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Use of calcifediol in gilthead seabream (*Sparus aurata*): Effects on growth performance, biochemical composition, skeletal anomalies and histology

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ABSTRACT

Vitamin D_3 is the typical vitamin D supplement in aquafeeds, but this form requires further metabolization to the active form. Calcifediol is used in livestock as an alternative, however it isn't common in aquaculture. This trial shows the effects of calcifediol in gilthead seabream. 600 seabream (12.8 ± 1.5 g) were fed 5 levels of calcifediol (<2.0, 99.7, 167.4, 298.6 or 825.4 ppb) for 15 weeks. 99.7 ppb of calcifediol improved growth, while 99.7–167.4 ppb reduced the FCR. 99.7 ppb of calcifediol increased the deposition of DHA and omega 3. The level of calcium in the vertebrae increased quadratically up to 99.7 and 167.4 ppb. No effect of the diet was observed on the prevalence of skeletal abnormalities. Mid-intestine fold length increased with 99.7 and 167.4 ppb of calcifediol compared to 825.4 ppb, while mid-intestine lamina propria width was thinner in fish fed 99.7 ppb than those without supplementation. 167.4 ppb calcifediol increased 50 % the percentage of goblet cells per surface area in the anterior intestine compared to those without supplementation. The results indicate that 99.7 ppb improved growth, FCR, whole-body omega3/omega6 ratio and calcium level in the vertebrae, while the highest levels of supplementation reduced growth, vertebrae calcium and mid-intestine fold length, suggesting a toxic effect of high levels of calcifediol in gilthead seabream juveniles.

1. Introduction

The implementation of novel nutritional strategies with more sustainable raw materials, that aim to reduce the dependence on fishmeal (FM) and fish oil (FO) in the feeds, is a key action for promoting sustainability and efficiency of aquaculture production. Although high incorporation of plant feedstuffs is a reality in aquaculture production, plant-based diets often lead to alteration in the vitamin profile of the feeds (Hansen, Hemre, 2013). Therefore, feeds must be supplemented with vitamins to cover fish nutritional requirements. Vitamin D is an essential fat-soluble vitamin in calcium homeostasis, acting in synergy with calcitonin and parathyroid hormone. Together they regulate calcium uptake and liberation from bone intervening in its remodelling (Halver and Hardy, 2002; NRC, 2011; Boglione et al., 2013). Little is known about the capacity of fish to synthesise vitamin D_3, with some authors suggesting the dietary source may play a significant role in this process, such as the case of salmonids (Fraser, 2018; Pierens and Fraser, 2015) Recent research suggest that *de novo* synthesis on fish reared indoors might be negligible (Rider et al., 2025), supporting the hypothesis that they require absorbing it directly from the diet (Lock et al., 2010). Vitamin D has been showed to have effects on fish bone (Darias et al., 2010; Dominguez et al., 2021; Sivagurunathan et al., 2022, 2024), immunity, nutrient metabolism, growth, and cell differentiation (Cerezuela et al., 2009; Lock et al., 2010; Pierens and Fraser, 2015; Fraser, 2018; Han et al., 2019; Knuth et al., 2020; Liu et al., 2022; Shao et al., 2022, Zhang et al., 2023).

Fish uptake of vitamin D mainly takes place through several sources including vitamin D_2 (ergocalciferol), mainly from phytoplankton and plants, and D_3 (cholecalciferol), mainly from zooplankton and animals (Rao and Raghuramulu, 1996). However, fish tend to possess low storage of vitamin D_2 in fish tissues, whereas vitamin D_3 is widely considered the main storage of the vitamin instead (Lock et al., 2010). Vitamin D_3 has also proven to have better effects on fish immunity (Soto-Dávila et al., 2020), thus, the traditional supplement of vitamin D in

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commercial aquaculture fish is D_3. Two hydroxylation steps are necessary to convert dietary D_3, via calcifediol (25-hydroxycholecalciferol, 25-OH-D3), into calcitriol (1,25-OH-D3). In several fish species (including some members of the cyprinidae, flat fish, gadidae, salmonidae, and scombridae families), further hydroxylation to form the most active metabolites calcitriol, [1 α, 25-Dihydroxyvitamin D3 (1,25 (OH) 2D3]and 24,25(OH)2D3 occurs in liver, kidney and several other tissues by 25-hydroxycholecalciferol-1-hydroxylase (Lock et al., 2010). However, vitamin D metabolism appears to lack uniformity in the different tissues and fish species. For instance, in salmonids, as opposed to mammals, 1,25-OH-D3 is detected in higher amounts than 25-OH-D3, likely because most metabolism occurs in the liver (Pierens and Fraser, 2015). Therefore, by supplementing directly with calcifediol, one of the two hydroxylation processes is spared, potentially improving the activity of the vitamin. Furthermore, 1 μg/kg 25-OH-D3 is equal to 40 IU D3, denoting their different activities. In fact, there are reports suggesting that dietary calcifediol in replacement or in addition to D_3 is effective in promoting performance, enhancing bone mineralization, reducing incidence of tibial dyschondroplasia (Atencio et al., 2005; Han et al., 2016) and modulating avian immunity (Chou et al., 2009; Gómez-Verduzco et al., 2013; Leyva-Jimenez et al., 2019). In fish, positive effects of the supplementation of calcifediol have been described in rainbow trout, including improved zootechnical performance and achieving maximal levels of active vitamin D in the blood to meet physiological demands, while being safe in terms of lack of significant effects on health indices, haematology, and blood chemistry, including calcium and phosphorus at high doses (Rider et al., 2023). However, this has not been tested in Mediterranean warm water marine species. The present work shows the first evidence of the effects of this compound on juvenile seabream.

2. Material and methods

2.1. Ethical statement

All the animal trials 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), and approved by the Animal Experimentation Ethics Committee of the University of Las Palmas de Gran Canaria under file number OEBA_ULPGC_11/2022.

2.2. Experimental diets

Five experimental diets were used, including four supplemented with increasing levels of calcifediol and a control without calcifediol supplementation. All the diets were supplemented with 3000 IU/kg vitamin D3. A negative control diet (<2.0) without calcifediol supplementation contained < 2.0 ppb analysed calcifediol in feed. The other four diets (99.7, 167.4, 298.6 and 825.4 ppb) were supplemented with calcifediol in increasing levels, reaching 99.7, 167.4, 298.6 and 825.4 ppb analysed calcifediol in feed respectively (Table 1). Feeds were extruded, and calcifediol was added after extrusion by lipid-coating. For the feeds, the conversion unit 1 μg/kg 25-OH-D3 is equal to 40 IU D3, was used. All the diets were manufactured by Huvepharma NV (Antwerp, Belgium), and were tested in triplicates ($n = 3$). The calcifediol levels tested were calculated following the recommendations for this species according to Dominguez et al. (2021)

2.3. Fish rearing and sampling

Six hundred gilthead seabream (*Sparus aurata*) juveniles, weighing 12.8 ± 1.5 g body weight, were distributed into 15 tanks in triplicate groups per diet and fed until apparent satiation thrice daily for 15 weeks under a natural photoperiod (12 h light). Water temperature (23.3 ± 0.9 ºC), oxygen (6.6 ± 0.2 mg kg^{-1}) and feed intake were monitored daily. Growth and productive parameters were measured along the trial. Before sampling, fish were previously fasted for 24 h and then, anesthetized with clove oil (Guinama S.L.U., Valencia, Spain). At the end of the 15-week trial all the fish were euthanized with overdose of anesthesia (clove oil, Guinama, Spain), sampled for weight, length, and viscero- and hepatosomatic indexes (VSI & HSI).

2.4. Biochemical composition

Tissues from 5 fish per tank were frozen (-80ºC) as samples for biochemical composition of liver and fillet. 5 whole-body per tank were kept at −80ºC for vitamin and mineral analysis.

Proximate composition analysis of feeds and fish samples was carried out according to the standardized procedures described by AOAC (2019), for protein, moisture, and ash. The total lipid content of the

Table 1
Ingredients and proximate composition of the experimental diets.

Ingredients, %	< 2.0	99.7	167.4	298.6	825.4
Soy protein concentrate	23.000	23.000	23.000	23.000	23.000
Wheat gluten	21.450	21.450	21.450	21.450	21.450
Corn gluten meal	14.900	14.900	14.900	14.900	14.900
Wheat meal	10.700	10.700	10.700	10.700	10.700
Fishmeal Super Prime	10.000	10.000	10.000	10.000	10.000
Faba beans (low tannins)	4.950	4.950	4.950	4.950	4.950
Fish oil	6.000	6.000	6.000	6.000	6.000
Rapeseed oil	2.150	2.150	2.150	2.150	2.150
Linseed oil	0.820	0.820	0.820	0.820	0.820
Palm oil	1.630	1.630	1.630	1.630	1.630
MCP (Monocalcium phosphate)	3.000	3.000	3.000	3.000	3.000
Vitamin and mineral premix	1.000	1.000	1.000	1.000	1.000
Choline chloride 50 % SiO2	0.200	0.200	0.200	0.200	0.200
Antioxidant	0.200	0.200	0.200	0.200	0.200
Vitamin D supplementation					
Calcifediol (28940 IU/g)	0	2788	8364	16728	47396
Vitamin D_3 IU/kg	3000	3000	3000	3000	3000
Total Units IU/kg Feed	3000	5788	11364	19728	50396
Proximate composition					
Protein (%)	49.4	49.8	48.9	50.5	50.9
Lipid (%)	14.4	12.3	12.3	12.7	12.7
Moisture (%)	7.4	5.7	6.6	5.7	4.5
Ash (%)	6.5	7.6	7.2	7.7	7.9
Analysed calcifediol in feed (ppb)	< 2.0	99.7	167.4	298.6	825.4

samples followed the method described by Folch et al. (1957). Fatty acid methyl esters were obtained by transmethylation of total lipids (Christie et al., 1989) and separated by gas chromatography following the conditions described by Izquierdo et al. (1989).

2.5. Vitamin and mineral composition

Samples from the feeds were collected for 25-hydroxycholecalciferol analysis through 25-hydroxycholecalciferol assay in feed PST-RD04-FPM-00052, HPLC by BIOVET laboratory for Feed Analysis (Bulgaria) with a LOD of 2ppb. 5 fish per tank were sampled for nutrient retention. Vitamin D_3 (cholecalciferol) content of whole-body and fillet samples was evaluated by Aquimisa S.L.U. (Salamanca, Spain) according to the European Standard UNE-EN 12821:2009. Homogenised and pooled 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 to a LOD of $<$ 1.0ppb. Mineral composition of the whole-body, fillet and vertebrae samples were determined by inductively coupled plasma mass spectrometry (ICP-MS). Approximately 0.20–0.25 g of samples with 0.5 mL of Milli-Q® water were digested using 2 mL of concentrated nitric acid (HNO3) by Milestone Ultrawave. Then digested samples were diluted to 25 mL with Milli-Q® water. Subsequently, the samples were introduced into nebulizer tube of the ICP-MS (iCapQ ICP-MS, Thermo Scientific, Waltham, USA) with an auto sampler (FAST SC-4Q DX, Elemental Scientific, Omaha, USA) and the mineral compositions were determined by ICP-MS.

2.6. Skeletal anomalies

20 fish per tank were frozen at −20ºC and X-ray were taken for osteological assessment of 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 were classified according to Boglione et al. (2001).

2.7. Histological evaluation

Histological analysis of the intestine was performed on five fish per tank. Samples, fixed in 10 % buffered formalin for 24 h, were collected from the anterior, middle, and posterior segments of the intestine and embedded using a Microm STP 120 Spin Tissue Processor (STP120; Thermo Fisher Scientific). Paraffin-embedded sections were cut at a thickness of 3 μm using a semi-automated microtome (Jung Autocut 2055, Leica, Germany) and mounted on SuperFrost-Plus slides. Sections were stained with Alcian Blue (pH 2.5) (Martoja and Martoja-Pierson, 1970), to differentiate goblet cells secreting acidic mucins. This staining technique was also used to measure mucosal fold area, height and width, as well as lamina propria thickness.

Stained sections were scanned using a MoticEasyScan Pro digital scanner (Motic, Xiamen, China) operated with Motic DS Assistant software (Motic VM V1 Viewer 2.0) and representative images of each section taken. Image analysis was performed (after calibration with the scale bars) using the analySIS® software package (Image Pro Plus® v4.5.0.29; Media Cybernetics, Silver Spring, MD, USA). Three folds from each section and intestinal segment (proximal, medial, and distal) were analyzed. The areas were manually delineated by tracing their perimeters, with the base defined by an imaginary line connecting the junctions of adjacent anterior and posterior folds. Mucosal fold height (from the villus apex to the traced base line), fold width, and lamina propria thickness (measured at the midpoint of the villus) were manually measured using the software's built-in tools. The goblet cell area within each delineated fold was automatically quantified using the eyedropper tool, and the percentage area was subsequently calculated.

2.8. Statistical analysis

All data were statistically analysed 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. Quadratic and lineal regressions and broken line analyses were conducted where possible. Significant differences were considered for $p < 0.05$. Weight gain, Specific Growth Rate (SGR), Feed Conversion Ratio (FCR) and Temperature Growth Coefficient (TGC) were calculated using the following formulae:

Weight gain (%)= 100× (final weight−initial weight)/initial weight

SGR (%)= $((Ln_{W2}\text{-}Ln_{W1}))$/days x 100

FCR = (Ingested food)/(generated biomass)

Where

W_1: initial body weight (g)

W_2 final body weight (g)

$$TGC = \frac{(W1^1/3 - W1^1/3)}{temp * days}$$

3. Results

3.1. Fish performance

Since the first intermediate sampling, there were significant differences between the treatments, where fish fed the diet without supplementation presented the lowest growth, while fish fed the diet supplemented to contain a total of 99.7 ppb calcifediol presented the highest growth, whereas fish fed diets with supplementation levels beyond this point presented intermediate values (Table 2). This trend was observed throughout the trial, and at the end of the 15 weeks the fish fed the diet with no supplementation and those fed the two highest levels of vitamin D (298.6 and 825.4 ppb calcifediol) had significantly lower weights when compared to fish fed the diet containing 99.7 ppb calcifediol, while those fed the diet containing 167.4 ppb calcifediol presented intermediate values (Table 2). At the end of the trial, no significant regressions were observed between the analysed calcifediol present in the feeds and the final weight.

Between the initial sampling and the first intermediate sampling fish presented a weight gain of over 149 %, growing from the initial 12.8 ± 1.5 g to up to 33.9 ± 3.9 (Table 1). No significant differences ($<$0.05) were observed between the treatments, except between the sampling period from day 37–72, where fish fed the diets with no supplementation performed significantly worse than those fed the diets containing 99.7 and 167.4 ppb calcifediol, while those fed diets with higher supplementation presented intermediate results. Regardless, during the entire duration of the trial, fish presented a total weight gain of up to 528.9 ± 27.3 (%) for the case of fish fed the diet with 99.7 ppb calcifediol (Table 2).

Fish accepted the feed from the beginning of the trial, although the feed tended to float more than usual, and some pellets remained on the surface for a longer period than usual. Regardless of this, the fish presented normal feeding behavior.

FCR data showed an overall improved performance after the 15-week trial of fish fed the diets supplemented with calcifediol regardless of the level of supplementation, except for those fed the highest level

Table 2
Gilthead seabream growth in terms of body weight and weight gain during the 15-week trial fed increasing levels of calcifediol.

	Analysed calcifediol in feed (ppb)	< 2.0	99.7	167.4	298.6	825.4	ANOVA (*p* value)
Mean weight (g)	day 0	12.8 ± 1.4	12.8 ± 1.5	12.8 ± 1.5	12.7 ± 1.5	12.8 ± 1.5	0.983
	day 37	32.1 ± 4.1^{a}	33.9 ± 3.9^{b}	33.0 ± 3.7^{ab}	32.5 ± 4.1^{ab}	33.4 ± 4.1^{ab}	0.005
	day 72	54.5 ± 6.9^{a}	58.8 ± 7.7^{c}	57.3 ± 6.3^{bc}	55.8 ± 7.2^{ab}	56.4 ± 6.8^{abc}	0.000
	day 105	75.0 ± 9.5^{a}	80.5 ± 11.9^{b}	77.9 ± 8.6^{ab}	76.7 ± 9.7^{a}	75.4 ± 9.2^{a}	0.000
Weight gain (%)	0–37 days	149 ± 6	165 ± 5	158 ± 11	156 ± 2	160 ± 12	0.247
	37–72 days	65 ± 4^{a}	73 ± 2^{b}	74 ± 0^{b}	72 ± 2^{ab}	69 ± 3^{ab}	0.021
	72–105 days	38.7 ± 0.6	36.9 ± 2.0	36.3 ± 1.5	36.2 ± 3.8	33.7 ± 3.0	0.246
	0–105 days	476.6 ± 19.4	528.9 ± 27.3	511.2 ± 20.5	497.6 ± 18.5	487.0 ± 23.1	0.100

Different letters in the same row indicate significant differences for the ANOVA analysis ($p < 0.05$).

of supplementation which presented intermediate values (Table 3). At the end of the trial no significant regressions were observed between the analysed calcifediol present in the feeds and the FCR for days 72–105 or 0–105.

SGR values tended to follow the trend described for FCR, although there were no statistical differences among the diets when compared with ANOVA (Table 3). However, at the end of the trial a significant lineal regression was observed between the analysed calcifediol present in the feeds and the SGR for the last period comprehending days 72–105 of the trial, denoting that an increase in the supplementation level caused a reduction in SGR, whereas this effect was not observed throughout the entire trial.

TGC was not significantly affected by the inclusion levels of calcifediol (Table 3).

3.2. Viscero- and hepatosomatic indexes (VSI & HSI)

At the end of the trial VSI and HSI were recorded, and the results showed that there were no significant effects of the supplementation of calcifediol on these parameters (Table 4). At the end of the trial no significant regressions were observed between the analysed calcifediol present in the feeds and the VSI or HSI.

3.3. Proximal composition

At the end of the trial proximal composition of the whole-body, fillet, and liver were analysed (Table 5, Figs. 1, 2, 3, 4, and 5). Whole-body lipid composition increased with increasing calcifediol supplementation, being significantly lower in those fish fed the diet without supplementation, and higher in those fed 167.4 ppb and beyond (Table 5, Fig. 1). Protein content followed an inverse trend, being highest in the diet without supplementation and decreasing significantly on those fed 167.4 and 298.6 ppb of calcifediol (Table 5, Fig. 2). Ash content in the fillet followed a significant quadratic regression being lower in fish fed the diets with lower levels of supplementation, and increased until the diet with 167.4 ppb, remaining unchanged with higher levels of supplementation (Fig. 3). Liver lipids increased linearly with increasing levels of supplementation following a significant regression (Fig. 4). Liver protein also followed a significant lineal regression, although with no significant effect (Fig. 5). The other parameters were not significantly affected by the diets.

Fatty acids composition remained not significantly affected by the different diets (not included), except for those described in Table 6. Whole-body 14:1n-7, 14:1n-5, 15:1n-5, 16:OISO tended to increase with increasing calcifediol supplementation. 20:1n-7 was lowest in fish fed diets containing 99.7 and 825.4 ppb of calcifediol, whereas the highest level was found in those fed the diet without supplementation. On the other hand, the ratios describing oleic acid/ Docosahexaenoic acid (DHA) and omega 3/ omega 6 ratios describe a higher deposition of DHA and omega 3 in the diet containing 99.7 ppb of calcifediol, whereas the contrary was observed for fish fed the highest level of calcifediol. In fillet, the only clear effect observed was on 18:2n-9, which tended to reduce with increasing calcifediol in the feed (Table 6). As for liver, the only fatty acid presenting a significant effect of calcifediol supplementation was 16:1n-5, which was higher in the fish fed the diet containing 99.7 ppb calcifediol, compared to the diet containing 167.4 ppb, while the other diets presented intermediate values (Table 6).

3.4. Vitamin and Mineral composition

Samples from whole-body, vertebrae and fillet were collected for calcium (Ca) and phosphorus (P), whereas vitamin D_3 was also evaluated on whole-body (Table 7, Figs. 6 and 7). Whole-body vitamin D_3 did not show significant differences when compared through ANOVA analysis, but there was a tendency to increase with dietary supplementation of Calcifediol following a significant lineal regression due to the high deposition of vitamin D_3 in one of the replicas (Fig. 6). Vertebrae calcium followed a significant quadratic regression, where the levels of calcium were higher in fish fed the diets containing 99.7 and 167.4 ppb calcifediol in feed (Fig. 7). The other parameters were not significantly

Table 3
Gilthead seabream FCR, SGR, and TGC during the 15-week trial fed increasing levels of calcifediol.

	Analysed calcifediol in feed (ppb)	< 2.0	99.7	167.4	298.6	825.4	ANOVA (*p* value)
FCR	0–37 days	1.60 ± 0.29	1.32 ± 0.09	1.34 ± 0.14	1.26 ± 0.01	1.31 ± 0.08	0.128
	37–72 days	1.51 ± 0.06^{b}	1.29 ± 0.04^{a}	1.29 ± 0.06^{a}	1.36 ± 0.03^{a}	1.34 ± 0.07^{a}	0.005
	72–105 days	1.20 ± 0.02	1.23 ± 0.02	1.20 ± 0.03	1.25 ± 0.17	1.19 ± 0.08	0.877
	0–105 days	1.31 ± 0.07^{b}	1.17 ± 0.03^{a}	1.16 ± 0.07^{a}	1.16 ± 0.04^{a}	1.19 ± 0.02^{ab}	0.019
SGR	0–37 days	2.47 ± 0.07	2.64 ± 0.05	2.56 ± 0.11	2.54 ± 0.02	2.58 ± 0.12	0.247
	37–72 days	1.43 ± 0.08^{a}	1.57 ± 0.04^{b}	1.58 ± 0.01^{b}	1.54 ± 0.03^{ab}	1.50 ± 0.05^{ab}	0.024
	72–105 days	0.99 ± 0.01	0.95 ± 0.05	0.94 ± 0.03	0.93 ± 0.09	0.88 ± 0.07	0.239
	0–105 days	1.67 ± 0.03	1.75 ± 0.04	1.72 ± 0.03	1.70 ± 0.03	1.69 ± 0.04	0.093
TGC	0–37 days	3.18 ± 0.11	3.44 ± 0.08	3.33 ± 0.17	3.28 ± 0.03	3.36 ± 0.18	0.238
	37–72 days	2.49 ± 0.17	2.74 ± 0.09	2.73 ± 0.05	2.66 ± 0.06	2.59 ± 0.13	0.082
	72–105 days	2.02 ± 0.02	2.01 ± 0.12	1.96 ± 0.05	1.93 ± 0.18	1.82 ± 0.12	0.259
	0–105 days	2.72 ± 0.07	2.90 ± 0.09	2.84 ± 0.07	2.79 ± 0.05	2.76 ± 0.08	0.087

Different letters in the same row indicate significant differences for the ANOVA analysis ($p < 0.05$).

Table 4
Gilthead seabream VSI and HSI after a 15-week trial fed with calcifediol.

	Analysed calcifediol in feed (ppb)	< 2.0	99.7	167.4	298.6	825.4	ANOVA (*p* value)
VSI (%)	day 105	8.67 ± 0.91	8.02 ± 0.79	8.68 ± 1.33	8.48 ± 0.74	8.34 ± 1.16	0.359
HSI (%)	day 105	1.04 ± 0.27	1.11 ± 0.25	0.96 ± 0.16	0.97 ± 0.36	0.93 ± 0.16	0.287

Different letters in the same row indicate significant differences for the ANOVA analysis ($p < 0.05$).

Table 5
Gilthead seabream fillet and liver proximal composition after a 15-week trial fed with calcifediol.

Proximal composition (% dry weight)		Analysed calcifediol in feed (ppb)					ANOVA (*p* value)	*p* value regression	R^2
		< 2.0	99.7	167.4	298.6	825.4			
Whole-body	Lipids	31.8 ± 1.4^{a}	35.7 ± 1.6^{ab}	38.2 ± 1.1^{b}	37.7 ± 2.2^{b}	38.3 ± 0.3^{b}	0.010	0.001q	0.687
	Ash	4.5 ± 0.1	4.2 ± 0.2	5.0 ± 0.6	4.4 ± 0.7	5.2 ± 0.2	0.082	0.060 l	0.246
	Protein	58.6 ± 1.0^{b}	54.8 ± 1.7^{ab}	53.4 ± 1.8^{a}	53.6 ± 2.5^{a}	55.5 ± 0.9^{ab}	0.021	0.009q	0.542
Fillet	Lipids	15.3 ± 2.6	13.6 ± 2.6	14.6 ± 1.4	17.1 ± 6.2	12.6 ± 2.2	0.624	0.432 l	0.048
	Ash	5.7 ± 0.1^{ab}	5.4 ± 0.3^{a}	6.4 ± 0.1^{c}	6.4 ± 0.4^{c}	6.2 ± 0.1^{bc}	0.000	0.029q	0.445
	Protein	79.1 ± 2.1	81.5 ± 3.5	78.9 ± 1.3	77.6 ± 4.1	82.2 ± 4.9	0.481	0.350 l	0.067
Liver	Lipids	33.3 ± 0.9	35.4 ± 0.4	34.2 ± 0.5	36.3 ± 4.8	39.6 ± 4.0	0.142	0.007 l	0.435
	Ash	2.9 ± 0.1	2.9 ± 0.2	3.0 ± 0.2	2.7 ± 0.4	2.9 ± 0.1	0.668	0.588 l	0.023
	Protein	43.2 ± 1.4	41.2 ± 1.9	44.4 ± 1.4	42.3 ± 3.2	39.3 ± 2.4	0.122	0.043 l	0.28

Different letters in the same row indicate significant differences for the ANOVA analysis ($p < 0.05$). Regression models applied were l: lineal and q: quadratic, p values of the most significant results are shown here.

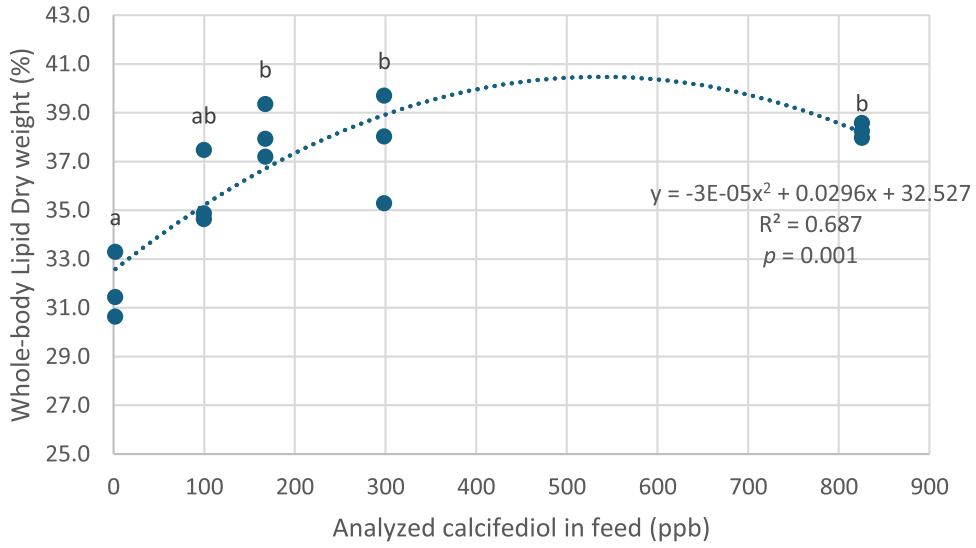

Fig. 1. Whole-body lipid composition (%) at the end of the 15-week trial. Line represents quadratic regression adjusted to the variable for the three replicas per treatment.

affected by the diets.

3.5. Prevalence of Skeletal Anomalies

20 fish per tank were x-rayed and evaluated for the presence of skeletal anomalies. At the end of the trial the main typologies observed were pre-haemal and haemal vertebrae lordosis and anomalous maxillary and/or pre-maxillary bones.

Skeletal anomalies were also classified according to the region being affected, being the cranium, pre-haemal and haemal vertebrae, those presenting the highest prevalence of anomalies. At the end of the trial the prevalence of anomalies ranged between 25.9 % and 33.0 %. However, no significant effects of the diets were observed on the prevalence of total anomalies, specific types of anomalies or regions (Table 8).

3.6. Histological evaluation

Intestinal folding patterns were mostly not significantly affected by the supplementation of calcifediol, except for the mid-intestine fold length, which were larger for fish fed the diets containing 99.7 and 167.4 ppb of calcifediol compared to those fed 825.4 ppb, whereas the other diets presented intermediate values (Table 9, Figs. 8, 9). This tendency followed a significant quadratic regression that showed that the fold achieved its maximum length in the intermediate diets compared to the lowest and highest levels of supplementation (Fig. 8). On the other hand, lamina propria width was significantly thinner in the mid-intestine of fish fed 99.7 ppb calcifediol than those fed the diet without supplementing, while the other diets presented intermediate values (Table 9).

Histological evaluation of the intestine showed a significant effect on the percentage area of the intestine occupied by goblet cells per fold (Fig. 10). Fish fed 167.4 ppb calcifediol presented a 50 % increase in the

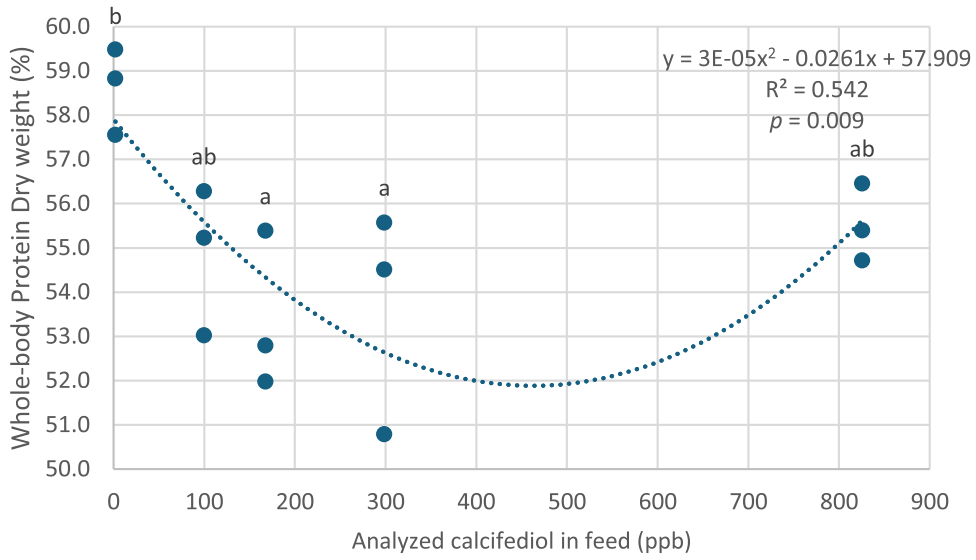

Fig. 2. Whole-body protein composition (%) at the end of the 15-week trial. Line represents quadratic regression adjusted to the variable for the three replicas per treatment.

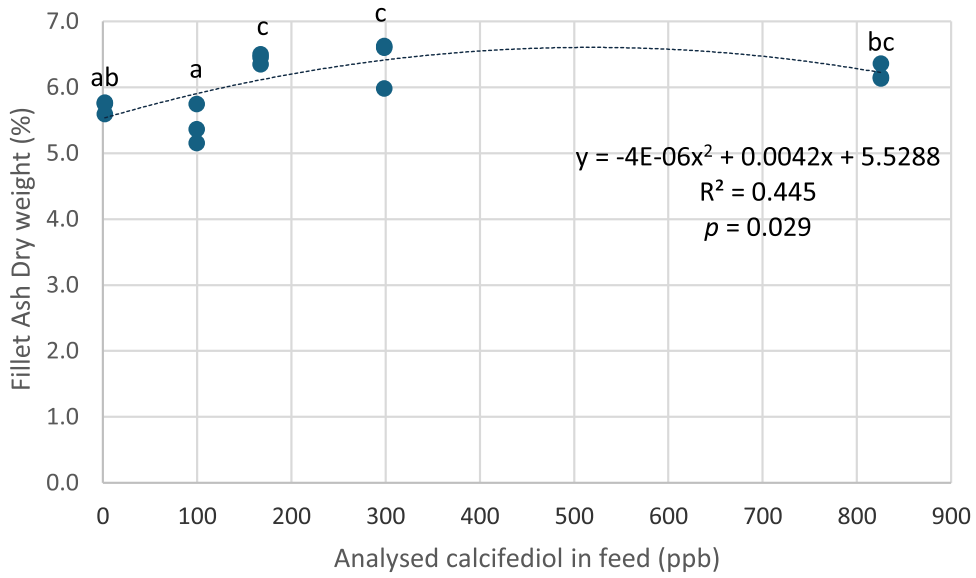

Fig. 3. Fillet ash composition (%) at the end of the 15-week trial. Line represents quadratic regression adjusted to the variable for the three replicas per treatment.

percentage of goblet cells per surface area in the anterior intestine compared to fish fed the diet without calcifediol supplementation, whereas the other diets presented intermediate values (Table 10). Posterior intestine observations showed an increase in the surface occupied by goblet cells with increasing calcifediol supplementation, being significantly higher in fish fed the diets containing 298.6 ppb and above compared to the diet without supplementation (Table 10).

4. Discussion

Vitamin D is an essential nutrient for fish. However, little attention has been paid to its requirements for marine warm water fish. In a recent study, 11.6 IU/g (290ppb) of vitamin D_3 appeared to reduce the incidence of skeletal anomalies, while further elevation of dietary vitamin D_3 levels increased the concentration of vitamin D_3 in liver while also increasing the prevalence skeletal anomalies in association to the up-regulation of *alp* and *bmp2* gene expression in gilthead seabream juveniles (Dominguez et al., 2021). Another study showed the sensitivity of gilthead seabream larvae to vitamin D_3 by demonstrating that supplementation levels of 25 and 30 ppb improved growth and bone mineralization rate, while further increase up to 384 ppb resulted in signs of toxicity such as hypercalcemia, hyperphosphatemia, and higher incidence of skeletal anomalies (Sivagurunathan et al., 2022). These results denote the differences between vitamin D_3 sensitivity in gilthead seabream at different development stages. Despite the recent advances in vitamin D nutrition in fish, the main source of the vitamin employed in aquaculture is vitamin D_3, whereas other sources of this vitamin have received little attention. In this sense, calcifediol is a conversion product of vitamin D_3 in the liver and has demonstrated its efficiency in other fish species recently as a useful and safe alternative to vitamin D_3 (Rider et al., 2023).

In the present study, feeding juveniles of gilthead seabream with a range of calcifediol levels between < 2.0 and 825.4 ppb for 15 weeks until fish had sextupled their weight, markedly altered gilthead seabream growth. In this sense, fish fed the diet containing 99.7 ppb calcifediol presented the highest final weight, whereas those fed the diet

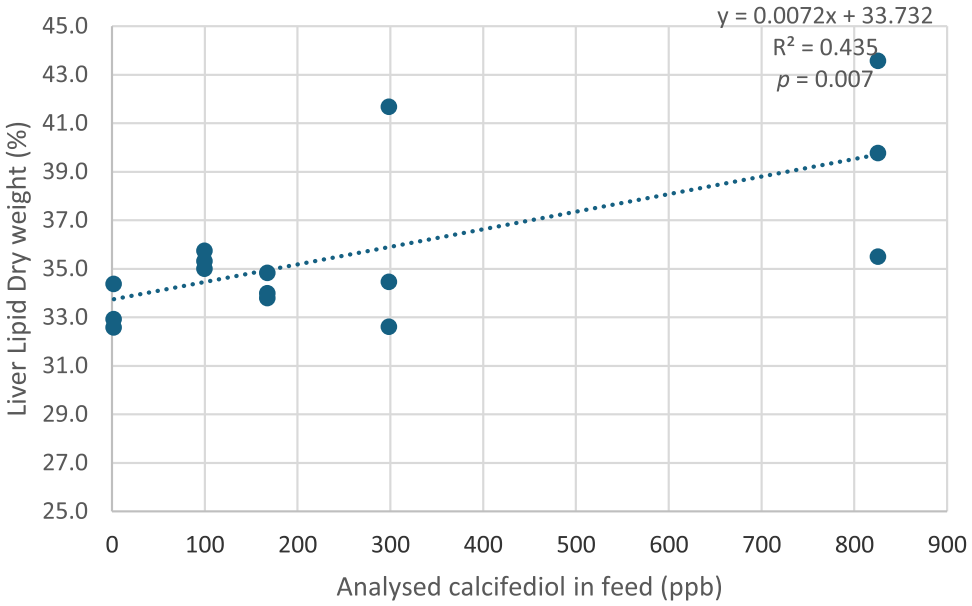

Fig. 4. Liver lipid composition (%) at the end of the 15-week trial. Line represents lineal regression adjusted to the variable for the three replicas per treatment.

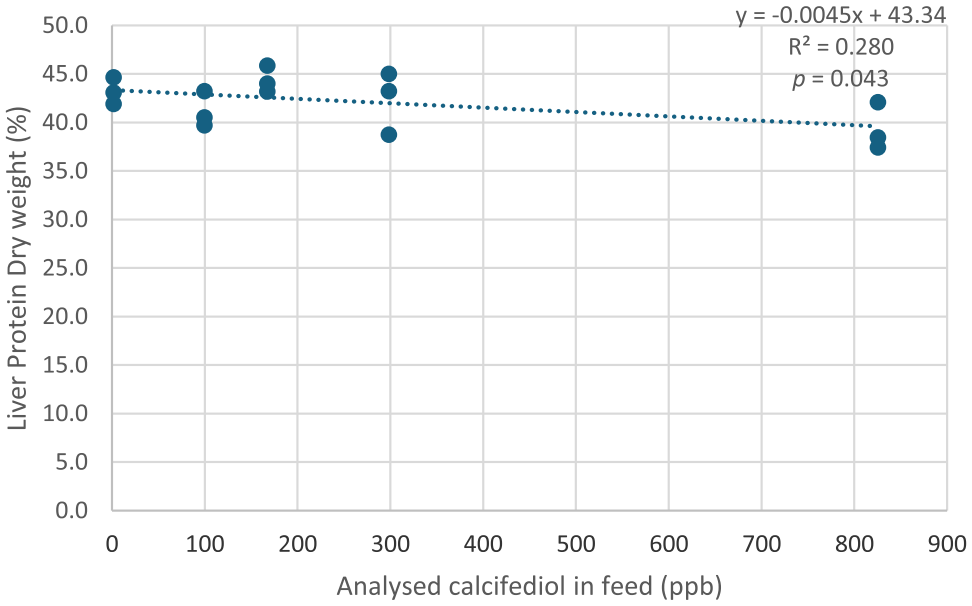

Fig. 5. Liver protein composition (%) at the end of the 15-week trial. Line represents lineal regression adjusted to the variable for the three replicas per treatment.

without supplementation and with levels at 298.6 ppb and beyond presented significantly lower weights. When compared to previous studies on the same species, and under similar conditions (Dominguez et al., 2021), the results show that calcifediol improves significantly the growth of this fish. In particular, this study showed effects of calcifediol already after 37 days of the trial, whereas the previous study didn't show any significant improvements in growth even after 70 days when seabream were fed vitamin D_3, suggesting that indeed calcifediol is a more efficient source of vitamin D than vitamin D_3 to improve growth in this species. When comparing to the only other studies conducted using calcifediol in fish (Rider et al., 2023, 2024, 2025), the results indicates that gilthead seabream is more sensitive to this compound than rainbow trout, since 686.7 μg/kg (ppb) calcifediol were needed to improve growth in this species of salmonid, compared to the 99.7 ppb in seabream, a mere 14.5 % of the supplementation required in trout. On the other hand, no negative effects were observed in growth on the trout fed the highest level of supplementation (6854 ppb), despite being a 23-fold increase in the maximum level of tolerable supplementation in seabream (298.6 ppb). This might be caused by differences in vitamin D metabolism of salmonids, as pointed out by these authors (Rider et al., 2023, 2025) and previous studies (Hilton and Ferguson, 1982; Graff et al., 2002; Lock et al., 2010). Despite the improvement in growth of the use of calcifediol in gilthead seabream, some findings suggest that an excess of this metabolite could be causing toxicity for this species. Indeed, the highest levels of calcifediol supplementation tested in this trial (>298.6 ppb) caused the fish to grow at a level on par with the level without supplementation, suggesting a possible toxicity of this compound.

Whole-body lipid and protein contents were significantly and inversely affected by the increase in calcifediol supplementation. A decrease in the lipid content coupled with an increase in protein content has already been observed in other trials where gilthead seabream juveniles presented reduced growth and could be a symptom of reduced

Table 6
Fatty acids composition of total lipids from fillet, liver and whole-body of gilthead seabream fed diets with different supplementation of calcifediol (% total identified fatty acids).= .

Tissue	Fatty acid	Analysed calcifediol in feed (ppb)					ANOVA (*p* value)
		2.0	99.7	167.4	298.6	825.4	
Whole-body	14:1n−7	0.026 ± 0.002^{a}	0.030 ± 0.004^{a}	0.032 ± 0.006^{ab}	0.034 ± 0.006^{ab}	0.044 ± 0.003^{b}	0.008
	14:1n−5	0.027 ± 0.004^{a}	0.034 ± 0.004^{ab}	0.033 ± 0.008^{ab}	0.034 ± 0.004^{ab}	0.046 ± 0.007^{b}	0.033
	15:1n−5	0.029 ± 0.002^{a}	0.033 ± 0.001^{ab}	0.031 ± 0.006^{a}	0.032 ± 0.003^{ab}	0.041 ± 0.003^{b}	0.017
	16:OISO	0.020 ± 0.003^{a}	0.024 ± 0.004^{ab}	0.023 ± 0.001^{ab}	0.023 ± 0.004^{ab}	0.029 ± 0.002^{b}	0.022
	20:1n−7	1.036 ± 0.051^{b}	0.872 ± 0.012^{a}	0.951 ± 0.053^{ab}	0.921 ± 0.079^{ab}	0.886 ± 0.028^{a}	0.016
	20:5n−3	3.418 ± 0.180	3.569 ± 0.082	3.415 ± 0.375	3.298 ± 0.248	3.171 ± 0.206	0.382
	22:6n−3	9.607 ± 0.739	9.896 ± 0.212	9.193 ± 0.805	9.416 ± 0.694	8.303 ± 0.252	0.068
	OLEIC/DHA	3.435 ± 0.345^{ab}	3.189 ± 0.090^{a}	3.518 ± 0.318^{ab}	3.287 ± 0.243^{ab}	3.902 ± 0.118^{b}	0.041
	OMEGA3/ OMEGA6	1.198 ± 0.070^{ab}	1.237 ± 0.011^{b}	1.154 ± 0.070^{ab}	1.178 ± 0.036^{ab}	1.079 ± 0.023^{a}	0.026
Fillet	18:2n−9	0.609 ± 0.033^{b}	0.518 ± 0.063^{ab}	0.508 ± 0.035^{ab}	0.506 ± 0.042^{ab}	0.465 ± 0.041^{a}	0.027
	20:5n−3	2.710 ± 0.889	3.441 ± 0.112	3.187 ± 0.226	3.009 ± 0.071	3.289 ± 0.147	0.319
	22:6n−3	7.603 ± 3.173	10.660 ± 0.762	9.429 ± 1.067	8.460 ± 0.263	10.125 ± 0.823	0.200
Liver	16:1n−5	0.092 ± 0.008^{ab}	0.099 ± 0.009^{b}	0.075 ± 0.012^{a}	0.095 ± 0.003^{ab}	0.086 ± 0.009^{ab}	0.044
	20:5n−3	2.883 ± 0.103	2.833 ± 0.328	2.841 ± 0.432	2.389 ± 0.086	2.623 ± 0.092	0.167
	22:6n−3	10.381 ± 1.311	11.971 ± 1.758	12.409 ± 2.382	9.692 ± 1.198	10.494 ± 1.369	0.292

Table 7
Vitamin D_3, calcium and phosphorus composition in gilthead seabream after a 15-week trial fed with calcifediol.

Tissue	Analysis	Initial	Analysed calcifediol in feed (ppb)					ANOVA (*p* value)	*p* value regression	R^2
			< 2.0	99.7	167.4	298.6	825.4			
Whole-body	Vitamin D_3 (µg/100 g)	< 1.00	< 1.00 ± 0.00	1.74 ± 0.79	2.06 ± 1.00	< 1.00 ± 0.00	4.39 ± 3.16	0.109	0.012 l	0.393
	Ca (mg/100 g)	1412	1191 ± 32	818 ± 169	1325 ± 341	834 ± 39	1198 ± 565	0.219	0.602q	0.081
	P (mg/100 g)	890	790 ± 62	790 ± 157	790 ± 79	883 ± 179	630 ± 10	0.193	0.057q	0.379
Vertebrae	Ca (mg/100 g)	3353	1601 ± 313^{ab}	3123 ± 82^{bc}	3574 ± 704^{c}	2412 ± 505^{abc}	1173 ± 1013^{a}	0.004	0.021q	0.475
	P (mg/100 g)	1858	1787 ± 556	1210 ± 98	1440 ± 131	1360 ± 438	1230 ± 260	0.311	0.220 l	0.113
Fillet	Ca (mg/100 g)	64.0	42.7 ± 10.1	44.3 ± 12.4	43.0 ± 9.9	99.3 ± 62.7	39.7 ± 9.1	0.138	0.137q	0.282
	P (mg/100 g)	2660	2867 ± 12	2700 ± 44	3063 ± 378	2920 ± 173	2850 ± 40	0.286	0.712q	0.055

Different letters in the same row indicate significant differences for the ANOVA analysis ($p < 0.05$). Regression models applied were l: lineal and q: quadratic, p values of the most significant results are shown here. Vitamin D_3 (µg/100 g) analysis presented a LOD of < 1.0 (µg/100 g), and as such, this value has been included in the table.

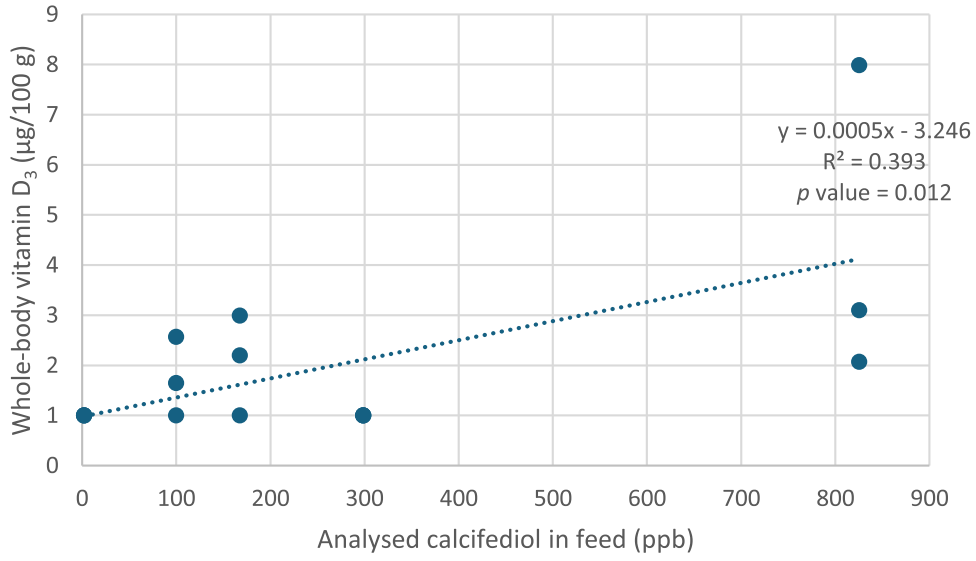

Fig. 6. Whole-body vitamin D3 content at the end of the 15-week trial. Line represents lineal regression adjusted to the variable for the three replicas per treatment.

overall growth (Vergara et al., 1999). Liver lipid content increased linearly with increasing calcifediol supplementation. Increased liver lipid content or liver steatosis has been found in gilthead seabream under suboptimal conditions, including thermal challenges (Ibarz, et al., 2007) and copper toxicity (Dominguez et al., 2019), thus possibly indicating a toxic effect of the highest levels of calcifediol supplementation.

Fatty acid composition was affected in whole-body, fillet, and liver, although the fatty acids affected were minor (presenting less than 1 % of

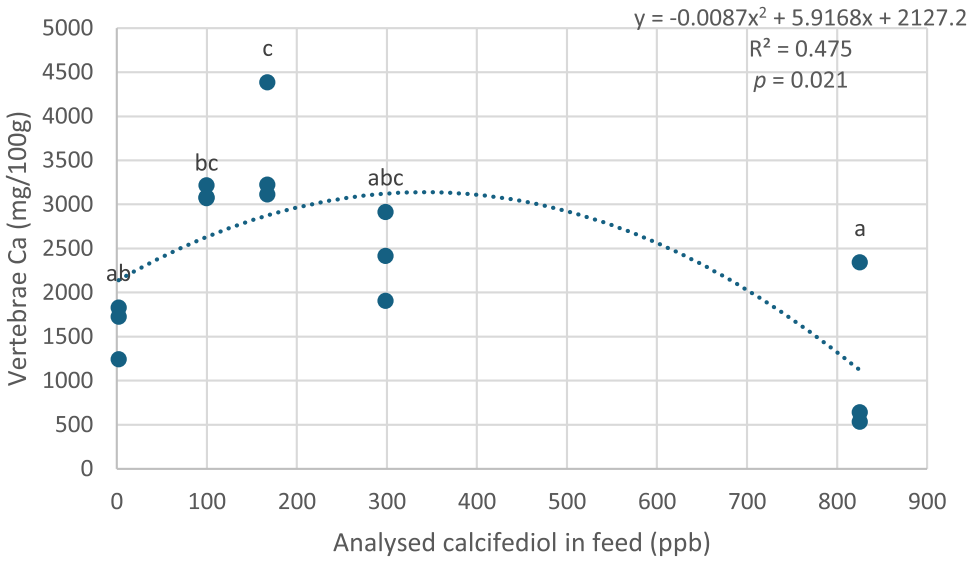

Fig. 7. Vertebrae calcium content at the end of the 15-week trial. Line represents quadratic regression adjusted to the variable for the three replicas per treatment.

Table 8
Prevalence of skeletal anomalies in gilthead seabream after a 15-week trial fed with calcifediol.

Anomalies Frequency (%)		Analysed calcifediol in feed (ppb)					ANOVA (*p* value)
		< 2.0	99.7	167.4	298.6	825.4	
Total Anomalies		33.0 ± 5.1	26.7 ± 2.9	25.9 ± 1.6	29.1 ± 9.6	26.7 ± 10.4	0.717
Typology	Pre-haemal lordosis	8.3 ± 10.4	6.7 ± 2.9	5.4 ± 5.6	11.9 ± 7.4	10.0 ± 8.7	0.831
	Haemal lordosis	12.6 ± 14.1	3.3 ± 2.9	6.9 ± 2.7	5.4 ± 5.6	8.3 ± 10.4	0.722
	Maxillary and/or premaxillary	10.6 ± 5.9	15.0 ± 8.7	15.6 ± 5.1	15.2 ± 9.7	15.0 ± 5.0	0.900
Region	Craneum	12.2 ± 6.3	16.7 ± 7.6	15.6 ± 5.1	15.2 ± 9.7	21.7 ± 12.6	0.751
	Pre-haemal Vertebrae	8.3 ± 10.4	8.3 ± 5.8	5.4 ± 5.6	11.9 ± 7.4	15.0 ± 15.0	0.762
	Haemal Vertebrae	15.9 ± 14.3	3.3 ± 2.9	8.5 ± 2.6	5.4 ± 5.6	8.3 ± 10.4	0.469

Different letters in the same row indicate significant differences for the ANOVA analysis ($p < 0.05$).

Table 9
Folding intestine patterns for gilthead seabream intestine after 15 weeks of calcifediol supplementation.

Intestine section	Measurement (μm)	Analysed calcifediol in feed (ppb)					ANOVA (*p* value)
		2	99.7	167.4	298.6	825.4	
Anterior Intestine	Fold length	1437 ± 161	1475 ± 290	1495 ± 286	1482 ± 147	1444 ± 150	0.918
	Fold width	190 ± 65	167 ± 26	174 ± 36	170 ± 25	164 ± 20	0.622
	Lamina propria width	59 ± 11^{b}	44 ± 10^{a}	48 ± 6^{ab}	47 ± 7^{ab}	55 ± 12^{ab}	0.021
Mid-intestine	Fold length	578 ± 59^{ab}	678 ± 45^{b}	672 ± 29^{b}	626 ± 45^{ab}	550 ± 8^{a}	0.012
	Fold width	146 ± 4	137 ± 18	155 ± 15	144 ± 19	131 ± 7	0.338
	Lamina propria width	58 ± 1	47 ± 5	45 ± 5	47 ± 3	52 ± 10	0.096
Posterior Intestine	Fold length	939 ± 166	1062 ± 166	1039 ± 202	1023 ± 96	883 ± 16	0.489
	Fold width	153 ± 8	159 ± 41	153 ± 19	150 ± 19	139 ± 7	0.871
	Lamina propria width	55 ± 5	51 ± 7	48 ± 6	48 ± 6	48 ± 16	0.902

the total identified fatty acids). Vitamin D affects lipid metabolism and fatty acid synthase (He et al., 2021, Lin et al., 2022). In several grouper species, the activities of enzymes such as LPL, were increased with optimum vitamin D supplementation levels, and authors suggested that these could be responsible for unsaturated fatty acid synthesis. The specific effects of calcifediol on lipid metabolism have been recently studied on Atlantic salmon, where its supplementation significantly reduced mesenteric adiposity, suggesting a redistribution of the lipids in the whole body, and an improved utilisation (Rider et al., 2024). In the present trial oleic/DHA and omega3/omega6 ratios improved with the diet containing 99.7 ppb calcifediol, which could be in line with these statements.

Calcifediol supplementation increases the deposition of whole-body vitamin D_3 content, suggesting its ability to spare the use of this vitamin for other metabolic uses or increasing vitamin D3 absorption. Vitamin D_3 is widely considered the main storage of vitamin D in fish (Lock et al., 2010). In this sense, seabream tends to deposit vitamin D_3 in several tissues when fed higher levels of supplementation of this vitamin, including liver (Dominguez et al., 2021) and whole-body (Sivagurunathan et al., 2022). However, the fact that despite feeding all the fish with the same level of vitamin D_3 tended to increase the retention for this vitamin when fed higher levels of calcifediol, appears to indicate that this metabolite is indeed sparing the vitamin D_3 present in the diet, and can therefore be used in other metabolic processes. Similarly, in rainbow trout dietary calcifediol increased plasma D_3 and calcitriol l (Rider et al., 2023).

Vertebral calcium content was also significantly affected by calcifediol supplementation. Vitamin D is involved in this mineral's absorption (Lock et al., 2010). Interestingly, the current results demonstrate higher calcium retention in fish fed the intermediate diets (99.7 and 167.4

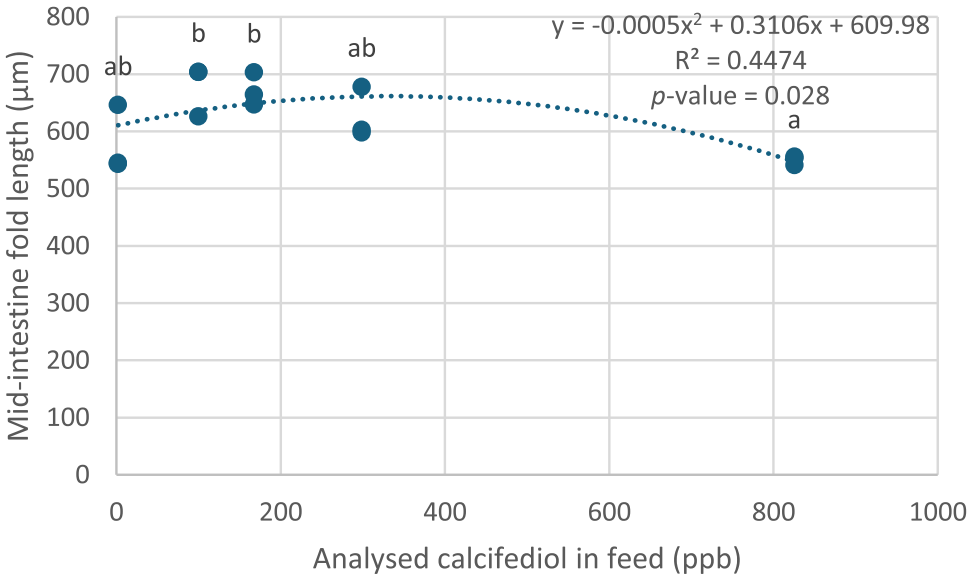

Fig. 8. Mid-intestine fold length of gilthead seabream fed calcifediol for 15 weeks.

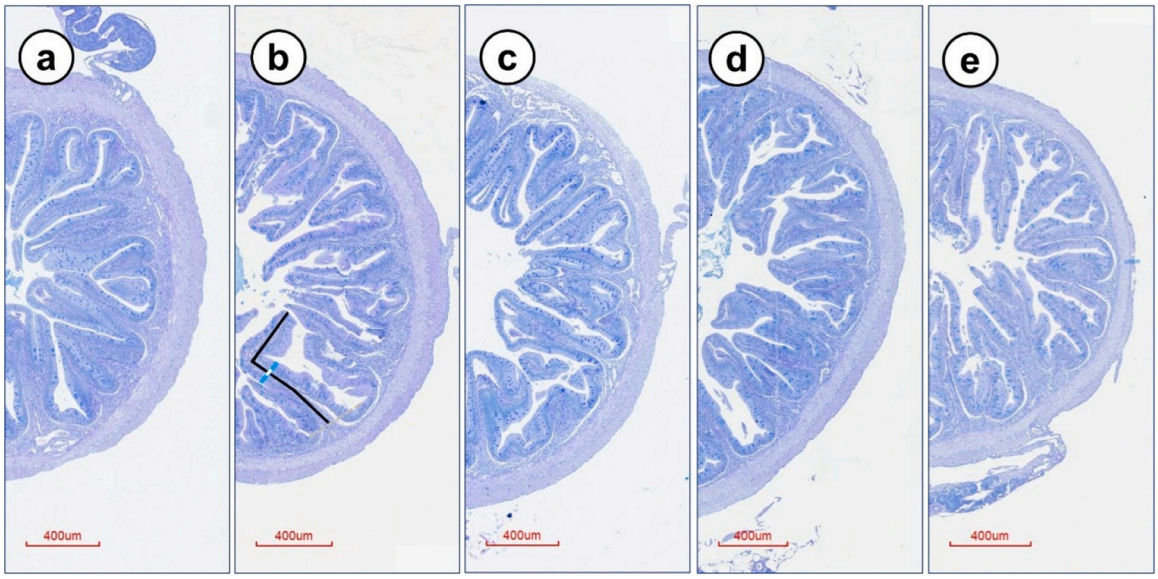

Fig. 9. Representative sections of mid-intestinal mucosal fold patterns in gilthead seabream after 15 weeks of calcifediol supplementation (ppb). a: < 2.0; b: 99.7; c: 167.4; d: 298.6; e: 825.4. Scale bar 400x. Lines in b indicate an example of length (black).

ppb), whereas fish fed the diet without or with the highest level of supplementation (825.4 ppb) presented the lowest retention of calcium. In mammals with cases of Vitamin D_3 exposure, calcifediol production continues to increase, leading to hypercalcemia (Plumlee, 2004). Vitamin D_3 metabolites increase plasma calcium by increasing the amount of calbindin (an intestinal calcium-binding protein). The amount of calcium absorbed is directly related to the amount of calbindin in the enterocytes. However, vitamin D_3 metabolites also stimulate calcium transfer from bone to plasma (Plumlee, 2004) and may result in fragile bones and poor growth (Halver, 2003), which can explain the reduction in the calcium level in vertebrae of fish fed the highest level of calcifediol in the present trial. In other cases however, this relation between vitamin D and plasmatic calcium was not observed, denoting physiological differences between fish species, such as those described in salmonids (Rider et al., 2023), denoting the importance of studying the species-specific mechanisms of vitamin D metabolism.

Despite the role of the D vitamers in calcium and phosphorus regulation, there were no significant effects on the prevalence of skeletal anomalies in the present trial. Fish tend to be more sensitive to present skeletal anomalies during earlier developmental stages, particularly during embryonic and post-embryonic periods, and are caused by biotic and abiotic factors (Georgakopoulou et al., 2007; Kourkouta et al., 2021; Sfakianakis et al., 2006; Villeneuve et al., 2005, 2006). The results from the present trial are contrary to those observed in previous studies with juvenile (Dominguez et al., 2021) and larvae (Sivagurunathan et al., 2022) gilthead seabream, where the highest levels of vitamin D_3 supplementation (300 and 384 ppb respectively) caused an increase in the total prevalence of skeletal anomalies and up-regulation of several bone-related molecular markers. However, these symptoms were never severe enough to cause an increase in mortality. When compared to the gilthead seabream larvae trial (Sivagurunathan et al., 2022), these symptoms did in fact cause an increase in mortality when supplementation levels of vitamin D_3 reached 384 ppb. This indicates that, even

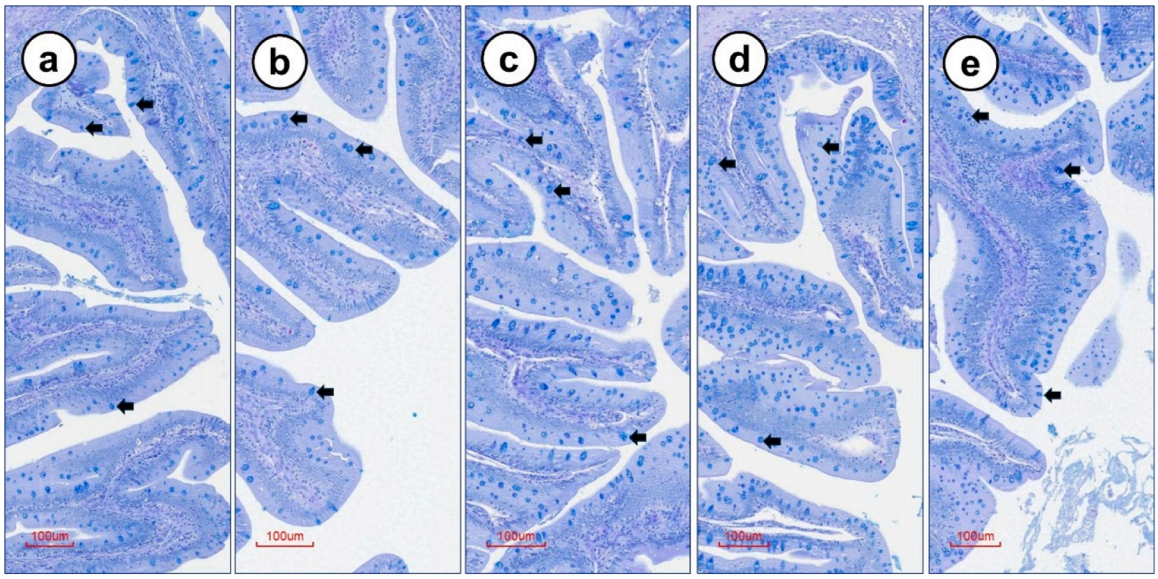

Fig. 10. Representative section of goblet cell occurrence in the intestinal folds (posterior intestine) of gilthead seabream after a 15-week calcifediol feeding trial (ppb). a: < 2.0; b: 99.7; c: 167.4; d: 298.6; e: 825.4. Scale bar 100x. Black arrows indicate.

Table 10
Percentage area occupied by goblet cells per intestinal fold in gilthead seabream after a 15-week trial fed with calcifediol.

Area occupied by goblet cells per intestinal fold (%)	Analysed calcifediol in feed (ppb)					ANOVA (*p* value)
	< 2.0	99.7	167.4	298.6	825.4	
Anterior Intestine	1.62 ± 0.24^{a}	2.16 ± 0.25^{ab}	2.43 ± 0.15^{b}	2.22 ± 0.15^{ab}	1.87 ± 0.35^{ab}	0.017
Posterior Intestine	1.28 ± 0.14^{a}	1.67 ± 0.10^{ab}	2.55 ± 0.46^{abc}	2.94 ± 0.20^{bc}	3.26 ± 0.98^{c}	0.003

though seabream appear to perform better with calcifediol than vitamin D_3, the safety margin of supplementation beyond which negative effects can be observed is similar to the other studies (<300 ppb). This has also been observed in other fish species, namely salmonids, where excess levels of dietary vitamin D_3 does not affect skeletal anomalies or mortality in brook trout (*Salvelinus fontinalis*) (Poston, 1969), while affecting other species such as flounders (Haga et al., 2004). This could be caused once again by the mentioned tolerance of salmonids to high doses of vitamin D (Hilton and Ferguson, 1982; Graff et al., 2002; Lock et al., 2010; Rider et al., 2023). This lack of negative effect on skeletal anomalies when fish are fed high levels of vitamin D has also been observed in other species (Cheng et al., 2023), and it could be another example of lower toxicity of calcifediol for seabream, compared to vitamin D_3.

Histological evaluation of the intestinal morphometry of seabream showed significant differences in the length of intestinal folds in the mid-intestine. The absence of significant differences in the length of intestinal folds of individuals fed different levels of calcifediol in the other intestinal segments can be explained by the fact that calcifediol is liposoluble, meaning that it is absorbed together with dietary lipids. Dietary fats and fat-soluble vitamins are absorbed and transported in chylomicrons, the main lipoproteins synthesized by the intestine, through the blood to the liver (Haddad, 1995). The epithelial cells of the mid-intestine possess specialized mechanisms for the absorption of fats and fat-soluble vitamins. Indeed, in a study on lipid absorption in the different segments of the gastrointestinal tract of Atlantic salmon, the results showed that lipid absorption is not uniform throughout the gastrointestinal tract of Atlantic salmon and that most of the lipid absorption takes place in the mid-intestine. The main location of lipid absorption in the mid-intestine is consistent with observations in other teleost fish species (Denstadli et al., 2004). Thus, vitamin D absorption might take place mainly in the mid-intestine, as suggested by the presence of significant differences only in the mid-intestine segment. Intestinal folds width can be affected by some ingredients, such as MOS (mannan-oligosaccharides), as shown by Torrecillas et al., (2011). The height, width and surface of the folds of the anterior intestinal mucosa of seabass (*Dicentrarchus labrax*) increased with MOS supplementation and the posterior intestine had shorter but wider folds than those of the control group, resulting in an increase in the total absorption surface of the intestine. An increase in the intestinal absorption surface is beneficial for the fish (Cerezuela et al., 2012). Another possibility that can explain the effect on the intestinal morphology is the role of vitamin D cell proliferation and differentiation (Samuel and Sitrin, 2008), however, this hypothesis is yet to be confirmed.

The width of the lamina propria allows to assess the health status of the intestinal segments. The lamina propria is a loose connective tissue underlying the epithelial tissue. In healthy conditions, this tissue is thin and elongated, but it undergoes morphological modifications when the gastrointestinal tract is agitated, including the widening of the lamina propria (Baeverfjord and Krogdahl, 1996). In the present study, significant differences were observed in the width of the lamina propria of the anterior intestine between a diet without calcifediol supply (<2 ppb) and a thinner lamina propria when fed a diet with a dose of 99.7 ppb. It is therefore appropriate to suggest that calcifediol has an effect on the gastrointestinal tract and that this effect resembles an improvement in gut morphology via the lamina propria. In a study by Sun et al. (2024), vitamin D_3 supplementation in grass carp reduced edema of the lamina propria compared to the vitamin D_3-deficient group in the middle and posterior segments. In a study on turbot (*Scophthalmus maxima*), shortened mucosal folds, disordered goblet cells, and a widened lamina propria were observed in the reference group. In contrast, the intestine of the groups supplemented with 1012.6 and 3978.2 IU/kg vitamin D_3 presented elongated mucosal folds, well-organized goblet cells, and a reduced thickness of the lamina propria, indicating an improvement in

intestinal morphology with optimal levels of vitamin D supplementation (Chen et al., 2022).

The area occupied by goblet cells per intestinal fold was significantly affected by calcifediol supplementation. This parameter is useful to confirm the previous observations and to ensure that variations in fold size do not affect the results. Goblet cells, present throughout the intestine, are responsible for the synthesis and secretion of the protective mucus layer that covers the epithelial surface. This mucus layer serves as a means of protection, lubrication, and transport between the luminal content and the epithelial lining, constituting an integral structural component of the intestine (Deplancke and Gaskins, 2001; Forstner, 1995). For this reason, the number of goblet cells in the posterior intestine, the segment in which the number of bacteria is highest (Jutfelt, 2011), can be affected by dietary modifications and show significant differences depending on the dose of vitamin D. Goblet cell presence can be similarly affected by other ingredients (Torrecillas et al., 2011). Indeed, the number of goblet cells secreting acidic mucins per surface unit, both in the anterior and posterior intestine, was increased by dietary supplementation with MOS (Torrecillas et al., 2011). Previous work has suggested that a wide range of luminal insults, including alterations of the normal microbiota, could lead to changes in the functions of goblet cells and in the chemical composition of intestinal mucus (Deplancke and Gaskins, 2001). These effects have been observed with the inclusion of probiotics and microalgae in the diets of seabream on histological alterations and microbial ecology of the gut (Cerezuela et al., 2012). The study assumes that the observed decrease in the total number of goblet cells could be due to the alteration of the epithelium and that a loss of goblet cells could lead to a reduced intestinal defense. One of the other conclusions is that a significant increase in the number of goblet cells could improve the protection of the intestinal mucosa against pathogens. Therefore, the significant differences observed in the present trial, indicating a higher number of goblet cells per fold in case of calcifediol supplementation at a certain level, suggesting a beneficial effect of calcifediol on intestinal physiology.

5. Conclusions

Calcifediol is an effective vitamin D source for gilthead seabream juveniles, with 99.7 ppb of supplementation improving growth, FCR, whole-body omega3/omega6 ratio, and vertebrae calcium retention, reducing anterior intestine lamina propria width, increasing mid-intestine fold length when compared to the diet without calcifediol supplementation, proving the essentiality of the vitamin for seabream. On the other hand, the highest levels of supplementation caused a reduction in growth, vertebrae calcium, mid-intestine fold length, evidence a toxic effect of high levels of calcifediol in gilthead seabream juveniles.

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CRediT authorship contribution statement

David Dominguez: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Pedro Castro:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation. **Manon Marmonier:** Writing – original draft, Visualization, Formal analysis, Data curation. **Daniel Arana:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Paula Arriagada:** Writing – review & editing, Resources, Funding acquisition. **Daniel Montero:** Writing – review & editing, Resources, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Daniel Arana reports a relationship with Huvepharma NV that includes: employment. Paula Arriagada reports a relationship with Huvepharma NV that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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