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Research Article

Effects of Menadione Sodium Bisulphite (Vitamin K_3) Supplementation of the Diets Based on Plant Feed Ingredients on Growth and Bone Health of Gilthead Seabream (*Sparus aurata*) Fingerlings

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The nutritional profile of aquaculture feeds can be altered with substitution of fish-based ingredients, including the vitamin contents, ultimately affecting the vitamin supply to fish. Requirements for several vitamins have been described for early life stages of species including some as salmonids and carps, but optimum levels for gilthead seabream fingerlings have not been described for most vitamins. In order to evaluate the essentiality of vitamin K in gilthead seabream fingerlings, a basal diet with low levels of marine ingredients (FM 10% and FO 6%) designed to contain five supplementation levels of vitamin K_3 (0.1, 2.8, 6.3, 12.0, and 23.0 mg kg^{-1}) was tested for 70 days. Growth and productive parameters were monitored along the trial and samples for X-ray analyses, and bone and liver molecular markers, histology, and proximal composition were taken. At the end of the experiment, growth, *grp* expression, and the reduced prevalence of skeletal disorders suggested that dietary vitamin K_3 levels for gilthead seabream fingerlings should be above 12 mg kg^{-1} in diets containing a larger proportion of plant protein and vegetable oils.

1. Introduction

The essentiality of vitamins in fish nutrition is widely accepted, and their quantitative dietary requirements are known for certain fish species [1]. Information on quantitative requirement and bioavailability of most vitamins for commercially farmed marine gilthead seabream (*Sparus aurata*) are limited [2]. In recent years, the availability of finite quantities of fish meal (FM) and fish oil (FO) of marine origin has resulted in the wider use of plant proteins and oils in fish feed. In this sense, some of the most widely used alternative oils in fish feed contain significantly higher levels of VK than traditional marine ingredients (0.01–1.0 mg kg^{-1} wet weight); these include soybean (2.7 mg kg^{-1}) and canola oil (1.1 mg kg^{-1}), among others [3–5]. However, it is still a common practice in the feed industry for gilthead seabream to supplement these feeds with vitamin K. A need for bioavailability of micronutrients from feed ingredients of plant origin as well as assessment of micronutrient supplements to improve growth, development, and health of fish is widely recognized. Studies on the pathogenesis of skeletal disorders in marine fish and salmonids have shown a link between nutritional status of vitamin K in skeletal metabolism [6–9].

Vitamin K is a fat-soluble vitamin that occurs in two natural active forms: (1) K_1 (phylloquinones; 2-methyl-3-phytyl-1,4-naphthoquinone), mainly found in green leafy

vegetables and vegetable oils and (2) K_2 (menaquinones; 2-methyl-1,4-naphthoquinones), synthesized by intestinal bacteria. Apart from natural sources, the most common source of vitamin K employed as a supplement in feeds is the synthetic derivative menadione or vitamin K_3, either as menadione sodium bisulphate (MSB), its complex (MSBC), or menadione nicotinamide bisulphite (MNB) [10]. However, the supplementation of VK in fish feeds' premixes can cause significant losses after feed processing or storage, which can account to a reduction of analyzed menadione of up to 50-90% in the final feed [9, 11].

In order to be biologically active for tissue metabolism, vitamin K_3 must be converted to menaquinone-4 (MK-4) [12–15]. This vitamin is an essential cofactor in vitamin K-dependent protein carboxylation [16]. The vitamin K-dependent enzyme γ-glutamyl carboxylase uses the oxygenation of vitamin K to convert glutamyl residues to carboxylated glutamyl residues in vitamin K-dependent proteins (VKDPs), including clotting factors (factors II, VII, IX, and X), anticoagulants (proteins C, S, and Z), and proteins involved in bone mineralization such as osteocalcin and Matrix Gla protein (Rishkavy and [17]). The gamma carboxylation of VKDP is required for these proteins to bind to Ca^{2+} and regulates the blood clotting process and mineralization of bone and cartilaginous tissues [16, 18–20].

Vitamin K deficiency can derive in altered blood clotting, thus increasing clotting time, haemorrhages, anaemia, and also skeletal anomalies [9, 21, 22]. Alteration in the vitamin K-dependent (VKD) activities or degree of VKD protein carboxylation has been used as markers for suboptimal vitamin K status [10]. To date, the vitamin K requirements of the following fish species have been reported: Atlantic cod (*Gadus morhua*) 0.2 mg kg^{-1} [23], Atlantic salmon (*Salmo salar*) 0.1 mg kg^{-1} [13], Jian carp (*Cyprinus carpio* var. Jian) 3.13 mg kg^{-1} [24], lake trout (*Salvelinus namaycush*) 0.5-1.0 mg kg^{-1} [25], and large yellow croaker (*Pseudos ciaena crocea*) < 3.45 mg kg^{-1} [26]. High dietary levels of MSB (2.0-2.4 g kg^{-1}) had no negative effects on growth, survival, and blood clotting of brook (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) [25, 27]. Amago salmon (*Oncorhynchus rhodurus*) fed a much lower level of menadione (40 mg kg^{-1} of diet) also showed no toxicity symptoms [28]. However, Atlantic salmon fed a diet supplemented with 30 mg kg^{-1} of MSB over 28 weeks showed reduced growth and increased mortality as compared to fish fed an equivalent quantity of vitamin K_1 [29]. Thus, further research is needed to ascertain the bioavailability, stability during processing and storage, and toxicity of these compounds over prolonged periods of feeding.

Since the levels of vitamin K present in the feeds are altered with the current trend to substitute marine ingredients with plant-based oils, we hypothesize that the levels present in the diet for gilthead seabream do not contain enough vitamin K to cover requirements, and that further supplementation to the feeds is needed. The present study was undertaken to determine the optimum amount of vitamin K required in a diet based on a high proportion of plant feed ingredients for gilthead seabream fingerlings for optimum growth and prevention of skeletal disorders.

Table 1: Composition of the basal diet.

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

†Proprietary composition Skretting ARC, vitamin and mineral supplementation as estimated to cover requirements according to NRC [1] except for vitamin K that was added separately.

2. Material and Methods

Experimental feeding trials involving vertebrate animals (gilthead seabream) were conducted at ECOAQUA Institute of University of Las Palmas de Gran Canaria (Canary Islands, Spain) in accordance to the European Union Directive (2010/63/EU) and Spanish legislation (Royal Decree 53/2013) concerning experiments with live animals.

2.1. Experimental Diets. Five practical plant-based diets with low levels of FM (10%) and FO (6%) containing increasing levels of vitamin K_3 (0.1, 2.8, 6.3, 12.0, and 23.0 mg kg^{-1}) were formulated (Table 1) following recommendations from a previous trial conducted in the same facilities with gilthead seabream juveniles (manuscript in preparation). The experimental diets were produced by Skretting Aquaculture Research Centre AS, Stavanger, Norway, by extrusion process and formulated to be isonitrogenous and isocaloric containing all known essential nutrients for gilthead seabream; thus, the energy (gross energy = 22 mJ/kg) and nitrogen composition were similar among diets. Menadione sodium bisulphite was used as vitamin K source and was supplied by Brother Enterprises Holding Company (Haining, China), while dietary levels were confirmed by vitamin K analysis [30].

2.2. Experimental Conditions and Fish. Fish were randomly distributed into 15 250 l conical tanks, so as to contain 30 gilthead seabream (*Sparus aurata*) fingerlings per tank. Initial weight was 20.3 ± 0.2 g, and fish were fed manually by an operator until apparent satiation three times on a daily basis under a natural photoperiod (12 h light) for 70 days. Triplicate groups were used for each experimental diet, adding up to a total of 90 fish per treatment. Feed intake was monitored daily, whereas growth and other productive parameters were tracked three times along the duration of the trial after being anesthetized with clove oil (Guinama S.L.U., Valencia, Spain). Water was supplied though a flow-through system, and the temperature (21.9 ± 0.2°C) and oxygen (>5.8 ppm) were checked daily in each tank with a multiparametric probe (Oxy Guard, Zeigler Bros,

Table 2: Sequences of primers used for gene expression analysis.

Gene	Nucleotide sequence (5′-3′)	Accession number
Beta-actin (*bact*)	F: TCTGTCTGGATCGGAGGCTC R: AAGCATTTGCGGTGGACG	X89920
Elongation factor 1α (*ef1a*)	F: CATGGTTGTGGAGCCCTTCT R: TCCTGCACGACCATTCATTTC	AF184170
Gla-rich protein (*grp*)	F: CGCGATGAGTCAGATGAGAG R: AAGGTGATGCTCCGAAGAGA	EU022752.1
Alkaline phosphatase (*alp*)	F: AGAACGCCCTGACGCTGCAA R: TTCAGTATACGAGCAGCCGTCAC	AY266359
Runt-related transcription factor 2 (*runx2*)	F: GCCTGTCGCCTTTAAGGTGGTTGC R: TCGTCGTTGCCCGCCATAGCTG	AJ619023
Osteocalcin (*oc*)	F: GGCAGCCATCTGTCTGACTT R: GGTCCGTAGTAGGCCGTGTA	AF048703
Bone morphogenic protein 2 (*bmp2*)	F: GTGGCTTCCATCGTATCAACATTTT R: GCTCCCCGCCATGAGT	JF261172.1

Gardners, USA). Once the 10-week trial had ended, all fish were fasted for 24 h, euthanized using ice, and then sampled for weight and length. 10 fish per tank were stored frozen (-20°C) for proximal composition analysis of whole body, whereas livers from 5 fish in each tank were taken for vitamin concentration analysis; vertebrae from 5 fish per tank were frozen in liquid nitrogen and kept at -80°C for further gene expression analyses of bone molecular markers; histological evaluation was conducted on liver samples from 5 fish per tank and which were stored in 10% buffered paraformaldehyde after sampling; the 10 remaining fish were stored frozen (-20°C), and X-rays were taken for osteological assessment of skeletal deformities.

2.3. Proximal Composition and Vitamin K Evaluation. Biochemical composition of diets and muscle was evaluated through standard procedures [31], where extraction by the chloroform:methanol method was employed to assess crude lipid [32] while ash composition was measured by combustion in a muffle furnace at 600°C for 12 h. On the other hand, the Kjeldahl method [31] was employed to evaluate the protein content ($N \times 6.25$), whereas dry matter content was determined by drying the sample until reaching constant weight in an oven at 105°C.

Vitamin K_1 (phylloquinone) content was analyzed in liver. Homogenized and pooled liver samples were submitted to enzymatic saponification, and the fat-soluble fraction was extracted with n-hexane. A reverse-phase HPLC with postcolumn reduction was used to quantify vitamin K_1 using fluorometric detection according to the European Norm EN 14148 from July 2003.

2.4. Gene Expression

2.4.1. RNA Extraction. 60 mg of vertebrae was used for total RNA extraction using TRI Reagent Solution (Life Technologies, Carlsbad, CA, USA) and purified on RNeasy Mini Spin Columns (Qiagen, Hilden, Germany). A Nanodrop 1000 (Thermo Fisher, Waltham, USA), and a 1.4% agarose electrophoresis was used to check RNA quality and quantity.

2.4.2. Reverse Transcription. Reverse transcription of 1 μg total RNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) was performed from each experimental sample. Briefly, 1 μg total RNA and nuclease-free water to a final volume of 15 μl was heated at 65°C for 10 min and cooled in ice. This was followed by adding a mix consisting of 4 μl of 5× iScript reaction mix and 1 μl of iScript reverse transcriptase, to end up with a total final reaction volume of 20 μl. The reaction mix was kept for 5 min at 25°C, 30 min at 42°C, and followed by 5 min at 85°C to inactivate reverse transcriptase. The reverse transcription reactions were diluted 1 : 10 for gene quantification.

2.4.3. Quantitative PCR. Real-time PCR for gene expression quantification using IQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) was conducted using a total of 2 μl of diluted cDNA in duplicates for the target and the housekeeping gene in a reaction volume of 20 μl. Housekeeping genes beta actin (*bact*) and elongation factor 1-alpha (*ef1α*) were used to normalize the expression of the target genes in vertebrae. Table 2 shows the nucleotide sequences of primers used in this study. Real-time quantitative PCR was performed using the iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR conditions included are as follows: 95°C for 3 min and 30 sec, followed by 40 cycles of 95°C for 15 sec, 58.1°C for 30 sec, 72°C for 30 sec, 95°C for 1 min, and a final denaturation step from 58 to 95°C for 10 sec. The $2^{-\Delta\Delta Ct}$ method was used in order to compare the relative changes in gene expression.

2.5. Skeletal Anomalies. Skeletal anomaly evaluation was conducted using X-ray analyses by 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)). Digital processing

Table 3: Performance of fingerling gilthead seabream fed diets containing different levels of vitamin K_3 for 70 days.

Performance	Dietary vitamin K_3 level (mg kg^{-1})†					p value linear regression	p value quadratic regression
	0.1	2.8	6.3	12.0	23.0		
Initial body weight (g)	20.1 ± 0.1	20.4 ± 0.1	20.3 ± 0.2	20.3 ± 0.0	20.4 ± 0.4	0.226	0.455
Final body weight (g)	50.5 ± 1.7	50.1 ± 3.7	50.4 ± 0.7	53.6 ± 1.7	51.5 ± 1.0	0.174	0.010
Survival (%)	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	96.7 ± 3.3	100.0 ± 0.0	0.512	0.191
Weight gain	151 ± 8	156 ± 17	147 ± 5	165 ± 9	153 ± 7	0.645	0.573
Specific growth rate	1.35 ± 0.05	1.38 ± 0.09	1.33 ± 0.03	1.43 ± 0.05	1.37 ± 0.04	0.629	0.571
Feed conversion ratio	1.07 ± 0.02	1.16 ± 0.15	1.09 ± 0.03	1.08 ± 0.02	1.09 ± 0.04	0.746	0.951

†Analyzed vitamin K_3 content of the diet.

was conducted on these X-rays by means of the Onis 2.4 software (DigitalCore Co. Ltd, Tokyo, Japan), and the classification and prevalence of skeletal deformities were conducted according to Boglione et al. [33]. Only severe skeletal anomalies were considered.

2.6. Histological Studies. During sampling, tissues were segmented to allow a faster penetration of alcohol in histology cassettes. A Histokinette 2000 (Leica, Nussloch, Germany) was used for sample dehydration by gradually increasing alcohol grades from 70° to 100° and ending with xylene and paraffin. 3 μm thick slices of the paraffin block were prepared using a Leica RM 2135 microtome (Leica, Nussloch, Germany) and fixed to a slide. Haematoxylin–eosin staining [34] was employed for optical evaluation by pair evaluators to determine signs of hepatopancreas damage, including signs of inflammation and presence of eosinophyls, accumulation of fat, and bile duct obstruction, using a 0-3 scale, where 0 was absence of observation and 3 was widespread presence in the tissue.

2.7. Statistical Analysis. Software SPSS v21 (IBM Corp., Chicago, IL, USA) was employed to statistically analyze all data, and means ± SD was calculated for every parameter measured, and results were presented to three significant figures. Kolmogorov–Smirnov test was used to test data were tested for normality. Quadratic and lineal regressions, as well as broken line analyses, were conducted where possible, selecting the analysis that presented a higher significance to describe the results. The error analysis presented in these figures revealed that the ratio lack-of-fit to pure error variances gives evidence to support the adequacy of the models described by the equations presented throughout the manuscript. Significant differences were considered for $p \leq 0.05$. Weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR) were calculated using the following formulae:

$$\text{Weight gain}\,(\%) = 100 \times \frac{(\text{final weight} - \text{initial weight})}{\text{initial weight}},$$
$$\text{SGR}\,(\%) = \frac{((\text{Ln}W_2 - \text{Ln}W_1))}{\text{days}} \times 100, \quad (1)$$

$$\text{FCR} = \frac{(\text{ingested food})}{(\text{generated biomass})}, \quad (2)$$

where W_1 is the initial body weight (g) and W_2 is the final body weight (g).

3. Results

3.1. Growth and Productive Parameters. No signs of feed rejection were observed in any experimental, and the only mortalities recorded were due to escapees. At the end of the trial period, fish fed the diet containing 12 mg kg^{-1} vitamin K_3 showed a significantly higher whole body weight than those fed diets containing lower vitamin K_3 levels, which was similar to that of fish fed 23 mg kg^{-1} vitamin K_3 (Table 3). Furthermore, a significant quadratic regression ($y = -0.0129x^2 + 0.3836x + 49.694$, $p = 0.01$, and $R^2 = 0.526$) was observed between final body weight and the dietary vitamin K_3 levels. When a broken-line model was applied to this parameter, the intersection appears at 13.4 mg vitamin K_3 kg^{-1}. As for other growth parameters including WG, SGR, and FCR, there were no significant differences among the different treatments (Table 3).

3.2. Muscle and Liver Composition and Vitamin K Analyses. At the end of the trial, muscle proximal composition was not affected by the level of vitamin K_3 supplementation in terms of protein, lipids, and ash contents (Table 4). On the other hand, liver vitamin K_1 concentration was not significantly affected by the increase in dietary vitamin K_3 dietary content, although there was a clear tendency to increase with increasing dietary vitamin K ($p = 0.058$).

3.3. Gene Expression. Liver gene expression analyses revealed that an increase in dietary vitamin K_3 caused an increase in the expression of *grp*, and when a broken-line model is used to calculate this variable, the intersection appears to be at 9.99 mg vitamin K_3 kg^{-1} (Figure 1). On the other hand, analyses from bone markers conducted in vertebrae showed that there were no effects of vitamin K_3 supplementation.

3.4. Skeletal Anomalies. Evaluation of skeletal anomalies showed that there were no significant effects of vitamin K_3 supplementation for most of the typologies described except for caudal vertebra anomalies, which followed a significant ($p = 0.01$) quadratic regression where 12 and 23 (mg kg^{-1}) vitamin K_3 supplementation reduced the incidence of this anomaly (Table 5).

Table 4: Lipid, protein, and ash content of muscle and liver K_1 (phylloquinone) contents of gilthead seabream fed different levels of vitamin K_3 for 70 days.

Analyses	Dietary vitamin K_3 level (mg kg^{-1}) †					p value linear regression	p value quadratic regression
	0.1	2.8	6.3	12.0	23.0		
Muscle lipids (% dry weight)	12.8 ± 0.6	14.1 ± 0.5	13.5 ± 0.7	13.4 ± 0.6	14.1 ± 0.9	0.210	0.453
Muscle ash (% dry weight)	5.3 ± 0.1	5.2 ± 0.2	5.4 ± 0.0	5.4 ± 0.0	5.3 ± 0.1	0.254	0.220
Muscle protein (% dry weight)	81.3 ± 0.8	80.5 ± 0.4	81.3 ± 0.4	80.8 ± 0.8	80.7 ± 0.2	0.357	0.661
Liver vitamin K_1 (mg/100 g)	0.33 ± 0.02	0.30 ± 0.01	0.33 ± 0.04	0.32 ± 0.05	0.37 ± 0.02	0.058	0.093

†Analyzed vitamin K_3 content of the diet.

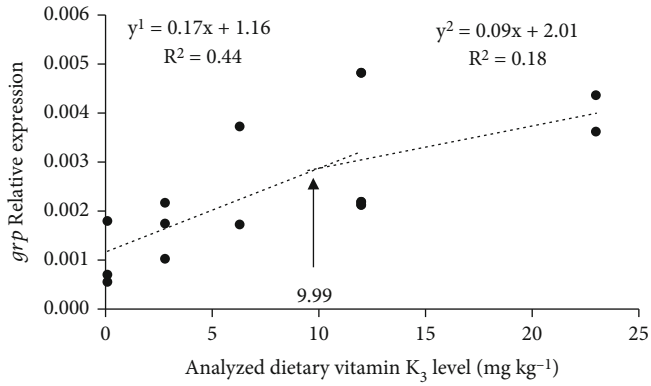

Figure 1: Liver gene expression analyses of gilthead seabream fed increasing levels of dietary vitamin K for 70 days.

Table 5: Prevalence of skeletal anomalies (%) in seabream fed increasing levels of dietary vitamin K_3 for 70 days.

Prevalence of skeletal anomalies (%)	Dietary vitamin K_3 level (mg kg^{-1})†					p value linear regression	p value quadratic regression
	0.1	2.8	6.3	12.0	23.0		
Total severe anomalies	35.2 ± 17.6	36.1 ± 12.7	26.3 ± 11.6	22.5 ± 3.5	21.7 ± 4.4	0.075	0.117
Prehaemal lordosis	19.4 ± 12.7	30.6 ± 21.0	23.5 ± 13.0	18.3 ± 2.4	15.6 ± 2.7	0.328	0.598
Prehaemal anomaly	0.0 ± 0.0	2.8 ± 4.8	2.8 ± 4.8	0.0 ± 0.0	0.0 ± 0.0	0.493	0.661
Haemal lordosis	3.7 ± 6.4	8.3 ± 8.3	0.0 ± 0.0	0.0 ± 0.0	6.1 ± 6.3	0.853	0.482
Haemal vertebral fusion	2.8 ± 4.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.335	0.312
Caudal vertebra anomaly	7.4 ± 1.6	5.6 ± 9.6	2.6 ± 4.4	0.0 ± 0.0	0.0 ± 0.0	0.065	0.010
Anomalous maxillary and/or pre-maxillary	1.9 ± 3.2	0.0 ± 0.0	5.1 ± 8.9	4.2 ± 5.9	4.2 ± 7.2	0.496	0.702

†Analyzed vitamin K_3 content of the diet.

3.5. Histological Studies. Histological analyses demonstrated that dietary vitamin K_3 did not alter liver morphology significantly, in particular liver steatosis (2.71 ± 0.28), microhaemorrhages (0.80 ± 0.42, Figure 2), congestion (1.67 ± 0.75, Figure 3), inflammatory infiltrate (0.58 ± 0.44), or obstruction of the bile ducts (1.26 ± 0.45). A similar trend was observed for pancreatic haemorrhages (1.33 ± 0.69), fat deposition (0.64 ± 0.19), presence of inflammatory infiltrate (0.39 ± 0.24), or melanomacrophages (1.25 ± 0.62).

4. Discussion

The results of this study show that 12 mg kg^{-1} vitamin K_3 was required to achieve maximum growth of gilthead seabream. Further increases in vitamin K_3 levels had no significant effect on growth. Studies on large yellow croaker fed 12.82 mg kg^{-1} of MSB also showed the highest growth response [26]. Other fish species seem to require lower levels of vitamin K to achieve optimum growth such as Atlantic salmon (0.1, mg vitamin K kg^{-1}, [13]). For Atlantic cod, Grahl-Madsen and Lie [23] indicated that fish presented higher body weights when fed 6.5 and 9.8 mg MSB kg^{-1}, while it is worth noting that they observed a tendency towards a reduction in growth in Atlantic cod fed 21.5 mg kg^{-1} of MSB for 23 weeks as compared to fish fed 9.8 mg kg^{-1} of MSB, but the authors stated that the short period of time did not allow this dietary group to test this response statistically; thus, it may be more realistic to

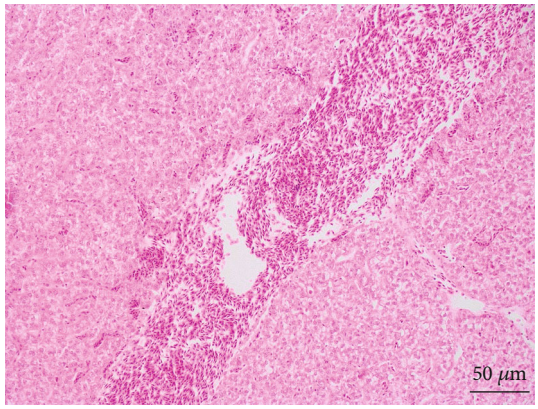

Figure 2: Microscopic view of liver of a gilthead seabream juvenile stained with H&E presenting microhaemorrhages (20x).

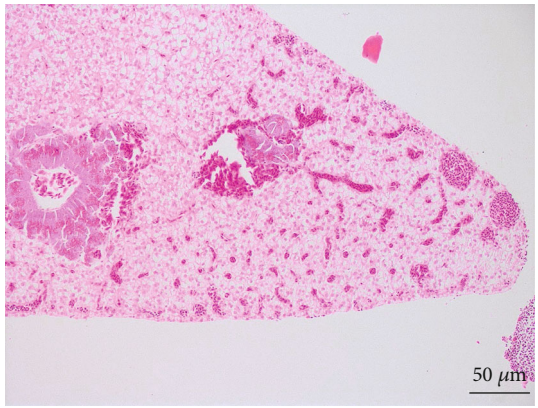

Figure 3: Microscopic view of liver of a gilthead seabream juvenile stained with H&E presenting congestion (20x).

describe a requirement for this species at the next higher level, 6.5 mg MSB kg^{-1}. This suggests a possible toxic effect of vitamin K, whereas similar results were obtained in the present trial, where seabream fed 23 mg vitamin K_3 kg^{-1} had slightly lower growth than fish fed 12 mg vitamin K_3 kg^{-1}. However, the relatively short experimental period (10 weeks) may have been one of the factors by which no adverse effects on growth of fish fed higher levels of this vitamin were observed. Krossøy et al. [13] did not find a significant effect of vitamin K on growth of Atlantic salmon in an experiment of a longer duration (28 weeks). These authors suggested a vitamin K requirement for Atlantic salmon of <10 mg kg^{-1} based on weight gain and other parameters such as mortality, blood coagulation time, and prevalence of bone deformities, instead of defining it at the lowest level of vitamin K in the diet. They indicated that approximately 0.1 mg kg^{-1} K_1 was enough for good growth, bone strength, and health of Atlantic salmon fry from the onset of start feeding. The matter of the substitution of marine oils by those of plant origins could be behind the results obtained in the present trial when compared to other trials. For example, the study conducted by Krossøy et al. [13] in Atlantic salmon used fish meal and fish oil, but also soyconcentrate and wheat, although the percentages of inclusion are not stated, whereas Grahl-Madsen and Lie [23] tested diets based on squid mantle (920 g kg^{-1}) and a very low level of sardine oil (1.8 g kg^{-1}) for cod. Thus, the low level of marine fish oil in the study by Grahl-Madsen and Lie [23] could be the reason behind the improvement in growth for those fish fed diets supplemented with vitamin K, as is the case in the present trial.

Vitamin K_3 supplementation did not have a significant effect on muscle proximate composition; however, liver vitamin K_1 content tended to increase with increasing vitamin K_3 concentration in the diet. This effect has been observed in other fish species, such as Atlantic cod [23] and large yellow croaker [25], while studies conducted with Atlantic salmon demonstrated that there was a similar relation between dietary vitamin K and its accumulation in whole fish [13]. Interestingly, in Atlantic salmon fed a multinutrient package containing increasing levels of several vitamins, minerals, amino acids, and cholesterol, whole body vitamin K_1 accumulation remained unaltered, whereas the level of MK_4 increased with vitamin K_3 levels [35]. In a similar study, Taylor et al. [36] found no differences in liver MK_4 accumulation in diploid or triploid salmon, whereas an effect of the ploidy of these fish on MK_4 liver accumulation could be observed. In the present trial, the maximum level of vitamin K_3 in the diet was 23 mg kg^{-1}, whereas the maximum accumulation level achieved in liver described in the previously discussed trials was found at 9.8 mg vitamin K kg^{-1} [23] and 12.82 mg vitamin K kg^{-1} [26], while the whole body maximum vitamin K accumulation level was found beyond 50 mg MNB kg^{-1} feed [13]. It is worth noting that the only vitamin that could be analyzed in liver in the present trial was K_1, the major circulating form of vitamin K, which is also excreted at a far slower rate than K_3 [37]. On the other hand, menaquinone-4 was measured in Atlantic cod [23], Atlantic salmon [13, 36], and large yellow croaker [26]; thus, it would be of interest to analyze the effects of vitamin K supplementation in seabream on the rates of retention of the different forms of vitamin K in the future.

Dietary vitamin K_3 supplementation had a significant effect on molecular markers such as Gla-rich protein, a member VKDP family [18, 38]. Gla-containing proteins are known to occur in several tissues including bone, kidney, and pancreas [17, 39, 40]. In the present trial, this parameter was closely related to the dietary vitamin K_3 level in the experimental diets and demonstrated the essentiality of this vitamin for proper function of this molecular marker.

Vitamin K functions as a coenzyme for vitamin K-dependent carboxylase, which is required for the synthesis of proteins involved in bone metabolism such as osteocalcin [41]. Nevertheless, no effects of vitamin K_3 supplementation were found on gene expression of vertebrae from gilthead seabream. In accordance with these results, no clear effects of vitamin K_3 were observed on the prevalence of skeletal disorders except for those affecting the caudal vertebrae, which showed a decrease with increasing levels of dietary vitamin K_3. In any case, there is still no standard recommendation on the quantity of vitamin K required for optimal bone health; however, there are reports that indicate its essentiality in reducing the prevalence of skeletal anomalies in fish species [9, 21, 39, 42], while other studies showed

no significant effect on bone markers such as stiffness [13]. Results from osteological evaluations show that there is a slight tendency to improve bone health with vitamin K_3 supplementation at $12\,mg\,kg^{-1}$; however, further research is needed to confirm this observation.

Vitamin K is mainly absorbed through the intestine and transported by chylomicrons to the liver, which is considered to be the main storage organ for this vitamin [40]. This tissue is also the main site of gamma-carboxylation of vitamin K-dependent clotting factors, and inadequate vitamin K supplementation may lead to liver disorders causing cholestasis [40]. The liver of gilthead seabream consists of pancreatic acinus scattered through the tissue, forming a hepatopancreas. Vitamin K is also tightly associated to blood coagulation by playing a key role in the coagulation cascade (Rishkavy and [17]); thus, any symptom of altered blood clotting (such as microhaemorrhages or congestion) should have been observed in the liver. However, the morphology and status of this tissue, as analyzed from a histological point of view, showed no alterations regardless of dietary level of vitamin K_3, thus confirming the lack of effect of vitamin K supplementation for this function.

In summary, growth, *grp* expression, and the reduced prevalence of skeletal disorders suggest that dietary vitamin K_3 levels for gilthead seabream fingerlings should be above $12\,mg\,kg^{-1}$ in diets based on the major proportion of plant protein and vegetable oils.

Data Availability

The majority of data used to support the findings of this study are included within the article. The histological analysis data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

This output reflects the views only of the authors, and the European Union cannot be held responsible for any use which may be made of the information contained therein.

Conflicts of Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: R. Fontanillas is an employee of Skretting AS, Stavanger, Norway.

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