGTAGGCCGTGTA	AF048703
Bone morphogenic protein 2 (*bmp2*)	F: GTGGCTTCCATCGTATCAACATTTT R: GCTCCCCGCCATGAGT	JF261172.1

2.6. Statistics

All data were statistically analyzed using SPSS v21 (IBM Corp., Chicago, IL, USA) and means ± SD were calculated for every parameter measured. Data were tested for normality with the one-sample Kolmogorov–Smirnov test. For normally distributed data, one-way analysis of variance (ANOVA) was used to determine the effects of the different diets. Data were tested for homogeneity of variances and post-hoc analysis was carried out using Tukey test if variances were homogeneous or Games-Howell test whenever variances were different. When data did not follow a normal distribution, logarithmic or arcsin transformation was carried out and the non-parametric tests of Kruskal-Wallis was used. A series of quadratic and lineal regression, as well as broken line analyses were conducted where possible to describe the effects of vitamin D_3 on the fish. Significant differences were considered for $p < 0.05$. Weight gain (WG), Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR) were calculated using the following formulae:

$$\text{Weight gain } (\%) = 100 \times (\text{final weight} - \text{initial weight})/\text{initial weight}$$

$$\text{SGR } (\%) = (\text{Ln}_{W2} - \text{Ln}_{W1})/\text{days} \times 100$$

$$\text{FCR} = \text{Ingested food}/\text{generated biomass}$$

where

W_1: initial body weight (g)
W_2 final body weight (g)

3. Results

3.1. Feeding trial and growth performance

Fish readily accepted the experimental diets from the beginning of the trial, and no mortalities were recorded along the trial. After 70 days of feeding there were no significant differences in final body weight, weight gain or SGR, among the mean values of fish fed the different levels of vitamin D_3 (Table 3). Nevertheless, weight gain and SGR showed a trend towards higher values with increased dietary vitamin D_3 following significant linear relations ($p = 0.05$; $R^2 = 0.26$ and $p = 0.05$; $R^2 = 0.264$, respectively).

Table 3
Performance of juvenile gilthead seabream fed diet different levels of vitamin D_3 for 70 days.

Dietary vitamin D_3 (IU g^{-1})	5.8	17.0	20.0	22.0	26.0	*p*-value
Final weight (g)	49.2 ± 5.1	49.5 ± 5.8	51.3 ± 5.4	49.3 ± 5.2	51.2 ± 6.5	n.s.
WG	138.7 ± 13.8	138.8 ± 6.4	151.3 ± 5.9	151.1 ± 10.6	151.3 ± 7.2	n.s.
SGR	1.28 ± 0.09	1.28 ± 0.04	1.36 ± 0.04	1.35 ± 0.06	1.36 ± 0.04	n.s.
FCR	1.09 ± 0.02	1.12 ± 0.03	1.15 ± 0.05	1.11 ± 0.06	1.10 ± 0.04	n.s.

n.s.: non-significant: *p*-value > 0.05.

3.2. Vitamin D contents and proximate composition

Vitamin D_3 content in liver significantly increased following a quadratic regression ($p = 0.00$; $R^2 = 0.982$) with the dietary vitamin D_3 levels (Table 4, Fig. 1), whereas muscle proximate composition was not affected by dietary vitamin D_3.

3.3. Skeletal anomalies

Skeletal anomalies were predominantly found in the anterior region including cranium and, predominantly, pre-haemal vertebrae (Table 5). The incidences of anomalies from the maxillary and/or pre-maxillary (Fig. 2), and of pre-haemal vertebral fusions and followed a quadratic regression ($R^2 = 0.94$, $p = 0.05$ and $R^2 = 0.93$, $p = 0.07$, respectively) with the level of dietary vitamin D_3 showing the lowest incidences of anomalies around 11.6–15.5 IU g^{-1} vitamin D_3. Moreover, the incidences of pre-haemal fusion, anomalies from the maxillary and/or pre-maxillary, and caudal anomalies also followed a quadratic regression with the liver contents in vitamin D_3 ($R^2 = 0.94$, $p = 0.059$; $R^2 = 0.93$, $p = 0.074$; and $R^2 = 0.48$, $p = 0.19$, respectively), with the lowest incidences of anomalies 11.6–15.5 IU g^{-1} vitamin D_3 in diet.

3.4. Gene expression

Analyses conducted in vertebrae to evaluate bone molecular markers showed a quadratic regression between dietary vitamin D_3 and *bmp2* ($R^2 = 0.76$, $p = 0.015$) and *oc* ($R^2 = 0.58$, $p = 0.073$), both with inflection points around 12.8 IU g^{-1} vitamin D_3. Besides, a strong linear regression was observed between the expression of *bmp2* and maxillary ($R^2 = 0.99$, $p = 0.05$) or caudal anomalies ($R^2 = 1.00$, $p = 0.001$), as well as between *alp* and caudal anomalies ($R^2 = 1.00$, $p = 0.03$). On the other hand, *runx2* was not significantly affected by dietary vitamin D_3 levels (Table 6).

3.5. Histological studies

Study of hepatic morphology showed very similar characteristics among livers of fish fed the different vitamin D_3 levels and a comparable degree of steatosis (Table 7). However, there was a non-significant ($p = 0.073$) increase in certain signs of myocarditis, such as cardiac congestion and swollen cardiac muscle, with the increase in dietary vitamin D_3 (Table 7). Moreover, there were significant potential regressions between the dietary vitamin D_3 levels and cardiac congestion ($R^2 = 0.67$, $p = 0.034$) or swollen cardiac muscle ($R^2 = 0.92$, $p = 0.00$). Equally, there was a significant potential regression between liver vitamin D_3 contents and swollen cardiac muscle ($R^2 = 0.963$, $p = 0.018$).

Table 4
Muscle composition (% dry weight) and liver D_3 contents of gilthead seabream fed increasing contents of vitamin D_3 for 70 days.

Dietary vitamin D_3 (IU g^{-1})	5.8	17.0	20.0	22.0	26.0	*p*-value
Liver vitamin D_3 (mg kg^{-1})	0.16 ± 0.03[a]	0.62 ± 0.07[b]	0.68 ± 0.02[bc]	0.69 ± 0.05[bc]	0.87 ± 0.07[c]	0.00
Muscle lipids (% d.w.)	13.2 ± 0.6	14.1 ± 0.7	12.3 ± 0.6	13.5 ± 0.8	13.7 ± 0.5	n.s.
Muscle ash (% d.w.)	1.6 ± 0.0	1.6 ± 0.1	1.6 ± 0.0	1.5 ± 0.0	1.6 ± 0.0	n.s.
Muscle protein (% d.w.)	21.0 ± 0.1	21.0 ± 0.3	21.2 ± 0.2	20.9 ± 0.4	21.4 ± 0.7	n.s.

Different letters in the same row indicate significant differences, $p < 0.05$, n = 3. n.s.: non-significant: *p*-value > 0.05.

4. Discussion

Despite the importance of vitamin D for bone and trace mineral metabolism in fish, there is no information about the vitamin D requirements of gilthead seabream, especially when practical diets are used. In the present study, feeding juveniles of this species with a range of vitamin D_3 levels between 5.8 and 26.0 IU g^{-1} for 70 days until fish had double their weight, did not markedly altered gilthead seabream growth. These results may suggest that the basal dietary levels were sufficient to cover the requirements of this species for growth. However, weight gain and SGR values increased between 6 and 9% following a linear regression with dietary vitamin D_3, which could also indicate that feeding these vitamin D_3 levels for longer periods of time could have improved growth.

Increase dietary vitamin D_3 significantly raised the liver contents in vitamin D_3 in a dose-dependent manner following a potential regression. This result was in agreement with the increase in liver vitamin D_3 found in first-feeding fry of Atlantic salmon (*Salmo salar*) (Graff et al., 2002) or Siberian sturgeon (*Acipenser baerii*) (Wang et al., 2017) fed dietary vitamin D_3 levels of 0.2–57.0 mg kg^{-1} and 60–1.0 × 10^5 IU kg^{-1}. Moreover, the pattern of increase of vitamin D_3 in gilthead seabream liver was similar to that found in liver and other tissues of Atlantic salmon (Horvli et al., 1998), denoting a nonspecific accumulation of the vitamin even when fed at high dietary doses (0.04–28.68 mg kg^{-1}). Recent studies found that the whole body contents of vitamin D_3 in Atlantic salmon reach a saturation level around 0.06–0.09 mg kg^{-1} dietary vitamin D_3 (Antony Jesu Prabhu et al., 2019). However, it must be considered that in the study by Antony Jesu Prabhu et al. (2019) an increase in dietary vitamin D_3 was concomitant with the increase in other nutrients, which could interact with vitamin D_3 deposition in body tissues.

Dietary vitamin D_3 did not affect the proximate composition of gilthead seabream muscle, in agreement with the lack of effect found also in Atlantic salmon fed up to 57 mg kg^{-1} vitamin D_3 for 90 days (Graff et al., 2002). On the contrary, whole body or muscle proximate composition is markedly affected by vitamin D_3 in rainbow trout (Barnett et al., 1982), Wuchang bream (Ling-Hong et al., 2015) or Siberian sturgeon (Wang et al., 2017), lipid contents being increased in vitamin D deficient fish.

Vitamin D seems to have a limited role in Ca and P homeostasis in fish (Vielma et al., 1998). There are evidences of the interaction between vitamin D and Ca metabolism in fish indirectly linked to P and bone metabolism (Lall and Lewis-McCrea, 2007), particularly at early developmental stages, when fish are more susceptible to vitamin D imbalances (Hamre et al., 2013). However, excessive levels of dietary vitamin D also increased the incidence of skeletal anomalies in Japanese flounder (Haga et al., 2004). In agreement, the incidence of skeletal anomalies in gilthead seabream followed a quadratic regression with dietary vitamin D_3 levels, with the lowest incidence suggested at 11.6–15.5 IU g^{-1} vitamin D_3, whereas higher vitamin D_3 levels led to a greater incidence in skeletal anomalies. Indeed, despite vitamin D_3 supplementation increases bone mineralization in fish, it may also have bone catabolic effects (Fleming et al., 2005; Wendelaar Bonga et al., 1983). Bones being a main store of calcium phosphate, vitamin D directly affects both osteoblast activity and osteoclast formation (Anderson and Atkins, 2008). The skeletal anomalies found in gilthead seabream also followed a quadratic regression with vitamin D_3 contents in liver, with the lowest values of anomalies corresponding also to 11.6–15.5 IU g^{-1}dietary vitamin D_3. Moreover, expression of *bmp2* and *alp*, biomarkers of osteoblast differentiation and mineralization, increased in relation to dietary vitamin D_3 and showed a high linear correlation to caudal and maxillary anomalies. These results are in agreement with the up-regulation of *bmp4* in European seabass fed increased dietary vitamin D levels (Darias et al., 2010) and the enhanced ALP synthesis and activity found in bone of other vertebrates fed increasing dietary vitamin D_3 (Manolagas et al., 1981; Witkowska-Sędek et al., 2018). Bone ALP activity is sensitive to

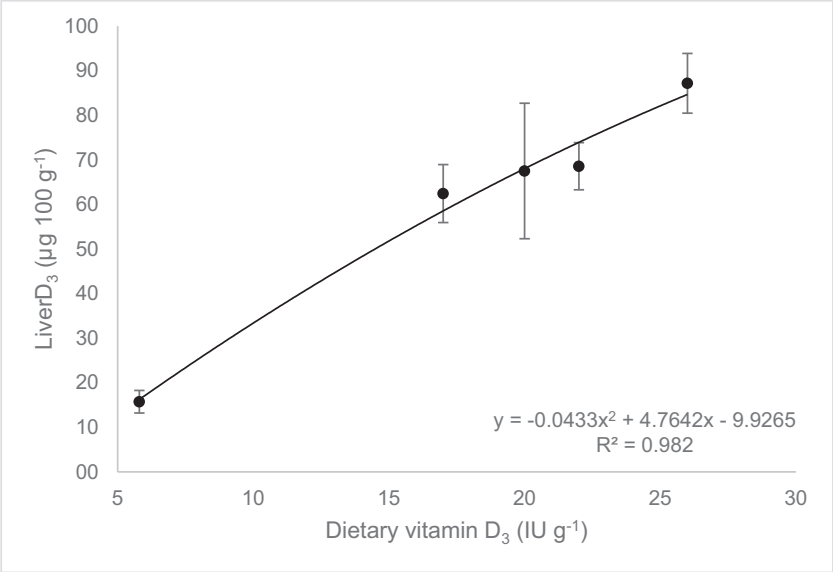

Fig. 1. Effect of dietary vitamin D_3 level on liver vitamin D_3 content of gilthead seabream. Values correspond to means per diet. The liver vitamin D_3 content increased following a quadratic regression ($p = 0.00$; $R^2 = 0.982$) with the dietary vitamin D_3 levels.

Table 5
Prevalence of skeletal anomalies (%) in gilthead seabream fed increasing levels of dietary vitamin D_3 for 70 days.

Dietary vitamin D_3 (IU g^{-1})	5.8	17.0	20.0	22.0	26.0	R^2 and *p*-value
Anomalies from the maxillary and/or pre-maxillary	5.8 ± 5.0	5.6 ± 4.9	4.8 ± 8.2	8.3 ± 14.4	15.3 ± 5.6	R^2 = 0.93, *p* = 0.07
Pre-haemal lordosis	17.4 ± 8.0	28.2 ± 18.7	24.2 ± 9.5	11.4 ± 4.6	26.4 ± 21.2	n.s.
Pre-haemal fusion	3.0 ± 5.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.8 ± 4.8	R^2 = 0.94, *p* = 0.05
Haemal lordosis	2.8 ± 4.8	5.6 ± 4.9	0.0 ± 0.0	5.6 ± 4.8	0.0 ± 0.0	n.s.
Haemal partial vertebral fusion	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3 ± 5.2	0.0 ± 0.0	n.s.
Haemal anomaly	0.0 ± 0.0	2.6 ± 4.4	2.1 ± 3.6	0.0 ± 0.0	0.0 ± 0.0	n.s.
Caudal anomaly	0.0 ± 0.0	3.0 ± 5.2	0.0 ± 0.0	3.0 ± 5.2	9.7 ± 10.0	n.s.

n.s.: non-significant: *p*-value > 0.05.

different metabolic forms of vitamin D_3 in a dose and time dependent manner (Hale et al., 1986). Moreover, although ALP promotes bone mineralization by releasing phosphates, it may also inhibit bone mineralization by the breakdown of pyrophosphates (Omelon and Grynpas, 2008), explaining that in the present study up-regulation of *alp* as a result of an increasing in dietary vitamin D_3 levels was associated to the highest incidence of skeletal anomalies. On the contrary, no relation between dietary vitamin D_3 and *runx2* or *oc* could be found. Certain metabolic forms of vitamin D, such as 1,25-$(OH)_2$ D_3 enhance calcium and phosphate absorption stimulating bone osteoblasts to secrete *oc* (Davideau et al., 1996). However, other factors such as *Jun–Fos* or *Msx-2* are involved in regulation of *oc* expression, leading to dissimilar expression patterns for *oc* with higher vitamin D_3 levels (Davideau et al., 1996). This may explain the lack of effect of dietary vitamin D_3 levels on *oc* expression found in the present study.

Among different histological alterations, unbalances in dietary vitamin D may cause atrophied hepatocytes and oedema of cardiac muscle fibres (Taveekijakarn et al., 1996). In agreement, in gilthead seabream, the increasing of dietary vitamin D_3 up to 20.0 IU g^{-1} significantly increased cardiac congestion and swollen cardiac muscle. In higher vertebrates, it has been demonstrated that despite the clear cardiovascular protective action of vitamin D, excess levels in this nutrient may also induce cardiovascular calcification and inflammation (Khanna et al., 2016; Mangge et al., 2014; Pilz et al., 2016). Among other cardiovascular protective actions, vitamin D regulates myocardial cell hypertrophy and modulates macrophage activity and cytokine generation (Adamczak, 2017). In the present study, the occurrence of myocarditis signs in fish fed vitamin D_3 levels of 20.0 IU g^{-1} or more denote the toxic effects of these dietary levels. These results, together with the higher occurrence of skeletal anomalies in seabream fed the highest dietary vitamin D_3 levels, suggest initial signs of hypervitaminosis D, despite growth was not significantly affected. Signs of hypervitaminosis D are very rare in fish and only in few studies there was a clear growth inhibition by excessive vitamin D levels (2500–2,500,000 IU kg^{-1}, Vielma et al., 1998). Even when dietary vitamin D_3 levels caused hypercalcemia in brook trout (*Salvelinus fontinalis*), growth was not affected (Poston, 1968).

Overall, the results of this study, suggested that the vitamin D_3 levels present in the basal diet (5.8 IU g^{-1}) were sufficient to cover the requirements of vitamin D for growth maintenance in gilthead seabream juveniles. Besides, increase in dietary vitamin D_3 up to 11.6–15.5 IU g^{-1} would contribute to reduce the incidence of skeletal anomalies, whereas further increase up to 20.0 IU g^{-1} negatively affected cardiac tissue and skeletal anomalies incidence. Thus, the recommended dietary levels for gilthead seabream juveniles would be between 5.8 and 11.6 IU g^{-1} vitamin D_3. These levels are close to those recommended for Atlantic salmon in practical diets without vitamin D_3 supplementation (<0.2 mg kg^{-1}, Graff et al., 2002) or Amago salmon (*Oncorhynchus rhodurus* 20,000 IU kg^{-1}, Taveekijakarn et al., 1996). In contrast, much higher dietary vitamin D levels have been recommended for rice field eel (5000 IU kg^{-1}; Tan et al., 2007) or Siberian sturgeon (1683–1403 IU kg^{-1}; Wang et al., 2017). Nevertheless, vitamin D requirements are much lower in other fish species such as juvenile hybrid tilapia (*O. niloticus* X *O. aureus*) (374 IU kg^{-1}; Shiau and Hwang, 1993) or channel catfish (*Ictalurus punctatus*, 0.05 mg kg^{-1}, (Brown and Robinson, 1992)). In practice, it is desirable to produce diets for gilthead seabream containing sufficient levels of vitamin D in the basal ingredients since EU legislation restricts the supplementation of vitamin D_3 in aquafeeds (Lock et al., 2010).

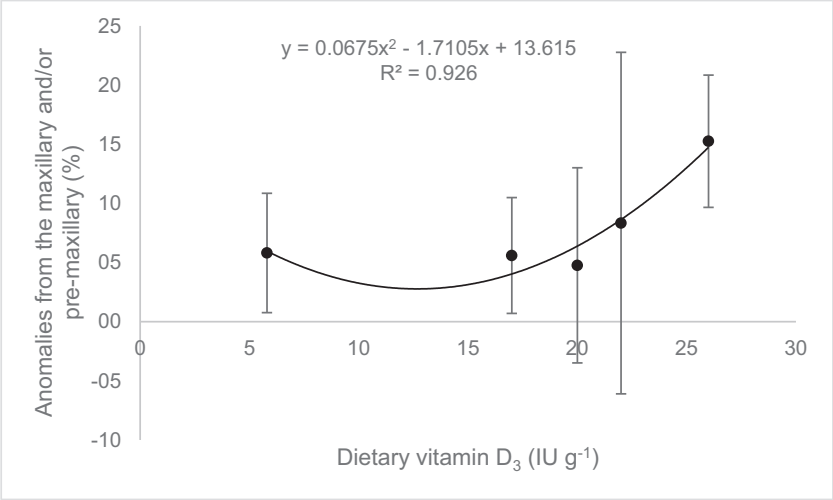

Fig. 2. Effect of dietary vitamin D_3 level on the prevalence of anomalies from the maxillary and/or pre-maxillary in gilthead seabream. Values correspond to means per diet. The incidences of anomalies from the maxillary and/or pre-maxillary followed a quadratic regression ($R^2 = 0.93$, $p = 0.05$) with the level of dietary vitamin D_3 showing the lowest incidences of anomalies around 11.6 g^{-1} vitamin D_3.

Table 6
Vertebra gene expression analyses of gilthead seabream fed increasing levels of dietary vitamin D_3 for 70 days.

Dietary vitamin D_3 (IU g^{-1})	17.0	20.0	26.0	*p value*	R^2
runx2	1.08 ± 0.54	1.34 ± 0.21	1.06 ± 0.23	n.s.	0.159
bmp2	1.49 ± 1.40[a]	1.48 ± 0.33[a]	9.73 ± 4.46[b]	0.015	0.756
alp	1.08 ± 0.53	1.10 ± 0.13	1.64 ± 0.54	n.s.	0.196
oc	1.10 ± 0.63	1.59 ± 0.39	0.55 ± 0.18	n.s.	0.583

n.s.: non-significant: *p*-value > 0.05.

Table 7
Liver and heart histological analyses of gilthead seabream fed increasing levels of dietary vitamin D for 70 days.

Vitamin D_3 (IU g^{-1})	5.8	17.0	20.0	26.0	p value
Liver steatosis		2.3 ± 0.6	2.9 ± 0.1	2.3 ± 0.3	n.s.
Cardiac congestion	1.06 ± 0.63[a]	1.39 ± 0.42[ab]	2.06 ± 0.25[b]	1.61 ± 0.10[ab]	p = 0.034
Swollen cardiac muscle	1.69 ± 0.46[a]	2.28 ± 0.25[ab]	2.67 ± 0.17[b]	2.83 ± 0.29[b]	p = 0.00
Eosinophils in cardiac tissue	0.11 ± 0.19	0.44 ± 0.51	0.44 ± 0.51	0.33 ± 0.58	n.s.

Different letters in the same row indicate significant differences, $p < 0.05$, $n = 3$.
n.s.: non-significant: *p*-value > 0.05.

5. Conclusions

Increased dietary vitamin D_3 levels up to 11.6 IU g^{-1} may reduce the incidence of skeletal anomalies, particularly caudal and maxillary and/or pre-maxillary anomalies, whereas further elevation of dietary vitamin D_3 levels increased the concentration of vitamin D_3 in liver as well as skeletal anomalies in association to the up-regulation of *alp* and *bmp2* gene expression. Thus, the recommended level for vitamin D_3 for gilthead seabream juveniles fed diets containing high levels of plant ingredients was suggested to be 11.6 IU g^{-1}.

Funding

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 727610 PerformFISH project. This output reflects the views only of the authors, and the European Union cannot be held responsible for any use which may be made of the information contained therein.

Author contributions

- David Dominguez: Conceptualization, Data curation, Formal analysis, investigation, Project administration, Writing - original draft, Writing - review & editing.
- Daniel Montero: Data curation, Funding acquisition, Project administration, Resources.
- M.J. Zamorano: Data curation, Funding acquisition, Project administration.
- Pedro Castro: Data curation, Methodology, Resources, Writing - review & editing.
- Ramón Fontanillas: Conceptualization, Funding acquisition, Resources, Writing - review & editing.
- P. Antony Jesu Prabhu: Writing - original draft, Writing - review & Editing.
- Marisol Izquierdo: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

R. Fontanillas is an employee of Skretting AS, Stavanger, Norway.

References

Adamczak, D.M., 2017. The role of toll-like receptors and vitamin D in cardiovascular diseases—a review. Int. J. Mol. Sci. 18. https://doi.org/10.3390/ijms18112252.
Anderson, P.H., Atkins, G.J., 2008. The skeleton as an intracrine organ for vitamin D metabolism. Mol. Aspects Med. 29, 397–406.
Andrews, J.W., Murai, T., Page, J.W., 1980. Effects of dietary cholecalciferol and ergocalciferol on catfish. Aquaculture 19, 49–54.
Antony Jesu Prabhu, P., Lock, E.-J., Hemre, G.-I., Hamre, K., Espe, M., Olsvik, P.A., Silva, J., Hansen, A.-C., Johansen, J., Sissener, N.H., Waagbø, R., 2019. Recommendations for dietary level of micro-minerals and vitamin D 3 to Atlantic salmon (*Salmo salar*) parr and post-smolt when fed low fish meal diets. PeerJ 7, e6996.
AOAC (Ed.), 2000. Official Methods of Analysis of the Association of Analytical Chemistry, 15th ed. AOAC, Arlington, VA, USA.

Barnett, B.J., Cho, C.Y., Slinger, S.J., 1982. Relative biopotency of dietary ergocalciferol and cholecalciferol and the role of and requirement for vitamin D in rainbow trout (*Salmo gairdneri*). J. Nutr. 112 (11), 2011–2019.

Boglione, C., Gagliardi, F., Scardi, M., 2001. Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead seabream (*Sparus aurata* L. 1758). Aquaculture 192, 1–22.

Boglione, C., Gisbert, E., Gavaia, P., Witten, P.E., Moren, M., Fontagné, S., Koumoundouros, G., 2013. Skeletal anomalies in reared European fish larvae and juveniles. Part 2: Main typologies, occurrences and causative factors. Rev. Aquac. 5, 121–167. https://doi.org/10.1111/raq.12016.

Brown, P.B., 1988. Vitamin D Requirement of Juvenile Channel Catfish Reared in Calaciym-free Water (Ph.D. Dissertation). Texas A&M University, College Station, Texas.

Brown, P.B., Robinson, E.H., 1992. Vitamin D studies with channel catfish (Ictalurus punctatus) reared in calcium-free water. Comparative Biochem. Physiol., 103A, 213–219.

Cerezuela, R., Cuesta, A., Meseguer, J., Ángeles Esteban, M., 2009. Effects of dietary vitamin D_3 administration on innate immune parameters of seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 26, 243–248.

Darias, M.J., Mazurais, D., Koumoundouros, G., Glynatsi, N., Christodoulopoulou, S., Huelvan, C., Desbruyeres, E., Le Gall, M.M., Quazuguel, P., Cahu, C.L., Zambonino-Infante, J.L., 2010. Dietary vitamin D_3 affects digestive system ontogenesis and ossification in European sea bass (*Dicentrachus labrax*, Linnaeus, 1758). Aquaculture 298, 300–307.

Davideau, J.-L., Papagerakis, P., Hotton, D., Lezot, F., Berdal, A., 1996. In situ investigation of vitamin D receptor, alkaline phosphatase, and osteocalcin gene expression in oro-facial mineralized tissues. Endocrinology 137.

Fleming, A., Sato, M., Goldsmith, P., 2005. High-throughput in vivo screening for bone anabolic compounds with zebrafish. J. Biomol. Screen. Off. J. Soc. Biomol. Screen. 10, 823–831.

Folch, J., Lees, M., Stanley, H.S., 1957. A simple method for the iso-lation and purification of total lipids from animal tissues. J. Biolog. Chem. 226, 497–509.

Graff, I.E., Høie, S., Totland, G.K., Lie, 2002. Three different levels of dietary vitamin D_3 fed to first-feeding fry of Atlantic salmon (*Salmo salar* L.): effect on growth, mortality, calcium content and bone formation. Aquac. Nutr. 8, 103–111.

Haga, Y., Takeuchi, T., Muruyama, Y., Ohta, K., Fukunga, T., 2004. Vitamin D3 compounds induce hypermalanosis on the bling side and vertebral deformity in juvenile Japanese flounder Paralichthys olivaceus. Fish. Sci. 70, 59–67.

Hale, L.V., Kemick, M.L.S., Wuthier, R.E., 1986. Effect of vitamin D metabolites on the expression of alkaline phosphatase activity by epiphyseal hypertrophic chondrocytes in primary cell culture. J. Bone Miner. Res. 1, 489–495. https://doi.org/10.1002/jbmr.5650010602.

Halver, E.J., 2002. The vitamins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition, 3rd edn. Academic Press, San Diego, CA, USA, pp. 62–141.

Hamre, K., Krossøy, C., Lock, E.J., Moren, M., 2010. Roles of lipid-soluble vitamins during ontogeny of marine fish larvae. Aquac. Res. 41, 745–750.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E.C., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. Rev. Aquac. 5, S26–S58.

Hansen, A.C., Waagbø, R., Hemre, G.I., 2015. New B vitamin recommendations in fish when fed plant-based diets. Aquac. Nutr. 21, 507–527.

Hilton, J.W., Ferguson, H.W., 1982. Effect of excess vitamin D_3 on calcium metabolism in rainbow trout *Salmo gairdneri* Richardson. J. Fish Biol. 21, 373–379.

Horvli, O., Lie, Aksnes, L., 1998. Tissue distribution of vitamin D_3 in Atlantic salmon *Salmo salar*: effect of dietary level. Aquac. Nutr. 4, 127–131.

Khanna, R., Kapoor, A., Soni, N., 2016. A heart set in stone: a case of extensive cardiac calcification. Heart Views 17 (3), 100–102. https://doi.org/10.4103/1995-705X.192557.

Lall, S.P., Lewis-McCrea, L.M., 2007. Role of nutrients in skeletal metabolism and pathology in fish — an overview. Aquaculture 267, 3–19.

Ling-Hong, M., Xian-Ping, G., Jun, X., Bo, L., Ke-Bao, W., Jian, Z., Ming-Chun, R., Qun-Lan, Z., Liang-Kun, P., Ru-Li, C., 2015. Dietary vitamin D_3 requirement of Wuchang bream (*Megalobrama amblycephala*). Aquaculture 436, 104–109.

Lock, E.J., Waagbo, R., Wendelaar Bonga, S., Flik, G., 2010. The significance of vitamin D for fish: a review. Aquac. Nutr. 16, 100–116.

Lovell, R.T., Li, Y.-P., 1978. Essentiality of vitamin D in diets of channel catfish (*Ictalurus punctatus*). Trans. Am. Fish. Soc. 107, 809–811.

Mangge, H., Weghuber, D., Prassl, R., Haara, A., Schnedl, W., Postolache, T.T., Paulmichl, K., Fuchs, D., 2014. The role of vitamin D in atherosclerosis inflammation revisited. More bystander than player? Appetite 76, 215. https://doi.org/10.1016/j.appet.2014.01.070.

Manolagas, S.C., Burton, D.W., Deftos, L.J., 1981. 1,25-Dihydroxyvitamin D3 stimulates the alkaline phosphatase activity of osteoblast-like cells. J. Biol. Chem. 256, 7115–7117. https://doi.org/10.1016/s0021-9258(19)68933-7.

Martoja, R., Martoja-Pierson, M., 1970. Histoquímica. In: Toray-Masson, S.A. (Ed.), Técnicas de histología animal. Toray-Masson, Barcelona, pp. 156–184.

NRC, 2011. National Research Council (NRC): Nutrient Requirements of Fish and Shrimp. Natl. Acad. Press, Washington, D.C.

Omelon, S.J., Grynpas, M.D., 2008. Relationships between polyphosphate chemistry, biochemistry and apatite biomineralization. Chem. Rev. 108, 4694–4715. https://doi.org/10.1021/cr0782527.

Pilz, S., Verheyen, N., Grübler, M.R., Tomaschitz, A., März, W., 2016. Vitamin D and cardiovascular disease prevention. Nat. Rev. Cardiol. 13, 404–417. https://doi.org/10.1038/nrcardio.2016.73.

Poston, H.A., 1968. Effects of massive doses of vitamin D3 on fingerling brook trout. Fish Res. Bull. 32, 48–50.

Saleh, R., Betancor, M.B., Roo, J., Montero, D., Zamorano, M.J., Izquierdo, M., 2014. Selenium levels in early weaning diets for gilthead seabream larvae. Aquaculture 426–427, 256–263. https://doi.org/10.1016/j.aquaculture.2014.02.011.

Shiau, S.-Y., Hwang, J.-Y., 1993. Vitamin D requirements of juvenile hybrid tilapia *Oreochromis niloticus x O. aureus*. Nippon Suisan Gakkaishi 59, 553–558.

Tan, Q., He, R., Xie, S., Xie, C., Zhang, S., 2007. Effect of dietary supplementation of vitamins A, D_3, E, and C on yearling rice field eel, *Monopterus albus*: serum indices, gonad development, and metabolism of calcium and phosphorus. J. World Aquac. Soc. 38, 146–153.

Taveekijakarn, P., Miyazaki, T., Matsumoto, M., Aral, S., 1996. Histopathological and haematological changes in amago salmon, *Oncorhynchus rhodurus* (Jordan & McGregor), fed a vitamin-D-free diet. J. Fish Dis. 19, 289–294.

Vielma, J., Lall, S.P., Koskela, J., Mattila, P., 1999. Influence of low dietary cholecalciferol intake on phosphorus and trace element metabolism by rainbow trout (*Oncorhynchus mykiss*, Walbaum). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 122, 117–125.

Vielma, J., Lall, S.P., Koskela, J., Schöner, F.J., Mattila, P., 1998. Effects of dietary phytase and cholecalciferol on phosphorus bioavailability in rainbow trout (Oncorhynchus mykiss). Aquaculture 163, 309–323. https://doi.org/10.1016/S0044-8486(98)00240-3.

Wang, L., Xu, H., Wang, Y., Wang, C., Li, J., Zhao, Z., Luo, L., Du, X., Xu, Q., 2017. Effects of the supplementation of vitamin D_3 on the growth and vitamin D metabolites in juvenile Siberian sturgeon (*Acipenser baerii*). Fish Physiol. Biochem. 43, 901–909.

Wendelaar Bonga, S., Lammers, P.I., van der Meij, J.C.A., 1983. Effects of 1,25- and 24,25-dihydroxy vitamin D_3 on bone formation in the cichlid teleost *Sarotherodon mossambicus*. Cell Tissue Res. 228, 117–126.

Witkowska-Sędek, E., Stelmaszczyk-Emmel, A., Majcher, A., Demkow, U., Pyrzak, B., 2018. The relationship between alkaline phosphatase and bone alkaline phosphatase activity and the growth hormone/insulinlike growth factor-1 axis and vitamin D status in children with growth hormone deficiency. Acta Biochim. Pol. 65, 269–275. https://doi.org/10.18388/abp.2017_2541.

Woodward, B., 1994. Dietary vitamin requirements of cultured young fish, with emphasis on quantitative estimates for salmonids. Aquaculture 124, 133–168.