



Effects of vitamin D₃ supplementation in gilthead seabream (*Sparus aurata*) juveniles fed diets high in plant based feedstuffs

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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 D₃ (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 D₃ levels between 5.8 and 26.0 IU g⁻¹ for 70 days did not markedly alter growth. Increase dietary vitamin D₃ significantly raised the liver contents in vitamin D₃ in a dose-dependent manner following a potential regression. Increased dietary vitamin D₃ 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 D₃ levels increased the concentration of vitamin D₃ 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 D₃ 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 D₃ levels, suggest initial signs of hypervitaminosis D. Thus, the recommended level for vitamin D₃ 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 D₃ is known to modulate bone and trace mineral metabolism in fish (Vielma et al., 1999). Thus, vitamin D₃ 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

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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 D₃ essentiality on preventing the onset of skeletal anomalies was proved in European seabass (*Dicentrarchus labrax*), where diets containing low (11.2 IU VD₃/g diet) and high vitamin D₃ levels (42 IU VD₃/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* × *O. aureus*; Shiao 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 D₃ kg⁻¹; Barnett et al., 1982) or Atlantic salmon (0.06 or < 0.2 mg vitamin D₃ 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 D₃ 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 D₃), 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

Table 1

Ingredients of the experimental diets supplemented with increasing levels of D₃ 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.

designed to cover all known nutritional requirements for this species. Feeds were manufactured by extrusion process by Skretting Aquaculture Research Centre AS (Stavanger, Norway).

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 D₃ (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 D₃ 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 (*ef1a*) 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

Table 2
Sequences of primers used for gene expression analysis.

Gene	Nucleotide sequence (5'-3')	Accession number
Beta-actin (<i>bact</i>)	F: TCTGTCGTGATCGGAGGCTC R: AAGCATTTCGGGTGGAGC	X89920
Elongation factor 1a (<i>ef1a</i>)	F: CATGGTTGTGGAGCCCTTCT R: TCCTGCACGACCATTTCATTTTC	AF184170
Alkaline phosphatase (<i>alp</i>)	F: AGAAGCCCTGACGCTGCAA R: TTCAGTATACGAGCAGCCGTAC	AY266359
Runt-related transcription factor 2 (<i>runx2</i>)	F: GCCTGTCGCCTTTAAGGTGGTTGC R: TCGTCGTTGCCCGCATAGCTG	AJ619023
Osteocalcin (<i>oc</i>)	F: GGCAGCCATCTGTCTGACTT R: GGTCCGTAGTAGGCCGTGTA	AF048703
Bone morphogenic protein 2 (<i>bmp2</i>)	F: GTGGCTTCCATCGTATCAACATTTT R: GCTCCCGCCATGAGT	JF261172.1

(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.

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 D₃ 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:

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

$$\text{SGR (\%)} = (\text{Ln}_{W_2} - \text{Ln}_{W_1}) / \text{days} \times 100$$

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

where

W₁: initial body weight (g)

W₂ 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 D₃ (Table 3). Nevertheless, weight gain and SGR showed a trend towards higher values with increased dietary vitamin D₃ 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 D₃ 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 D₃ content in liver significantly increased following a quadratic regression ($p = 0.00$; $R^2 = 0.982$) with the dietary vitamin D₃ levels (Table 4, Fig. 1), whereas muscle proximate composition was not affected by dietary vitamin D₃.

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 D₃ showing the lowest incidences of anomalies around 11.6–15.5 IU g⁻¹ vitamin D₃. 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 D₃ ($R^2 = 0.94$, $p = 0.059$; $R^2 = 0.93$, $p = 0.074$; and $R^2 = 0.48$, $p = 0.19$, respectively), with the lowest incidences of anomalies 11.6–15.5 IU g⁻¹ vitamin D₃ in diet.

3.4. Gene expression

Analyses conducted in vertebrae to evaluate bone molecular markers showed a quadratic regression between dietary vitamin D₃ 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 D₃. 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 D₃ levels (Table 6).

3.5. Histological studies

Study of hepatic morphology showed very similar characteristics among livers of fish fed the different vitamin D₃ 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 D₃ (Table 7). Moreover, there were significant potential regressions between the dietary vitamin D₃ 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 D₃ contents and swollen cardiac muscle ($R^2 = 0.963$, $p = 0.018$).

Table 4

Muscle composition (% dry weight) and liver D₃ contents of gilthead seabream fed increasing contents of vitamin D₃ 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 D₃ 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 D₃, which could also indicate that feeding these vitamin D₃ levels for longer periods of time could have improved growth.

Increase dietary vitamin D₃ significantly raised the liver contents in vitamin D₃ in a dose-dependent manner following a potential regression. This result was in agreement with the increase in liver vitamin D₃ 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 D₃ levels of 0.2–57.0 mg kg⁻¹ and 60–1.0 × 10⁵ IU kg⁻¹. Moreover, the pattern of increase of vitamin D₃ 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 D₃ in Atlantic salmon reach a saturation level around 0.06–0.09 mg kg⁻¹ dietary vitamin D₃ (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 D₃ was concomitant with the increase in other nutrients, which could interact with vitamin D₃ deposition in body tissues.

Dietary vitamin D₃ 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 D₃ for 90 days (Graff et al., 2002). On the contrary, whole body or muscle proximate composition is markedly affected by vitamin D₃ 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 D₃ levels, with the lowest incidence suggested at 11.6–15.5 IU g⁻¹ vitamin D₃, whereas higher vitamin D₃ levels led to a greater incidence in skeletal anomalies. Indeed, despite vitamin D₃ 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 D₃ contents in liver, with the lowest values of anomalies corresponding also to 11.6–15.5 IU g⁻¹ dietary vitamin D₃. Moreover, expression of *bmp2* and *alp*, biomarkers of osteoblast differentiation and mineralization, increased in relation to dietary vitamin D₃ 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 D₃ (Manolagas et al., 1981; Witkowska-Sędek et al., 2018). Bone ALP activity is sensitive to

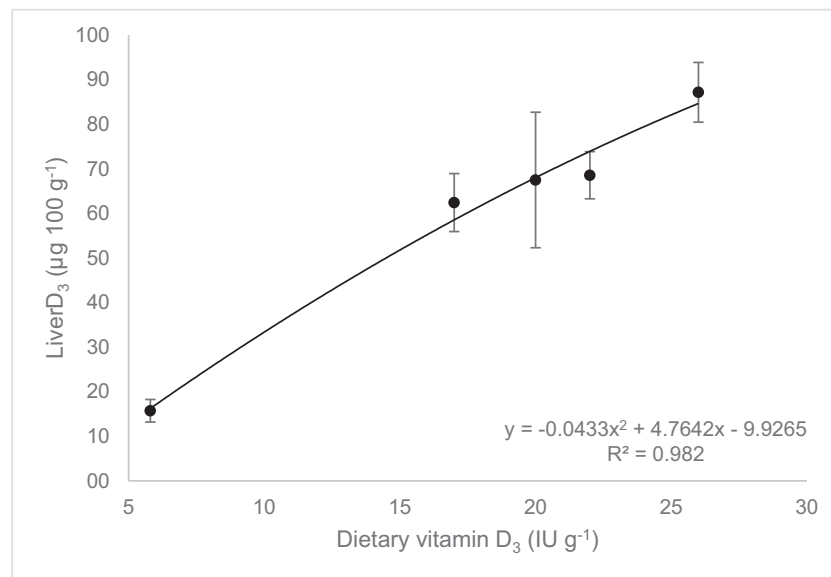


Fig. 1. Effect of dietary vitamin D₃ level on liver vitamin D₃ content of gilthead seabream. Values correspond to means per diet. The liver vitamin D₃ content increased following a quadratic regression ($p = 0.00$; $R^2 = 0.982$) with the dietary vitamin D₃ levels.

Table 5
Prevalence of skeletal anomalies (%) in gilthead seabream fed increasing levels of dietary vitamin D₃ 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 D₃ 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 D₃ levels was associated to the highest incidence of skeletal anomalies. On the contrary, no relation between dietary vitamin D₃ and *runx2* or *oc* could be found. Certain metabolic forms of vitamin D, such as 1,25-(OH)₂ D₃ 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 D₃ levels (Davideau et al., 1996). This may explain the lack of effect of dietary vitamin D₃ 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 D₃ 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 D₃ 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 D₃ 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 D₃ levels caused hypercalcemia in brook trout (*Salvelinus fontinalis*), growth was not affected (Poston, 1968).

Overall, the results of this study, suggested that the vitamin D₃ 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 D₃ 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 D₃. These levels are close to those recommended for Atlantic salmon in practical diets without vitamin D₃ 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 D₃ in aquafeeds (Lock et al., 2010).

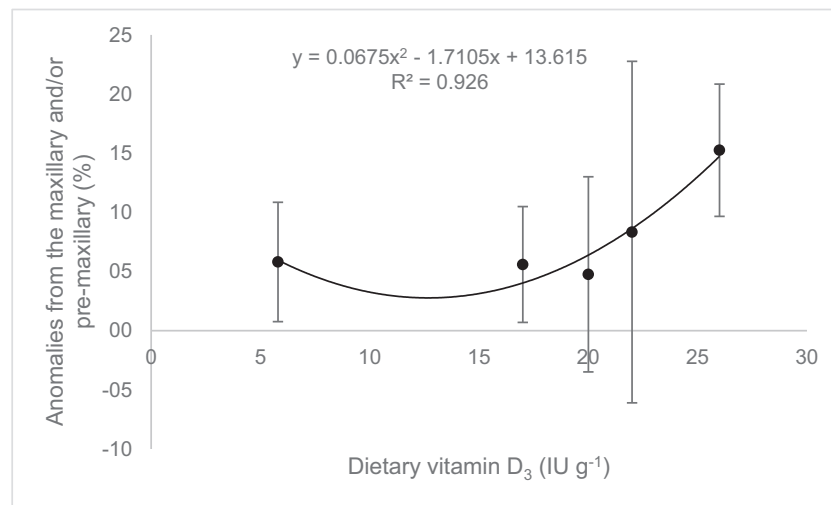


Fig. 2. Effect of dietary vitamin D₃ level on the prevalence of anomalies from the maxillary and/or pre-maxillary in gilthead seabream. Values correspond to means per diet. The incidences of anomalies from the maxillary and/or pre-maxillary followed a quadratic regression ($R^2 = 0.93$, $p = 0.05$) with the level of dietary vitamin D₃ showing the lowest incidences of anomalies around 11.6 g⁻¹ vitamin D₃.

Table 6

Vertebra gene expression analyses of gilthead seabream fed increasing levels of dietary vitamin D₃ for 70 days.

Dietary vitamin D ₃ (IU g ⁻¹)	17.0	20.0	26.0	<i>p</i> value	R ²
<i>runx2</i>	1.08 ± 0.54	1.34 ± 0.21	1.06 ± 0.23	n.s.	0.159
<i>bmp2</i>	1.49 ± 1.40 ^a	1.48 ± 0.33 ^a	9.73 ± 4.46 ^b	0.015	0.756
<i>alp</i>	1.08 ± 0.53	1.10 ± 0.13	1.64 ± 0.54	n.s.	0.196
<i>oc</i>	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	<i>p</i> 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 D₃ 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 D₃ levels increased the concentration of vitamin D₃ 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 D₃ for gilthead seabream juveniles fed diets containing high levels of plant ingredients was suggested to be 11.6 IU g⁻¹.

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- Pedro Castro: Data curation, Methodology, Resources, Writing - review & editing.
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Declaration of Competing Interest

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

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