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Effects of dietary vitamin D_3 levels on survival, mineralization, and skeletal development of gilthead seabream (*Sparus aurata*) larvae

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ABSTRACT

Vitamin D is an essential fat soluble micronutrient that helps in growth, bone development, calcium homeostasis and other metabolic process. The study on effect of vitamin D_3 in marine fish larvae were very scarce irrespective of species. The present study determines the impacts of dietary vitamin D_3 on growth performance, calcium absorption, mineralization, and skeletal anomalies during the development of gilthead seabream (*Sparus aurata*) larvae was assessed until 47 days post hatching. Diets containing four levels of vitamin D_3 (0, 25, 30, 384 μg kg^{-1} or 11.6, 1000, 1200, 15,360 IU kg^{-1}) were formulated to determine the effect of vitamin D_3 at deficient, excess, and optimum levels. The gilthead seabream larvae in the present study fed with this wide range of vitamin D_3 presented a constant growth with all the diets but presented signs of toxicity in excess level, affecting the survival, calcium uptake, and bone biomarker mechanism in larvae, which resulted in increased skeletal anomalies and mortality. An increase of dietary vitamin D_3 up to 384 μg kg^{-1} significantly raised the whole body vitamin D_3 content, calcium, and phosphorus intake and increased the incidence of skeletal anomalies, particularly cranial anomalies. The appearance of skeletal anomalies in larvae fed 384 μg kg^{-1} vitamin D_3 was in association with the upregulation of *bmp2, alp,* and *oc* gene expression. However, larvae fed 0, 25, 30 μg kg^{-1} vitamin D_3 showed higher survival than the group fed 384 μg kg^{-1} vitamin D_3. Meanwhile vitamin D_3 deficient diet 0 μg kg^{-1} presented with lower mineralization rate and increase incidence of maxillary anomaly. Thus, the current study revealed the evidence of vitamin D_3 deficiency as well as toxicity in gilthead seabream larvae during the developmental process and conclude that the recommended dietary vitamin D_3 level for gilthead seabream larvae may range between 25 and 30 μg kg^{-1} which improves larval survival, calcium and phosphate level and vertebral mineralization with reduced incidence of skeletal anomalies in gilthead seabream larvae

1. Introduction

Gilthead seabream (*Sparus aurata*) production in Europe and rest of the Mediterranean accounts for 252,406 t in 2019 but a decrease of −1.3% is predicted by 2020 (APROMAR, 2020). Hence, there is an urgent need to improve the technical performance of gilthead seabream production to meet its demand by quantity and quality. Seed quality is an important factor that determines the performance of the subsequent grow-on stages, since it affects the growth, susceptibility to disease or survivorship (EATIP, 2016). In particular, the intensification techniques applied to optimize cost-efficiency of the production systems can markedly affect juveniles' quality. For instance, the occurrence of skeletal anomalies in hatchery reared gilthead seabream larvae may affect volume and value of the production (Georgakopoulou et al., 2010; Koumoundouros et al., 1997). Many factors affect the onset of skeletal anomalies in sparids, including environmental, genetic, microbial, or nutritional aspects (Izquierdo et al., 2010; Kourkouta et al., 2021; Lee-Montero et al., 2015; Negrín-Báez et al., 2015; Roo et al., 2010, 2009, 2005).

The data on nutritional requirements of marine fish larvae are scarce and differ from those of juveniles (Hamre et al., 2013; Izquierdo and Koven, 2011). In commercial hatcheries, marine fish larvae are fed with

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live feed such as rotifers and *Artemia*, which are not part of their natural diet (Curnow et al., 2006; Izquierdo et al., 2000). Besides, marine fish larvae undergo various functional and morphological changes during early development. Further, when these live feeds are incorporated into the rearing system, they may lack few required nutrients such as vitamins and micro-minerals (Hamre, 2016), that are essential to promote growth, survival, and skeletal development. Despite microdiets have been developed to satisfy the nutritional requirements of larvae during early weaning period, complete replacement of live feed with microdiets has not been yet achieved for larvae of most fish species. This is due to the lack of knowledge in nutritional requirements of larvae, which differs among different species and developmental stages (Cahu and Zambonino Infante, 2001; Hamre et al., 2013; Kolkovski, 2013). Thus, some nutritional unbalances, particularly related to micronutrients, could be expected during larval and post-larval stages (Hamre et al., 2013), at the onset of skeletal anomalies.

Skeletal anomalies may have a strong negative impact on gilthead seabream survival (Andrades et al., 1996), particularly those affecting the splanchnocranium and the vertebral column (Boglione et al., 2013). The most common anomalies observed in reared gilthead seabream larvae are skeletal axis malformations, such as scoliosis, lordosis and kyphosis, deformed or fused vertebrae, missing or additional fin rays, together with bent opercular plate or jaw malformations (Boglione et al., 2001; Divanach et al., 1996). Among the different nutrients that may affect the occurrence of skeletal anomalies in marine fish larvae, fat-soluble vitamins have been early studied by several researchers. However, fewer studies were also devoted to understanding the physiological and molecular mechanisms mediating the effects of these vitamins on the occurrence of skeletal anomalies (Fernández et al., 2009, 2008) or to determining their optimum dietary level for normal larval development (Mazurais et al., 2008).

Japanese flounder larvae (*Paralichthys olivaceus*) fed on *Artemia* enriched with vitamin A, as a retinoic acid and in other forms, resulted in hypervitaminosis and caused vertebral compression (Dedi et al., 1997; Takeuchi et al., 1998). Diet fed without vitamin K supplementation caused higher incidence of skeletal deformities in mummichog (*Fundulus heteroclitus*) larvae (Udagawa, 2004, 2001) and haddock (*Melanogrammus aeglefinus L.*) (Roy and Lall, 2007). Dietary vitamin E affect growth and immune response in gilthead seabream juveniles (Montero et al., 2001), and on bone development and oxidative damage in gilthead seabream larval development (Izquierdo et al., 2019, 2013). Despite many studies have been done in fat-soluble vitamins and its importance in aquaculture species, the requirement of vitamin D and its effect on marine fish larvae need to be studied.

Vitamin D is a fat-soluble micronutrient that has various functions in bone development (St-Arnaud, 2008), calcium absorption, mineralization, and cellular proliferation (Lock et al., 2010). Vitamin D_3 (cholecalciferol) is the most active form, which gets hydroxylated in liver of fish to form 1,25(OH)D_3 (calcitriol) that helps in bone formation through the binding and activation of vitamin D receptors (VDR). VDR have a direct effect on several other biomarkers related with osteoblast differentiation and mineralization (Fenwick et al., 1994; Fleming et al., 2005; Suda et al., 2003; Walters, 1992; Yamakawa, 1964). Vitamin D requirements in juveniles and adults of different fish species oscillate from 250 to 2400 IU kg^{-1} diet (NRC, 2011). Besides, dietary requirements of vitamin D_3 in fish may vary according to the developmental stage and species (Table 1).

The effect of optimum doses of vitamin D in fish results in maximum growth, increased serum calcium and phosphate, improved bone calcium, increased mineralization rate and better larval performance (Darias et al., 2010; Fleming et al., 2005; Shiau and Hwang, 1993; Sivagurunathan et al., 2020; Wang et al., 2017). Excess dose of vitamin D lead to impaired growth, lethargy, hypermelanosis, development of skeletal anomalies and increased mineral retention in soft tissues (Darias et al., 2010; Haga et al., 2004; Poston, 1969; Zhu et al., 2015). Besides, deficiencies in vitamin D turn out in poor growth, reduced weight gain, less plasma calcium and phosphate, hypocalcemia, reduced bone calcium, maximized skeletal anomalies and low survival rate (Darias et al., 2010; Ling-hong et al., 2015; Shiau and Hwang, 1993; Taveekijakarn et al., 1996).

Table 1
Dietary vitamin D_3 requirement studies in fish species.

Species	Developmental stage	Vitamin D_3 requirement	Reference
European seabass (*Dicentrarchus labrax*)	Larvae	27,600 IU kg^{-1} diet	(Darias et al., 2010)
Atlantic salmon (*Salmo salar*)	Post-smolt	2400–3600 IU kg^{-1} diet	(Prabhu et al., 2019)
Rice field eel (*Monopterus albus*)	Fingerling	5000 IU kg^{-1} diet	(Tan et al., 2007)
Grass carp (*Ctenopharyngodon idellus*)	Fingerling	1000 IU kg^{-1} diet	(Jiang et al., 2009)
Wuchang bream (*Megalobrama amblycephala*)	Fingerling	200 IU kg^{-1} diet	(Ling-hong et al., 2015)
Common carp (*Cyprinus carpio*)	Juvenile	708.3–769.8 IU kg^{-1} diet	(Zhang and XU QY, 2011)
Siberian sturgeon (*Acipenser baerii*)	Juvenile	1683–1403 IU kg^{-1} diet	(Wang et al., 2017)
Orange spotted grouper (*Epinephelus coioides*)	Juvenile	1000–2000 IU kg^{-1} diet	(Xie et al., 2019)
Orange spotted grouper (*Epinephelus coioides*)	Juvenile	1000–2000 IU kg^{-1} diet	(He et al., 2021)
Gilthead seabream (*Sparus aurata*)	Juvenile	11,600 IU kg^{-1} diet	(Dominguez et al., 2021)

There are few studies in gilthead seabream regarding vitamin D and focused on juveniles, showing the effect of dietary vitamin D_3 on immune parameters, particularly innate immunity (Cerezuela et al., 2009), or the increased occurrence of skeletal anomalies and myocarditis (Dominguez et al., 2021). Unfortunately, information regarding the effect of dietary vitamin D_3 levels for gilthead seabream larvae is very scarce. As observed for other essential nutrients, vitamin D_3 requirements for marine fish larvae could be higher than for juveniles or adults of the same species (Hamre et al., 2013).

Hence, considering this research gap, the main aim of the present study was to determine the dietary requirement and to evaluate the effect of four different levels of vitamin D_3 in practical microdiets (administered from 27 to 47 dph) on growth, survival, and skeletal development of gilthead seabream larvae under hatchery reared condition. The levels were formulated based on the vitamin D_3 contents in rotifers, which are the most frequent first feed used for marine fish larvae in hatcheries, may range from 2400 up to 3200 IU Kg^{-1} vitamin D_3 in non-enriched rotifers and from 2800 to 15,200 IU Kg^{-1} in enriched rotifers (Hamre, 2016).

2. Materials and methods

2.1. Larval rearing

Gilthead seabream larvae (27 days post-hatch (dph), 8.85 ± 0.85 mm total length and 0.86 ± 1.80 mg body weight) were obtained from natural spawns of the gilthead seabream selected broodstock (PROGENSA (Spanish National Breeding Program) project (Afonso et al., 2012) from GIA (Grupo de Investigación en Acuicultura, ECOAQUA Institute, Las Palmas de Gran Canaria University (ULPGC), Spain). Larvae, previously fed rotifers (*Brachinous plicatilis*) enriched with ORIGREEN (Skretting, Norway) until 27 dph, were randomly distributed in 12 cylindroconical fiberglass tanks (170 L) at a density of 2100 larvae/tank. All tanks were supplied with filtered seawater (36 g L^{-1} salinity) from a flow-through seawater system at an increasing rate of 0.3–1.0 L min^{-1}. Water entered from the tank bottom through a mesh and drained from the top to ensure water quality. Water was continuously aerated (125 mL min^{-1}) attaining 5–8 ppm dissolved oxygen with

60 to 80% dissolved oxygen saturation levels. Photoperiod was fixed at 12 h light:12 h dark by fluorescent lights and the light intensity was kept at 1700 lx (digital Lux Tester YF-1065; Powertech Rentals, Western Australia, Australia). Average water temperature and pH trial were recorded 20 ± 2 °C and 7.0 ± 0.6, respectively throughout the experimental period (21 days).

2.2. Experimental diets

Four experimental microdiets (pellet size, 250–500 μm) were formulated to be isoproteic (66.8 ± 0.89% wet weight) and isoenergetic (21.19 MJ/kg) and were supplemented with 4 varying levels of cholecalciferol (Sigma-Aldrich, CAS-67970) and named according to the analyzed cholecalciferol levels in μg kg^{-1} diet: Diet 0VD, Diet 25VD, Diet 30VD and Diet 384VD, i.e., 11.6, 1000, 1200, 15,360 IU kg^{-1} (Table 2). The microdiets were prepared by grinding and sieving the ingredients below 125 μm and then ingredients were mixed as follows, squid powder, water-soluble components, lipids, and fat-soluble vitamins. Finally, gelatin was dissolved in warm water and mixed with above ingredients to prepare a homogenized mix. Then the mix was compressed pelleted and dried in an oven at 38 °C for 24 h. Dried pellets were grounded and sieved to obtain a particle size between 250 and 500 μm. Sieved pellets were stored under refrigerated conditions throughout the trial. The diet formulation, proximate analysis (moisture, ash, and crude protein by AOAC, 1995), and crude lipid by (Folch et al., 1957) and vitamin D_3 analysis (CEN, 2009) are shown in Table 2. Each diet was tested in triplicates. Microdiets were fed for 21 days manually every 45 min from 8:00 to 20:00. Daily feed supply was 3 g per tank during the first week and gradually increased up-to 5 g per tank with increasing in pellet size to 250–500 μm. Larvae were periodically observed under the binocular microscope to determine the feed acceptance.

2.3. Larval growth and survival

Larvae were collected after the 8th, 15th and 21st days of feeding.

Table 2
Ingredients and analyzed proximate composition of the experimental diets supplemented with different levels of vitamin D_3.

Experimental Diet				
	0VD	25VD	30VD	384VD
Ingredients (%)				
Squid powder*	70.2	70.2	70.2	70.2
Krill oil†	13.0	13.0	13.0	13.0
Vitamin premix (Vitamin D_3 free)	6.0	6.0	6.0	6.0
Mineral premix	4.5	4.5	4.5	4.5
Gelatin	3.0	3.0	3.0	3.0
Attractants	3.0	3.0	3.0	3.0
SelPlex	0.3	0.3	0.3	0.3
Vitamin D_3° (μg kg^{-1}) (supplemented)	0	37.5	50	500
Proximate composition (% wet weight)				
Moisture	10.05 ± 0.57	9.71 ± 0.23	9.2 ± 0.05	9.63 ± 0.04
Ash	5.41 ± 0.04	5.37 ± 0.07	5.51 ± 0.06	6.48 ± 0.11
Lipid	17.64 ± 1.05	17.21 ± 0.23	16.46 ± 0.37	17.35 ± 0.51
Crude protein	66.19 ± 1.28	67.05 ± 0.44	67.69 ± 0.25	66.23 ± 0.43
Vitamin D_3 contents (μg kg^{-1} diet) (analysed)				
Vitamin D_3	0.29	24.58	29.94	384.02

° Signa-Aldrich, CAS 67970.
* Rieber & Son, Bergen, Norway.
† Qrill, high phospholipids, Aker BioMarine, Fjordalléen, Norway.

Growth was determined at each sampling point by measuring larval total length and dry body weight. Total length of 30 anesthetized larvae (using clove oil) from each tank was measured in a Profile Projector (Leica microsystem, using Leica Application Suite software, Germany). Whole body weight was determined by three replicates of 10 fast larvae washed with distilled water and dried in a glass slide at an oven at 110 °C, for approximately 24 h, followed by 1 h periods until constant weight was reached. Final survival rate was calculated by individually counting larvae at the beginning and end of the feeding trial. The total dead larvae were estimated by counting the number of dead larvae on each day for daily mortality rate and remaining live larvae for biomass calculation.

2.4. Proximate composition, vitamin D_3 and mineral analysis

All the remaining live larvae (excluded those destined for anatomical inspection) at the end of the experiment were collected from each tank after a fasting period of 12 h, euthanized with ice, counted, washed with distilled water, and stored at −80 °C in air free labelled plastic sampling bags until analysis. Moisture, ash, protein (AOAC, 2000) and crude lipid (Folch et al., 1957) contents of larvae and diets were analyzed. Vitamin D_3 contents in larvae were determined after lipid extraction by preparative HPLC - Isocratic pump: Spectra system P 1000, UV detector: Gilson 112, Injector with 200 μL loop: Rheodyne 7725, Lab data software: Chromeleon and analytical HPLC - Isocratic pump, LaChrom: Merck HITACHI L7100, UV/VIS detector, LaChrom: Merck HITACHI L7420, Integration software: Chromeleon, Autosampler, Gilson 234 (CEN, 2009) at Institute of Marine Research (IMR, Bergen, Norway). The calcium and phosphorus levels in larvae were determined at IMR, after acidification and digestion of the samples (Microwave digester; MarsXpress, CEM, Kamp-Lintfort, Germany), by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), using an auto sampler (FAST SC-4Q DX) (Julshamn et al., 2007).

2.5. Skeletal anomalies inspection

For these analyses, as many as 70 larvae from each tank fixed in 4% formalin in phosphate buffer, pH 7.2, 0.1 M. Fixed larvae were stained with alizarin red (Vandewalle et al., 1998), photographed and examined for mineralized vertebrae (in the direction of cranial to caudal) and for skeletal anomalies (Boglione et al., 2001). The list of the considered anomaly types is reported (Supplementary file, Table S1). The effects of different levels of vitamin D_3 on axial skeleton mineralization were evaluated presumptively by considering the total number of completely mineralized vertebral bodies within a larval size class (total length, mm, Table S2), for each dietary group.

2.6. Gene expression analysis

Larvae of 60–100 mg/tank were collected in pool for molecular studies during every sampling (the 8th, 15th and 21st days of experimental feeding) and preserved in 500 μL RNALater (SIGMA, Madrid, Spain) and stored at −80 °C. Molecular biology analysis was carried out at GIA laboratory, ULPGC, Spain. Total RNA from larvae samples were extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Whole body samples were homogenized using the TissueLyzer-II (Qiagen, Germany) with QIAzollysis reagent (Qiagen, Germany). Samples were centrifuged with chloroform for phase separation (12,000 g, 15 min, 4 °C). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into a RNeasy spin column, where total RNA bonded to a membrane and RW1 and RPE buffers (Qiagen, Germany) were used to wash away contaminants. Purified RNA was eluted with 25 μL of RNase-free water. The quality and quantity of RNA were analyzed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was conducted using the iScriptcDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to

manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:10, 1:100, 1:200 and 1:1000). The product size of the real-time qPCR amplification was checked by electrophoresis analyses using pBR322 cut with HAEIII as a control. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, USA) using Beta-actin (*β-actin*) as the housekeeping gene in a final volume of 20 μL per reaction well, and 100 ng of total RNA reverse transcribed to complementary cDNA. The PCR conditions were the following: 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 nucleotides of the housekeeping and target gene primers were listed in Table 3.

2.7. Data analysis

Results from the experiment were statistically analyzed using IBM SPSS Statistics v26.0. (IBM Corp., Chicago, IL, USA). Data are expressed as means ± S.D. for proximate composition, growth performance, vitamin D_3 analysis, mineral analysis, and gene expression analysis. All values were tested for normality with the Kolmogorov–Smirnov test. To determine the effect of diet, one-way analysis of variance (ANOVA) was used for the normally distributed data. Homogeneity of variances were determined using Levene's statistic and means were compared to understand the statistical difference among the groups using Tukey's post-hoc test ($p < 0.05$). When the variance is not homogenous and not followed the normal distribution, logarithmic or arcsine transformation was executed, and followed by the non-parametric tests of Kruskal-Wallis.

Data from skeletal anomalies, and vertebral mineralization were presented as percentage and a correspondence analysis (CA) was performed to visualize the relationship between different skeletal anomalies and the dietary groups. To evaluate the effect of dietary vitamin D_3 and larval body vitamin D_3 on skeletal anomalies, one-way analysis of variance (ANOVA) was carried out and statistical difference among the groups were compared using Tukey's post-hoc test.

2.8. Ethical statement

All the animal experiments were performed according to the European Union Directive (2010/63/EU) and Spanish legislation (Royal Decree 53/2013) on the protection of animal for scientific purposes at ECOAQUA Institute of University of Las Palmas de Gran Canaria (Canary Island, Spain).

3. Results

3.1. Larval growth and survival

Microscopic observation of larval gut showed that all the experimental diets were well accepted by gilthead seabream larvae. The larvae fed with 30VD diet showed the highest survival rate, without significant differences from those fed diets 25VD and 0VD (Table 4). However, those larvae fed with diet containing 384VD showed the significantly ($p < 0.05$) lowest final survival rate (Table 4) with the highest daily mortality rate recorded after the 15th day of feeding (Supplementary file, Fig. S1). Dietary vitamin D_3 levels did not significantly affect larval growth in terms of total length or body weight, but total larval biomass was recorded significantly ($p < 0.05$) lowest for larval group fed with 384VD diet (Table 4).

Table 3
Sequences of primers used in gene expression studies.

Gene	Nucleotide sequence (5′ -3′)	Accession number
Beta-actin (*β-actin*)	F: TCTGTCTGGATCGGAGGCTC R: AAGCATTTGCGGTGGACG	X89920
Bone morphogenic protein 2 (*bmp2*)	F: GTGGCTTCCATCGTATCAACATTTT R: GCTCCCCGCCATGAGT	JF261172.1
Alkaline phosphatase (*alp*)	F: AGAACGCCCTGACGCTGCAA R: TTCAGTATACGAGCAGCCGTCAC	AY266359
Osteocalcin (*oc*)	F: GGCAGCCATCTGTCTGACTT R: GGTCCGTAGTAGGCCGTGTA	AF048703

3.2. Proximate composition, vitamin D_3 and mineral analysis

The crude lipid contents of larvae at the end of the trial were unaffected ($p > 0.05$) by the dietary vitamin D_3 levels (Table 4). However, ash and protein contents were significantly ($p < 0.01$) increased in larvae fed the highest vitamin D_3 levels (Table 4). Vitamin D_3 level in larvae was significantly ($p < 0.05$) increased by the elevation of vitamin D_3 levels from 0VD to 30VD and from 30VD to 384VD (Table) and followed a highly correlated linear regression (Fig. 1, $R^2 = 0.98$, $p = 0.001$). Calcium and phosphorus contents in 384VD fed larvae were significantly ($p < 0.05$) higher than in larvae fed lower dietary vitamin D_3 levels (Table 4). Moreover, the ratio calcium:phosphorous in gilthead seabream larvae were also significantly ($p < 0.05$) higher in larvae fed with 384VD diet (Table 4).

3.3. Skeletal studies

In Fig. 2 some examples of normal (Fig. 2a) and deformed seabream

Table 4
Growth performance and whole-body composition of the gilthead seabream larvae supplemented with different levels of dietary vitamin D_3.

Experimental Diet						
Dietary vitamin D_3 (μg kg^{-1})	0VD	25VD	30VD	384VD	R^2	p-value (One-way)
Growth performance						
Survival (%)	88.33 ± 2.89^a	85.33 ± 2.31^a	91.00 ± 2.65^a	60.67 ± 5.13^b	0.96	0.001
Total length (mm)	11.89 ± 0.12	11.14 ± 0.11	11.42 ± 0.49	11.43 ± 0.36	0.02	n.s.
Dry weight (mg/larvae)	2.17 ± 0.06	1.82 ± 0.11	1.98 ± 0.43	1.74 ± 0.10	0.49	n.s.
Biomass (g)	4.02 ± 0.15^a	3.26 ± 0.12^a	3.79 ± 0.92^a	2.21 ± 0.31^b	0.90	0.001
Whole body composition (% dry weight)						
Moisture	0.17 ± 0.01^b	0.17 ± 0.00^b	0.17 ± 0.00^b	0.16 ± 0.01^a	0.95	0.001
Ash	10.16 ± 0.77^a	9.72 ± 1.72^a	9.10 ± 1.48^a	11.83 ± 0.47^b	0.82	n.s.
Crude lipid	16.93 ± 1.55	17.30 ± 1.69	16.43 ± 0.58	17.77 ± 3.12	0.59	0.001
Crude protein	75.52 ± 1.26^a	75.69 ± 2.05^a	77.15 ± 0.84^{ab}	78.89 ± 2.13^b	0.82	0.001
Vitamin D_3 contents (μg kg^{-1} larvae)						
Vitamin D_3	0.02 ± 0.01^a	4.00 ± 0.45^{ab}	7.78 ± 1.45^b	33.04 ± 2.84^c	0.98	0.001
Mineral contents (mg kg^{-1} larvae)						
Calcium (Ca)	11 ± 0.01^a	11.33 ± 0.58^a	10.33 ± 0.58^a	16 ± 0.01^b	0.96	0.001
Phosphorus (P)	15.67 ± 0.58^a	15.67 ± 0.58^a	15.67 ± 0.58^a	17.33 ± 0.58^b	0.99	0.001
Ca:P	0.70 ± 0.02^a	0.73 ± 0.06^a	0.66 ± 0.03^a	0.92 ± 0.03^b	0.93	0.001

Different letters in a row denote significant differences between groups fed different diets (mean ± SD, n = 3, $P < 0.05$). n.s. non-significant.

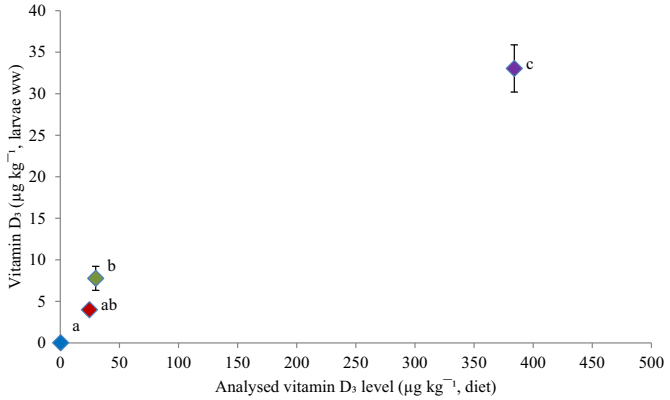

Fig. 1. Vitamin D_3 content in gilthead seabream larvae supplemented with different levels of vitamin D_3.*Different letters in the graph denote significant differences between groups fed different diets for a given feeding period (mean ± SD by one-way ANOVA, n = 3, $p = 0.01$, $R^2 = 0.978$).

larvae fed different levels of VD are shown. The percentage of completely mineralized vertebral column increased proportionally to the analyzed vitamin D_3 content in diet (Fig. 3, $R^2 = 0.93$, $p = 0.01$) and whole body (Fig. 4, $R^2 = 0.98$, $p = 0.01$), the average values being significantly highest in larvae fed with 384VD. Besides, larvae fed diet 0VD showed the lowest average number of completely mineralized vertebrae, regardless of the size class considered (Fig. S2), whereas the highest values were found in larvae fed with 384VD diet. Distribution of

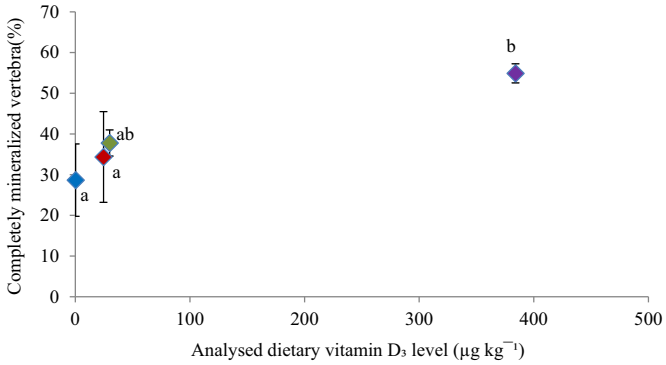

Fig. 3. Percentage of completely mineralized vertebra in gilthead seabream larvae supplemented with different levels of dietary vitamin D_3. *Different letters in the graph denote significant differences between groups fed different diets for a given feeding period (mean ± SD by one-way ANOVA, n = 3, $p = 0.01$, $R^2 = 0.928$).

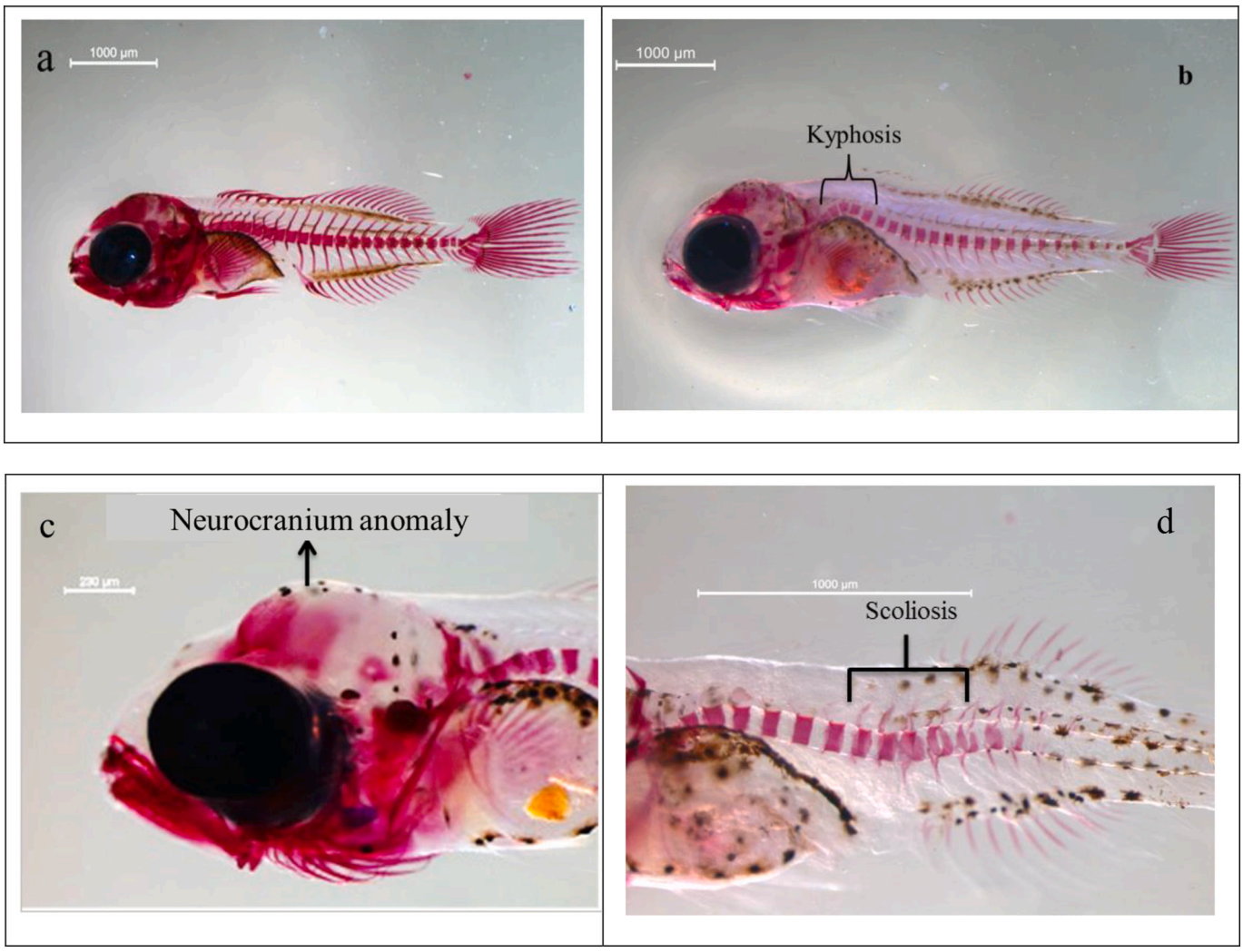

Fig. 2. Gilthead seabream larvae showing different types of skeletal deformities. (a) Normal and completely mineralized larvae, (b) Kyphosis (c) neurocranium anomaly, (d) scoliosis.

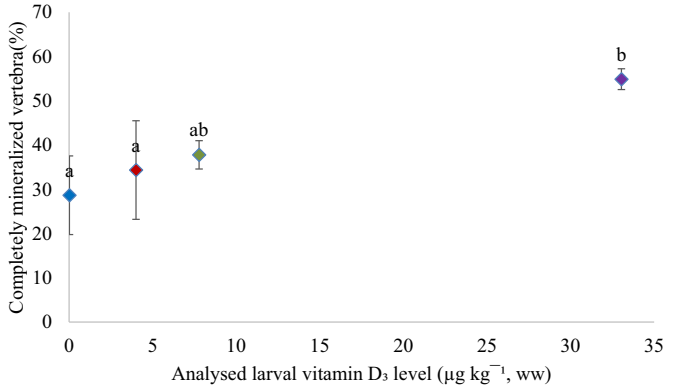

Fig. 4. Percentage of completely mineralized vertebra in relation with analyzed body vitamin D_3 of gilthead seabream larvae supplemented with different levels of dietary vitamin D_3. *Different letters in the graph denote significant differences between groups fed different diets for a given feeding period (mean ± SD by one-way ANOVA, n = 3, $p = 0.01$, $R^2 = 0.983$).

anomalies in each group (Table S3) and percentage frequency of gilthead seabream (Table S4) larvae with incidence of anomalies were observed, and it showed higher percentage of anomalies in group fed 384VD, and low frequency in diet fed 25VD. A correspondence analysis was performed for the distribution of anomalies in different group, and it shows, diet fed 384VD was far from other group by distribution but closer to neurocranium anomaly. (Fig. S3).

The observed trend of severe anomalies on the total observed anomalies in each group showed the significant ($p < 0.05$) lower values in larvae fed the intermediate levels of VD (25 and 30) and the significantly higher in 0VD and 384VD (Table 5). In the abdominal region of the vertebral column, the frequency of deformed vertebrae resulted significantly lower in the 25VD seabream with respect to all the other group, while kyphosis resulted significantly lower in 25VD than in all the other dietary group but not with 0VD. As far as cephalic anomalies are considered, we find that the prevalence of pre-maxillary and maxillary, with the highest significant values found in larvae fed 384VD ($R^2 = 0.99$, $p = 0.002$) (Table 5). There was a very low incidence of haemal lordosis and scoliosis, whose values followed similar patterns to the previous anomalies but did not show significant differences (Table 5). On the contrary, the highest percentage of maxillary anomalies was found in larvae fed 0 VD diet and the lowest in that fed with 384VD diet, without significant differences with larvae fed 25VD or 30VD (Table 5).

Table 5
Frequency (%) of different skeletal anomaly types on the total anomalies inspected in gilthead seabream lots fed increasing levels of dietary vitamin D_3.

Dietary vitamin D_3 (µg kg^{-1})	0VD	25VD	30VD	384VD	R^2	*p*-value
Severe anomalies	70.00 ± 8.91^{bc}	50.47 ± 10.71^{a}	57.63 ± 2.97^{ab}	87.17 ± 5.16^{c}	0.94	0.001
Anomalous maxillary and/or pre-maxillary	14.33 ± 3.22^{b}	8 ± 7.21^{ab}	8.67 ± 1.15^{ab}	0.00 ± 0.0^{a}	0.82	0.015
Other cephalic anomaly	24.28 ± 3.78^{a}	15.72 ± 5.15^{a}	14.76 ± 2.97^{a}	37.62 ± 4.59^{b}	0.99	0.002
Abdominal kyphosis	27 ± 2.00^{ab}	21.67 ± 3.22^{a}	27.67 ± 1.15^{b}	32.33 ± 1.53^{b}	0.60	0.002
Cephalic vertebrae	0.00 ± 0.0	0.95 ± 1.65	0.00 ± 0.0	0.00 ± 0.0	n.s.	n.s.
Abdominal vertebrae	41.43 ± 1.43^{b}	32.38 ± 3.59^{a}	42.38 ± 2.18^{b}	47.14 ± 2.86^{b}	0.51	0.001
Haemal vertebrae	2.38 ± 2.18	1.43 ± 2.48	0.48 ± 0.83	2.38 ± 0.83	n.s.	n.s.
Caudal vertebrae	1.90 ± 3.30	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	n.s.	n.s.

Different letters in a row denote significant differences between groups fed different diets (mean ± SD, $n = 3$, $P < 0.05$). n.s. non-significant.

3.4. Molecular studies

The relative gene expression of *bmp2* (Fig. 5) at different days post hatching for same dietary treatment was constant during larval development in 0VD and 30VD larvae, whereas the 25VD larvae presented higher expression at day 8 and 15, and larvae 384VD was higher at the 8th day of experimental feeding. The relative expression of *bmp2* gene during the experimental trials enhance a strong reduction in 384VD and in 25VD and not significant differences in 0VD and 30VD. These dietary groups showed intermediate expression at days 8 and 15, but the highest at day 21.

The expression of alkaline phosphatase (*alp*) (Fig. 6) was significantly higher ($p < 0.05$) at days 15 and 21 in 384VD larvae, when compared with day 8, but the differences with the among the dietary groups are significant only at the 15th day.

Osteocalcin (*oc*) gene expression (Fig. 7) among different days for same dietary treatment was significantly higher ($p < 0.05$) for 384VD larvae at days 8 and 15, but not at the 21st day. All the other levels of VD do not give any significant differences, even at different times.

4. Discussion

Skeletal anomalies are more prone to develop during the early ontogenetic stages, particularly during embryonic and post-embryonic periods. These anomalies may be related to biotic and abiotic factors (Georgakopoulou et al., 2007; Kourkouta et al., 2021; Sfakianakis et al., 2006; Villeneuve et al., 2005, 2006), including dietary nutrients, whose effect is not yet completely understood (Fernández et al., 2008). In sparids, skeletal anomalies have been associated to environmental, genetic, microbial, or nutritional factors (Izquierdo et al., 2010; Lee-Montero et al., 2015; Negrín-Báez et al., 2015; Roo et al., 2010, 2009, 2005). Among the different nutrients that may be related to the occurrence of skeletal anomalies, vitamin D is well known to affect bone development (St-Arnaud, 2008), but its effect on marine fish larvae and, particularly for gilthead seabream, has been scarcely studied.

In the present study, the increase in dietary vitamin D_3 levels up to 384 µg kg^{-1} proportionally raised the vitamin D_3 contents in gilthead seabream larvae, denoting good absorption and deposition in body tissues. In agreement with the relevant role of vitamin D on calcium absorption and mineralization (Lock et al., 2010), elevation of dietary

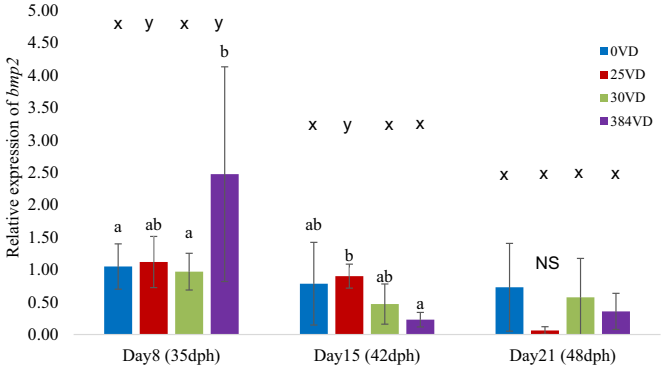

Fig. 5. Relative expression of the *bmp2* gene during the development of gilthead seabream larvae supplemented with different levels of vitamin D_3. *Different letters (a, b) above each bar denote significant differences between groups at the same date (mean ± SD, n = 3, $P < 0.05$). Different letters (x, y) grouped and lined above the bars denote significant differences between days for the same dietary treatment (mean ± SD, n = 3, $P < 0.05$), NS: non-significant.

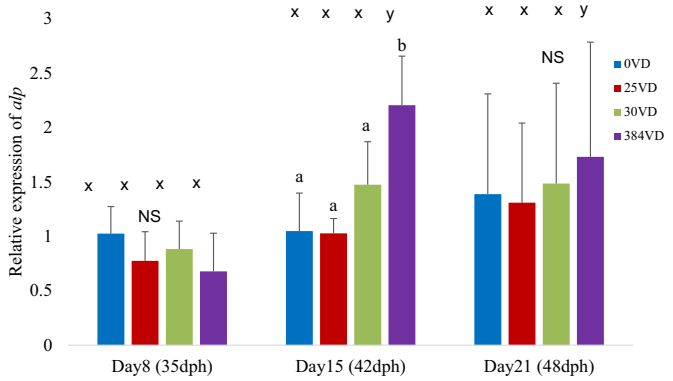

Fig. 6. Relative expression of the alkaline phosphatase gene (*alp)* during the development of gilthead seabream larvae supplemented with different levels of vitamin D_3. *Different letters (a, b) above each bar denote significant differences between groups at the same date (mean ± SD, n = 3, $P < 0.05$). Different letters (x, y) grouped and lined above the bars denote significant differences between days for the same dietary treatment (mean ± SD, n = 3, $P < 0.05$), NS: non-significant.

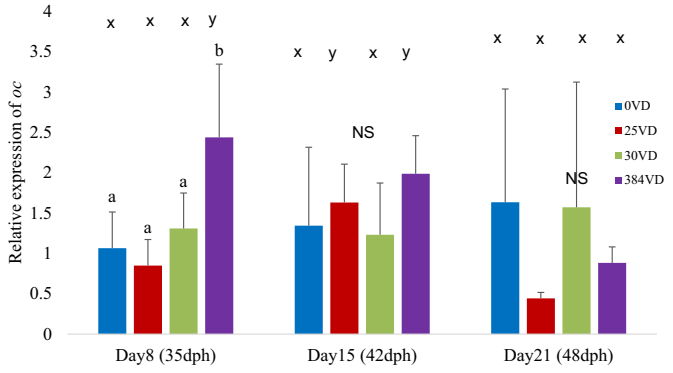

Fig. 7. Relative expression of the osteocalcin gene (*oc)* during the development of gilthead seabream larvae supplemented with different levels of vitamin D_3. *Different letters (a, b) above each bar denote significant differences between groups at the same date (mean ± SD, n = 3, $P < 0.05$). Different letters (x, y) grouped and lined above the bars denote significant differences between days for the same dietary treatment (mean ± SD, n = 3, $P < 0.05$), NS: non-significant.

vitamin D_3 levels proportionally increased calcium, phosphorous and calcium:phosphorous ratios in gilthead seabream larvae. This in agreement with previous studies on farmed halibut and carp, *Cyprinus carpio*, increased vitamin D_3 content in body with increase in dietary vitamin D_3 (Atsuko et al., 1991; Takeuchi, 2014) causing hypervitaminosis and hypercalcemia. Studies also suggest that, in Atlantic salmon, (*Salmo salar*), the deposition of vitamin D_3 in livers, intestine, kidney, spleen, gills, fillet, skin and in the plasma of the fish (Horvli et al., 1998). Several other studies were conducted in juvenile fishes, and it reported to be there is increase in vitamin D_3 storage with increase in dietary vitamin D_3, because vitamin D_3 is esterified with long-chain fatty acids for storage (Fraser, 2018).

Calcium, along with phosphorus plays vital roles in the development, maintenance, and stability of the skeletal system (Baeverfjord et al., 2019; Lall and Lewis-McCrea, 2007). Subsequently, it was observed an increase in bone mineralization when dietary vitamin D_3 levels were raised in the diet, in agreement with the increased bone mineralization found in zebrafish (*Danio rerio*) larvae submitted to increased levels of vitamin D_3 (Fleming et al., 2005). Similarly, in the present study when larvae were fed the diet non-supplemented in vitamin D_3 (0.29 μg kg^{-1}, basal vitamin D_3 content), they showed the lowest vitamin D_3 and calcium contents in whole body, as well as the lowest bone mineralization rates, together with an incidence of high skeletal anomaly. These were clear symptoms of vitamin D deficiency, suggesting that the basal dietary vitamin D_3 levels of the present diets was insufficient to meet gilthead seabream larvae requirements. Contrary to the growth reduction observed in European seabass larvae fed vitamin D_3 deficient levels (Darias et al., 2010), in the present study the seabream larvae fed non-supplemented vitamin D_3 diets showed good growth and survival. Also in Wuchang bream juveniles, a vitamin D_3 deficient diet reduces growth rate and plasma calcium (Ling-hong et al., 2015). However, in agreement with our study, Japanese flounder (*Paralichthys olivaceus*) juvenile shows good growth when fed with a vitamin D_3 deficient diet despite an increased incidence of hypermelanosis (Haga et al., 2004). Nevertheless, in gilthead seabream larvae lower basal vitamin D_3 levels or longer feeding periods than those tested in the present trial could lead to growth and survival reductions.

In this study, further increase in dietary vitamin D_3 up to 384 μgkg^{-1} led to marked mortalities in gilthead seabream larvae, denoting a negative hypervitaminosis effect. This hypervitaminosis effect could be related to the high bioavailability and binding capacity to vitamin D transport proteins of vitamin D_3 (Atsuko et al., 1991; Calvo et al., 2005; Graff et al., 2002b, 2002a; Hay and Watson, 1977; Horvli et al., 1998; Vielma et al., 1999), which may also cause hypercalcemia (Cusano et al., 2018). Indeed, the high phosphorous and, particularly, calcium and Ca:P contents in these larvae denote a hyperphosphatemia and a strong hypercalcemia that could be partly responsible for the mortalities observed in relation to the stress caused by unbalances of those minerals, as found in other fish species (Sundell et al., 1993; Swarup et al., 1984; Swarup and Srivastav, 1982). These results agree well with the low survival and hypercalcemia found in Japanese flounder larvae fed high vitamin D_3 levels (500 μgkg^{-1}, 20,000 IU) (Haga et al., 2004). Hypercalcemia in relation to excessive levels of dietary vitamin D_3 has also been found in ricefield eel (*Monopterus albus*) (Tan et al., 2007) or walking catfish (*Clarius batrachus*) (Abbink and Flik, 2007; Swarup et al., 1984). In gilthead seabream juveniles, high levels of calcium in water or diet raised cortisol and PTHrP levels (Abbink et al., 2004), denoting the stress conditions caused by such mineral imbalance.

In addition, larvae fed the diet non-supplemented in vitamin D_3 (0.29 μg kg^{-1}) presented a high incidence of skeletal anomalies, particularly in maxillary bones. Maxillary bones develop and mineralize early in gilthead seabream larvae (Faustino and Power, 1998), and are required for active predation on zooplankton to compensate their low egg-yolk reserves. This fact could explain the increased frequency of anomalies found in maxillary bones when seabream was fed a vitamin D_3 deficient diet, which showed lower calcium and phosphorous deposition and bone mineralization rates than larvae fed increased vitamin D_3 levels. These results agree well with the low mineralization and calcium contents in European seabass larvae fed deficient levels of vitamin D_3 and recorded the abnormal development of skeleton (Darias et al., 2010) during the developmental period. On the other hand, increase in dietary vitamin D_3 up to 25 μg kg^{-1}, not only reduced the skeletal anomalies in maxillary bones, but also lead to the lowest incidence of anomalies in the abdominal region, particularly, abdominal kyphosis. However, further increase up to 30 μg kg^{-1} did not reduce skeletal anomalies, suggesting that around 25–30 μg kg^{-1} vitamin D_3 was required in early weaning diets for gilthead seabream larvae to fulfill their requirements.

The highest dietary vitamin D_3 levels also led to the highest incidence of skeletal anomalies in agreement with previous studies in larvae of other species (Haga et al., 2004). However, excess levels of dietary vitamin D_3 does not affect skeletal anomalies or mortality in juveniles of other species such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) or brook trout (*Salvelinus fontinalis*) (Haga et al., 1999; Poston, 1969). Such lack of effect in comparison to the studies in larvae could be related to the species or the dietary vitamin D levels used, but also to a higher sensitivity of fish during earlier studies of

development, when bones are still being formed and mineralized. The high incidence of skeletal anomalies in fish larvae could be mediated by an increase production in calcitriol, the functional form of vitamin D (Haussler, 1986), which contributes to regulate calcium absorption (Graff et al., 1999). Indeed, feeding calcitriol to Japanese flounder larvae raised the incidence of vertebral deformities to the same levels found in larvae fed high vitamin D_3 diets (500 μg/100 g, 20,000 IU vitamin D_3/100g) (Haga et al., 2004). Calcitriol administration also increased calcium absorption in American eel (*Anguilla rostrata*) and Atlantic cod (*Gadus morhua*) (Fenwick et al., 1984; Sundell et al., 1993). Therefore, the hypercalcemia and increased skeletal anomalies found in seabream fed the highest dietary vitamin D_3 levels (384 μgkg^{-1}, 15,360 IU kg^{-1}) in the present study could be mediated by increase in the calcitriol levels in larvae.

Among the different types of skeletal anomalies studied, cephalic anomalies and abdominal kyphosis were particularly raised in seabream larvae fed the highest vitamin D_3 levels. In gilthead seabream larvae the cephalic region starts to develop immediately after hatching (Faustino and Power, 2001) and includes numerous bones that develop and ossified to form the cranium. A well-developed cranium improves the respiratory and feeding behavior of the larvae that helps in larval growth and improves survivability (Faustino and Power, 2001). In the present study, hypervitaminosis particularly caused an abnormal development of frontal bone, which was not observed in other larval groups and could negatively affect larval development and survival. In seabream juveniles, the main skeletal anomalies found in fish fed excess vitamin D_3 (0.00065 μg kg^{-1} diet) were maxillary or pre-maxillary anomalies, abdominal lordosis, abdominal fusion, and haemal lordosis. However, the studies on the effect of vitamin D on cranial abnormalities in fish are scarce and further studies are need regarding the effect of vitamin D_3 in cranial anomalies.

Vitamin D_3 is known to regulate the bone mineralization process through calcium and phosphate absorption (Christakos et al., 2011; DeLuca, 2016) and through osteoblast differentiation and maturation (Van Der Meijden et al., 2014; Woeckel et al., 2010) in animals and humans. Bone morphogenetic protein, such as *bmp2*, work with vitamin D_3 and promote osteoblast differentiation at earlier period and maturation at later stages (Song et al., 2011). In the present study, the expression of osteogenic factor *bmp2* increased in relation to dietary vitamin D_3 and reached a maximum level at earlier stages (day 8 of feeding, 35 dph) in larvae fed 384 μg kg^{-1}. This indicates the early differentiation of osteoblast cells in 384 VD larvae and could have results in mineralization of osteoblast cells by producing osteocalcin in 384 VD larvae earlier than the other dietary treatments. After day 8, *bmp2* expression was reduced in 384 VD group, which explains the possible reduction in proliferating osteoblast, that could be seen with increase in *alp* and *oc* expression (Jang et al., 2012). Osteocalcin (*oc*) promotes mineralization through its calcium binding ability (Cavaco et al., 2014) and increases bone mineral density. As expected, the expression of *oc* in the present study was also elevated at day 8 (35 dph) in larvae fed 384 VD diet and increased until day 15 (42 dph), while the expression of *oc* in other treatments remained consistent. Studies suggest that *bmp2* signalling increased Activating Transcription Factor 6 (ATF6) expression and cleavage, and the activated ATF6 increase *oc* expression (Jang et al., 2012). Moreover, in European seabass larvae the expression of *oc* was constant for different dietary groups except for the vitamin D deficient group (Darias et al., 2010) at 45 dph. Similarly, in gilthead seabream juveniles, the different levels of dietary vitamin D_3 did not affect the expression of *oc* (Dominguez et al., 2021). This suggests that the expression of *oc* depends on the level of vitamin D_3 in the diet, species, and its developmental stage. This expression of *oc* was continued until day 15, which also determines the presence of maturing and matured osteoblast that could be seen with the upregulation of *alp* gene on day 15. The up-regulation of *alp* and *oc* genes had been studied in rat and human osteoblast, which was induced by calcitriol (Pols and van Leeuwen, 2004; van Driel and van Leeuwen, 2017). Calcitriol is the active form of vitamin D_3 which has a direct effect on osteoblast to control extracellular matrix protein production (osteocalcin) and on the *alp* activity, which was an important biomarker of bone formation (van Driel and van Leeuwen, 2014). The activity of *alp* accelerates mineralization process (Shaw and Högler, 2012) and deposition of hydroxyapatite crystals (Saraç and Saygılı, 2007) for bone formation. An excess elevation of *alp* was the result of increased osteoblast activity (Irving and Jawad, 2005). In agreement, in the present study, there was an up-regulation of *alp* expression in the highest VD dietary group, which showed excess *bmp2* and *oc* expression. This increased expression of *alp* is associated with the progressive differentiation of osteoblasts which required for the maturation of bone extracellular matrix (Irving and Jawad, 2005). This increased expression of *bmp2, oc* and *alp* justifies the high mineralization percentage in gilthead seabream larvae fed 384 VD, which was higher than in the other dietary treatments. Meanwhile, the expression of *alp* was high until day 21 when compared with day 8, because hypervitaminosis D has been shown to elevate alkaline phosphatase in fish (Halver, 1982). Even though the bone biomarkers in the present study showed increased mineralization rates in the highest dietary vitamin D_3 group, the larvae in the group were severely affected with cranial frontal bone deformation and increased in mortality rate. In agreement, in juvenile gilthead seabream, an increased percentage of skeletal anomalies is found with the upregulation of *alp* and *bmp2* in relation with a high vitamin D_3 content in diet (Dominguez et al., 2021). In addition, increase in *bmp2* cause several adverse effects in animals and humans, such as osteoclast activation, cervical spine swelling, bone cyst formation, inflammation and even death (Boyne et al., 2005; Knox et al., 2011). Moreover, vitamin D_3 exerts toxic effects in animals when it is delivered several times higher than the normal requirement, denoted by hypercalcemia and increased synthesis of alkaline phosphatase (Guven et al., 1990). This result was in accordance with the high expression of *alp* and calcium and calcium:phosporous contents in seabream larvae fed the highest dietary vitamin D_3 diet. Thus, the results from the present study on gene expression, mineralization, and severe skeletal anomalies opened a pathway to further research in understanding the regulation of vitamin D_3 in bone mineralization and the physiological response of gilthead seabream larvae to vitamin D_3 toxicity.

Henceforth, the requirements of vitamin D_3 for various fish species such as *Dicentrarchus labrax* (Darias et al., 2010; Dioguardi et al., 2017), *Epinephelus coioides* (He et al., 2021), *Salmo salar* (Prabhu et al., 2019), *Megalobrama amblycephala* (Ling-hong et al., 2015), *Acipenser baerii,* (Wang et al., 2017) and *Labeo rohita* (Sivagurunathan et al., 2020) have been demonstrated and seem to vary with the level of vitamin D_3 administered, developmental stage, rearing condition, and physiological response. Unfortunately, very few studies have been conducted in marine fish larvae to determine the optimum dietary vitamin D_3 levels and fish physiological response. The results of the present study suggest that vitamin D_3 requirements are higher for gilthead seabream larvae than for European seabass larvae (0.00069 μg kg^{-1}, Darias et al., 2010). Moreover, even non-enriched rotifers (60–80 μg kg^{-1}, 2400 to 3200 IU kg^{-1}vitamin D) could fulfill the vitamin D_3 requirements for gilthead seabream larvae (Hamre, 2016). Besides, the requirements in the larvae seem to be higher than for juveniles, where vitamin D_3 requirements have been estimated to be 0.00029 μgkg^{-1} (Dominguez et al., 2021). Considering the limited knowledge available for gilthead seabream larvae on the effect of dietary vitamin D_3, the present study provides new insights to understand the importance of this nutrient in hatchery diets for larvae of this species.

5. Conclusion

The present study shows that gilthead seabream larvae are remarkably sensitive to dietary vitamin D_3 levels. This sensitivity differs from other fish species and developmental stages and alters the physiological response of the species. The current study demonstrated that a diet

without vitamin D_3 supplementation (0.29 μg kg^{-1}) showed better survival and growth rate but resulted in increased maxillary anomalies and lesser mineralization rates. When vitamin D_3 level increased to 25 and 30 μg kg^{-1}, the growth and mineralization rate were improved but further increase up to 384 μg kg^{-1} raised the vitamin D_3 levels in larvae and resulted in hypercalcemia, hyperphosphatemia, and higher incidence of skeletal anomalies, denoting a vitamin D toxicity. Thus, the recommended dietary level of vitamin D_3 for gilthead seabream larvae was suggested to be between 25 and 30 μg kg^{-1}.

CRediT authorship contribution statement

U. Sivagurunathan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **David Dominguez:** Conceptualization, Methodology, Validation, Project administration, Writing – review & editing, Supervision, Visualization. **Yiyen Tseng:** Data curation, Visualization. **Kamil Mert Eryalçın:** Data curation, Visualization. **Javier Roo:** Writing – review & editing, Resources. **Clara Boglione:** Writing – review & editing, Data curation, Methodology. **P. Antony Jesu Prabhu:** Writing – review & editing, Resources, Methodology. **Marisol Izquierdo:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Supervision, Visualization, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738505.

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