UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Modulation of bone metabolism and mineralization in gilthead seabream (*Sparus aurata*) fed various vegetable oil sources

Carmen Lucía Barrios Guzmán

Dissertação apresentada para a obtenção do grau de Mestre em Aquacultura e Pescas com especialização em Aquacultura

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Sob a supervisão de: Dr. Jorge Proença Dias

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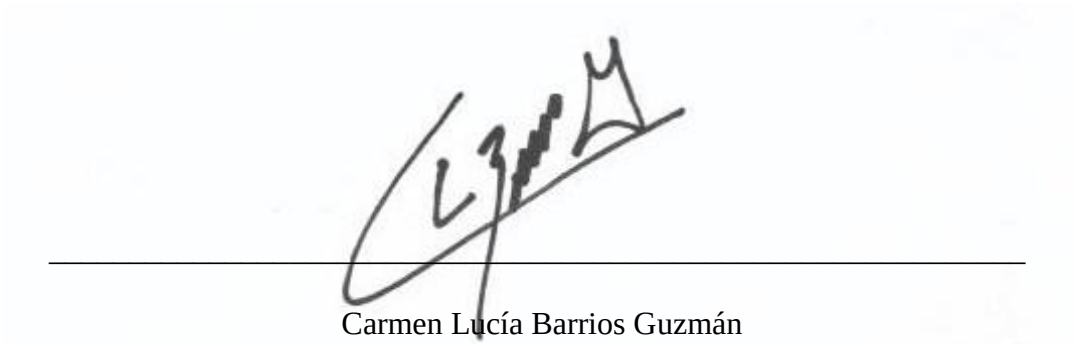
Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da lista de referências incluída.



Carmen Lucía Barrios Guzmán

Dedicatoria

A:

Dios, por darme la oportunidad de vivir y por estar conmigo en cada paso que doy, por fortalecer mi corazón e iluminar mi mente y por haber puesto en mi camino a aquellas personas que han sido mi soporte y compañía durante todo este periodo.

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Resumo

Este estudo visa avaliar o efeito de fontes de lípidos na dieta como elementos moduladores da composição óssea em ácidos gordos, da mineralização e metabolismo ósseo em juvenis de dourada. Doze grupos homogêneos de 25 juvenis de dourada (peso inicial médio de 39.3 ± 1.4 g) foram colocados em tanques de 90 L e alimentados durante 46 dias com uma de quatro dietas experimentais. Estas dietas experimentais eram isoprotéicas (proteína bruta, 46% de MS), isolipídicas (15% MS) e isoenergéticas, mas com fontes de óleos variáveis. As fontes de lípidos testadas foram: a) óleo de fígado de bacalhau (FO, uma fonte de n-3 LC-PUFA), b) óleo de soja (SBO, uma fonte de de ácidos gordos n-6), c) óleo de linhaça (LSO, uma fonte de ácidos gordos da série n-3, mas não LC-PUFA), e d) óleo de palma (PO, uma fonte de ácidos gordos saturados). Critérios associados ao crescimento em peso e à composição proximal do peixe inteiro não foram significativamente afetadas pelas dietas experimentais ($P>0,05$). A composição de ácidos gordos do osso foi alterada pelo perfil de ácidos gordos da dieta, mas sugere a existência de mecanismos de conservaçãos de alguns ácidos gordos específicos, nomeadamente do DHA. Embora o teor de cálcio do osso tenha sido pouco afetado pelos tratamentos alimentares, o nível de fósforo no osso de douradas alimentadas com a dieta de óleo de fígado de bacalhau foi significativamente superior ($P<0,05$) do que aqueles encontrados nos peixes alimentados com as dietas de óleo vegetal. A observação microscópica de tetraciclina marcado nas vértebras mostraram que a deposição mineral em vértebras de peixes alimentados com a dieta LSO foi significativamente menor ($P<0,05$) do que a encontrada nos peixes alimentados com dietas FO e SBO. Actividade da fosfatase ácida resistente ao tartarato (TRAP) em homogeneizado de osso foi positivamente correlacionada ($r = 0,658$) com ácido α-linolénico (ALA, 18:3 n-3), ao passo que um aumento da ingestão de EPA ($r = -0,478$) e DHA ($r = -0,536$) teve tendência a reduzir a atividade TRAP. Atividade de fosfatase alcalina (ALP) em homogeneizados de osso foi positivamente correlacionada com a ingestão de EPA ($r = 0,763$) e DHA ($r = 0,793$). Em comparação com os peixes alimentados com a FO e LSO dietas, aqueles alimentados com as dietas SBO e PO mostrou uma redução significativa ($P <0,05$) do total de fosfolipase A2 (PLA2) atividade em homogeneizados ósseos. No entanto, estas alterações não foram claramente associadas à actividade da PLA2 citosólica. Os resultados deste trabalho, confirmam que os lípidos alimentares desempenham um papel importante na modulação do metabolismo ósseo da dourada.

Palavras-chave: dourada, óleos alimentares, metabolismo ósseo, deposição mineral

Abstract

Little information exists on the role of dietary lipids on the bone metabolism in fish. A trial was undertaken to evaluate the effect of dietary lipid sources as modulators of bone fatty acid composition, mineralization and skeletal metabolism in fast-growing seabream juveniles. Twelve homogenous groups of 25 seabream juveniles each (mean initial body weight of 39.3 ± 1.4 g) were stocked in 90 L tanks and fed one of four semi-purified experimental diets formulated to be isonitrogenous (crude protein, 46% DM), isolipidic (15% DM) and isoenergetic, but with variable oils sources. The dietary lipid sources under testing were: a) cod liver oil (FO, a source of n-3 LC-PUFA); b) soybean oil (SBO, a source of n-6 fatty acids); c) linseed oil (LSO, a source of non-LC-PUFA n-3 fatty acids); and d) palm oil (PO, a source of saturated fatty acids). Overall growth performance criteria and whole-body composition of fish were not significantly affected by the various dietary experimental treatments ($P>0.05$). Bone fatty acid composition was altered by the dietary fatty acid profile, but suggests the existence of conservative mechanisms for some specific fatty acids, namely DHA. While the calcium content of bone was little affected by the dietary treatments, bone phosphorus level in seabream fed the cod liver oil diet was significantly higher ($P<0.05$) than those found in fish fed the vegetable oil diets. Microscopic observation of tetracycline-marked vertebrae showed that mineral deposition in vertebrae of fish fed the LSO diet was significantly lower ($P<0.05$) than that found in fish fed the FO and SBO diets. Tartrate-resistant acid phosphatase (TRAP) activity in bone homogenate was positively correlated ($r = 0.658$) with α-linolenic acid (ALA, 18:3n-3) intake, while an increased intake of EPA ($r = -0.478$) and DHA ($r = -0.536$) tended to reduce TRAP activity. Alkaline phosphatase (ALP) activity in bone homogenates was positively correlated with intake of EPA ($r = 0.763$) and DHA ($r = 0.793$). In comparison to fish fed the FO and LSO diets, those fed the SBO and PO diets showed a significant reduction ($P<0.05$) of total phospholipase A2 (PLA_2) activity in bone homogenates. However, such changes could not be clearly attributable to cytosolic PLA2. Overall data confirm that dietary lipids are important modulators of bone metabolism in a marine teleost, such as gilthead seabream.

Keywords: Gilthead seabream, oil sources, bone metabolism, mineral deposition.

1. Introduction

1.1 Replacement of fish oil by vegetable oil sources in Aquaculture feeds

According to estimates of the International Fishmeal & Fish Oil Organisation, in 2009 the aquaculture sector used about 81% of global fish oil (FO) production. Since industrial FO has to be shared with other users, such as land animal feeds, human nutritional supplements and pharmacology, the future expansion of aquaculture production can only continue if suitable and sustainable alternatives to FO are introduced in commercial fish feeds. Current trend on the aquafeed industry towards the lower usage of marine-derived ingredients promotes the use of vegetable oils devoid of n-3 long chain polyunsaturated fatty acids (LC-PUFA) (Drakeford and Pascoe, 2008; Miller et al., 2008; Turchini et al., 2009). In marine fish species, such as gilthead seabream, the use of vegetable oils as a sole lipid source is limited by the low ability of these species to convert linoleic (LA-18:2n-6) and ALA, abundant in many vegetable oils, into arachidonic (ARA – 20:4n-6), eicosapentaenoic (EPA – 20:5n-3) and docosahexaenoic acids (DHA – 22:6n-3) which are essential for marine fish (NRC, 2011). But the partial substitution (up to 60%) of FO by blends of vegetable oils (VO) does not seem to compromise growth, survival, feed utilization or major fillet organoleptic properties in seabream, although altering the fatty acid composition of flesh (Izquierdo et al., 2005; Benedito-Palos et al., 2009; Fountoulaki et al., 2009; Wassef et al., 2009; Benedito-Palos et al., 2010). Most studies involving FO replacement by VO sources show clear changes in the pattern and ratios of fatty acids circulating in plasma and stored in various tissues, but the effects at a metabolic level and on immune response of such changes are still poorly understood in fish (Caballero et al., 2004; Ganga et al., 2005; Montero et al., 2010; Montero and Izquierdo, 2010; Bouraki et al., 2011, Cruz-Garcia et al., 2011).

1.2 Skeletal bone in fish

Bone is a complex and multifunctional organ that consists of a structural framework of mineralized matrix that may contain heterogeneous populations of osteoblasts chondrocytes, osteoclasts, endothelial cells, monocytes, macrophages and lymphocytes. Bone modeling relies on the collective action of cells that produce, mineralize and resorb bone matrix. Osteoblasts produce and mineralize bone matrix, while bone resorption is performed by osteoclastic cells with hematopoietic origin. However in modern teleosts bone is mostly devoid of osteocytes, and osteoclasts are mostly mono-nucleated cells (Witten and Huysseune, 2009).

The combined and cooperative activities of osteoblasts and osteoclasts result in a bone architecture that provides mechanical support and protection for the body (Watkins et al., 2001). Similarly to land tetrapods, in teleost fish the skeleton is subject to remodelling, however showing several specific differences, related to the aquatic life of teleosts, to certain traits in conserved teleosts (but lost in mammals) and to several advanced characters that specifically evolved in the lineage of modern bony fish (Metscher and Ahlberg, 1999). Fish skeleton is a complex metabolically active tissue that undergoes continuous remodeling throughout their life. The skeletal system consists of bones and cartilage and serves multiple physiological functions. However, by far the most important function is to support the structural integrity of the body for normal posture, development and locomotion (Witten et al., 2004). Morphologically, fish bones consist of the dermal head bones, internal skeleton, and scales. However, fishes do not have any hematopoietic elements within the bones. Skeletal development in bony fish (Teleostei) follows the pattern that has been conserved during vertebrate evolution (Huysseune, A., 2000, Witten et al., 2004). There are two types of fish bones, cellular and acellular. The skeletal tissue of higher orders of teleost fish such as Perciformes is unique among vertebrates in that they lack osteocyte cells. Acellular bones are formed by osteoblast cells which move away from the site of mineralization as bone deposition occurs and are therefore incapable of extensive modeling (Moss, 1963). The tissues of acellular bone may not be directly involved in Ca metabolism under normal conditions (Taylor, 1985). Skeletal disorders are a reoccurring problem in fish hatcheries and commercial farms that affect growth, development and survival as well as market value of the final product (Lall and Lewis-McCrea, 2007).

1.3. The role of nutrients on bone remodeling

Protein is part of the organic matrix of bone for collagen structure and is essential to maintain the production of hormones and growth factors that modulate bone synthesis (Heaney, R. P. 1993). Approximately 60% of the magnesium in the body is in bone. As with calcium, magnesium is at risk for being deficient in the diet. Deficiency of this mineral could affect bone growth, osteoblastic and osteoclastic activity and alter calcium metabolism (Fatemi, et al., 1991). Manganese is needed for the biosynthesis of mucopolysaccharides in bone matrix formation and is a cofactor for several enzymes in bone tissue (Clegg, et al., 1998). Fish and other aquatic organisms absorb Ca and P from water and their Ca requirement is met by their ability to absorb this element directly from water.

However, the concentration of P is low in both freshwater (FW) and seawater (SW), The absorbed calcium is deposited in bone, scale and skin (Lall, 2002). Osteoblasts, cells involved in bone formation, are target cells for retinoids. Osteoclasts are cells that are responsible for bone resorption. These cells are stimulated by vitamin A, specifically all-trans retinoic acid and 9-cis-retinoic acid (Kindmark, et al. 1995). Vitamin E is important for proper skeletal development, especially to combat endogenous and exogenous free radicals that can cause damage to osteoblasts and stimulate osteoclasts (Arjmandi, et al., 2002) Both vitamins E and C are integral in the intracellular defence mechanism used to protect bone cells from free radicals (Xu, et al. 1995). Roy (2002) reported that dietary vitamin K supplementation had no detectable effect on the quantity of haddock bone matrix, however, a deficiency of this vitamin did affect the quality of bone matrix in haddock indicating its role in bone health of this species. Various studies supports the hypothesis that dietary lipids play an important role in skeletal biology and bone health in terrestrial vertebrates (Watkins et al., 2000; 2001; Poulsen et al. 2007; Lukas et al., 2011) and fish (Cahu et el., 2003; Villeneuve et al., 2006; Lall and Lewis-McCrea, 2007; Kjørsvik et al., 2009: Berge et al., 2009; Fjelldal et al., 2010; Gil-Martens et al., 2010; Sandel et al., 2010; Ytteborg et al., 2010; Viegas et al., 2012). The mechanism mediating such effect of dietary lipids on bone remodeling has been largely attributed to altered prostaglandin E_2 (PGE_2) synthesis in bone (Poulsen et al., 2007, Berge et al., 2009; Gil-Martens et al., 2010). PGE_2 is synthesized by the metabolism of phospholipase A2 (PLA_2)-derived arachidonic acid to prostaglandin G/H_2 via cyclooxygenase (COX) isoforms (COX-1 or COX-2), followed by the activity of prostaglandin E synthase (Calder, 2009). Similarly to what is found in humans, it is generally recognized that altering the amounts and types of dietary LC-PUFA from the n-6 and n-3 series can influence the spectrum of eicosanoids (leukotrienes, prostaglandins and thromboxanes) and docosanoids produced in fish (Bell et al., 1996; Mourente et al., 2005; Ganga et al., 2005; Petropoulos et al., 2009).

1.4. Objectives

The objective of this thesis was to evaluate the effect of various dietary lipid sources as modulators of bone remodeling in seabream juveniles. Study comprised the assessment of:

- Overall growth performance of fish
- Bone fatty acid and mineral composition
- Vertebrae mineralization pattern and activity of bone metabolic enzymes

2. Material and Methods

2.1. Experimental diets

Four isoproteic (crude protein, 46% DM) and isolipidic (15% DM) experimental diets were formulated to fulfill the known nutritional requirements of juvenile seabream. Diets were formulated with purified ingredients to allow a detailed control over its composition and isolate the target dietary changes. The dietary lipid sources under testing were: a) cod liver oil (FO, a source of n-3 LC-PUFA); b) soybean oil (SBO, a source of n-6 fatty acids); c) linseed oil (LSO, a source of n-3 fatty acids, but non LC-PUFA); and d) palm oil (PO, a source of saturated fatty acids). The vegetable oils were incorporated at the expenses of fish oil, at a 40% replacement level (Table 1).

Main ingredients were grinded (below 250 micron) in a micropulverizer hammer mill (Hosokawa Micron, SH1, The Netherlands). Powder ingredients and oil sources were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain). All diets were manufactured by temperature controlled-extrusion (pellet size: 2.0 mm) by means of a low shear extruder (Italplast P55, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 3 hours at 40°C. Throughout the duration of the trial, experimental feeds were stored refrigerated. Samples of each diet were taken for proximate composition and fatty acid analysis (Tables 1 and 2).

Table 1. Ingredients and proximate composition of experimental diets.

Ingredients, %	FO	SBO	LSO	PO
Casein [1]	40.00	40.00	40.00	40.00
Fish gelatin [2]	5.00	5.00	5.00	5.00
Wheat gluten [3]	5.00	5.00	5.00	5.00
Gelatinized starch [4]	12.00	12.00	12.00	12.00
Corn dextrine [5]	14.00	14.00	14.00	14.00
Cod liver oil [5]	15.50	9.00	9.00	9.00
Soybean oil [5]		6.50		
Linseed oil [5]			6.50	
Palm oil [5]				6.50
Vitamin & Mineral Premix [6]	2.00	2.00	2.00	2.00
Monocalcium phosphate [5]	4.00	4.00	4.00	4.00
L-Arginine [5]	0.50	0.50	0.50	0.50
Sodium alginate [5]	2.00	2.00	2.00	2.00
Proximate composition				
Dry matter, %	90.71	90.41	89.70	90.24
Crude protein, %DM	45.82	47.47	46.47	47.67
Crude fat, %DM	15.99	15.16	15.65	15.13
Ash, %DM	5.40	4.94	5.23	4.78
Gross energy, kJ/g DM	22.84	23.15	23.29	23.30
Total phosphorus, %DM	0.96	0.99	0.97	0.97

[1] Acid casein: 90% CP (PLIMON, Spain).
[2] Fish gelatin: 88 CP% CP (LAPI Gelatine SPA, Italy).
[3] VITEN: 85.7% CP, 1.3% CF (ROQUETTE, France).
[4] Paygel 290 wheat starch (ADM, The Netherlands).
[5] Sigma Aldrich Quimica S.A (Portugal).
[6] Standard premix for marine fish PVO40.01 (PREMIX Lda, Portugal).

Table 2. Fatty acid composition of the experimental diets (% of total FAME).

Fatty acid, %	FO	SBO	LSO	PO
Saturated (SFA)				
14:0	5.71	3.20	4.68	3.58
16:0	16.55	13.95	16.60	25.94
18:0	3.77	3.98	4.78	4.16
Other SFA	2.56	1.78	2.17	1.87
Monoenes (MUFA)				
16:1	7.85	4.25	5.70	4.32
18:1	25.26	23.66	28.63	30.60
20:1	7.80	4.74	4.89	4.71
Other MUFA	5.14	3.32	3.12	3.59
Polyenes (PUFA)				
18:2n6	4.94	23.19	9.37	6.69
18:3n3	1.14	3.59	12.84	0.90
18:4n3	2.07	1.14	0.66	1.13
20:4n6 - ARA	0.40	0.37	0.12	0.28
20:4n3	0.58	0.36	0.17	0.38
20:5n3 - EPA	6.59	4.32	1.62	4.40
22:5n3	0.64	0.48	0.00	0.53
22:6n3 - DHA	6.39	4.73	1.19	4.59
Other PUFA	2.61	1.68	1.61	1.67
Non ID	0.39	1.27	1.85	0.67
Total SFA	28.59	22.90	28.22	35.55
Total MUFA	46.05	35.97	42.35	43.22
Total PUFA	25.36	39.85	27.58	20.56
DHA/EPA	0.97	1.09	0.74	1.04
EPA/AA	16.61	11.60	13.39	15.75

2.2. Fish and rearing conditions

Homogenous groups of 25 seabream juveniles each, with a mean initial body weight of 39.3 g were stocked in 12 circular plastic tanks (volume: 90 L; water-flow rate: 3.5 $L \cdot min^{-1}$), supplied with flow-through seawater (temperature: 24±2°C; salinity: 33-34 $g \cdot L^{-1}$, dissolved oxygen above 6 $mg \cdot L^{-1}$). A 12/12 fluorescent light/dark cycle was adopted. Each dietary treatment was tested in triplicate tanks over 46 days. Fish were fed to apparent satiety, by hand, four times a day (twice a day during weekends) and feed intake was recorded on a weekly basis. Utmost care was taken to avoid feed losses. All fish were individually weighed at the beginning, bulk weighed every three weeks and at the end of the trial, following one day of feed deprivation. At the beginning and immediately before the end of the trial, fish from each tank were immersed for 5 min in a 0.1% tetracycline bath to label the mineral deposition in fish bone and to allow the subsequent assessment of the mineralization patterns associated to experimental feeding period. Six fish from the initial stock and from each tank at the end of the trial were sampled for subsequent analysis of whole-body composition. Vertebral column (freed of soft tissue) were collected in both initial and final fish (2 pools of three fish per replicate tank) and frozen at -80°C until subsequent analysis of bone of fatty acid profile, calcium and phosphorus content and assay activities of bone metabolic enzymes. Vertebrae from tetracycline marked fish were fixed in 4% phosphate buffered formalin until further histological processing.

2.3. Analytical methods

Diets were grinded prior to analysis of proximate composition. Frozen whole-body samples were minced, mixed, a representative sample freeze-dried and homogenized with a laboratory mill prior to analysis. The chemical composition analysis of the diets and whole fish was made using the following procedures: dry matter after drying at 105ºC for 24 h; ash by combustion at 550ºC for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428); fat by dichloromethane extraction (Soxhlet); gross energy in an adiabatic bomb calorimeter (IKA).

Analysis of total phosphorus in whole-body and vertebral bone ashed samples was done according to the ISO/DIS 6491 method using the vanado-molybdate reagent and calcium in ashed vertebral bone by spectrophotometry with the QuantiChrom Calcium Assay Kit (DICA-500).

Prior to assays of enzyme activities, vertebral bone samples were pulverized under liquid nitrogen, homogenised in a 0.1% Triton X-100 solution and then centrifuged at 16 000 x *g* for 2 minutes. Supernatants were used for assay of bone alkaline phosphatase (ALP) with p-nitrophenylphosphate as substrate according to Bessey et al. (1946) and bone tartrate resistant acid phosphatase (TRAP) using p-nitrophenol phosphate as substrate (Goto and Tsukamoto 2003). For the extraction of phospholipase A_2 fractions in bone homogenates, samples were incubated for 2 hours at 37ºC with a 0.1M Tris-HCL buffer, pH 7.8, containing 1mM EDTA, centrifuged at 10 000 x *g* for 15 minutes at 4ºC and supernatants used for enzymatic assays with commercial kits. Total (Total PLA_2) and cytosolic phospholipase A_2 ($cPLA_2$) using arachidonoyl thiol-PC as substrate (Kit 765021, Cayman Chemical) and secreted phospholipase A_2 ($sPLA_2$) using diheptanoyl thiol-PC as substrate (Kit 765001, Cayman Chemical). Protein was determined by means of the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Fatty acid composition of the diets and vertebral bone was determined through the analysis of methyl esters, according to the procedure described by Lepage and Roy (1986) and modified by Cohen et al. (1988). Acid catalyzed transmethylation of fatty acids was done through methylation of 300 mg of sample in 5 ml of acetyl chloride:methanol (1:19, v/v). Following vigorous vortexing, samples were placed in a water bath at 80 °C for 1 h. After cooling, 1 ml of water and 2 ml of n-heptane were added to the samples, which were then vortexed and left to rest, to allow separation of the liquid and organic phases. The organic phase was collected and filtered through a column prepared with cotton and sodium sulphate anhydrous, to remove any moisture, and placed in a vial for subsequent analysis by gas–liquid chromatography. The separation of the different methyl esters was performed using a Varian Star CP 3800 GC (Walnut Creek, CA), equipped with auto sampler and flame ionization detector. Samples were vaporized in the injector at 250 °C using helium as carrier gas (100:1 split ratio) in a polyethylene glycol capillary column DB-WAX (Folsom, CA, USA) (30 m x 0.25 mm id, film thickness: 0.25 µm) kept at 180 °C for 2 min, heating at 4 °C min^{-1} for around 10 min and holding at 220 °C for 25 min. The separated compounds were detected at 250 °C. Identification of fatty acid methyl esters was achieved by comparison of their retention times with those of mixed chromatographic standards (Sigma Aldrich). Peak areas were determined using the Varian software. Results were expressed as relative percentage of total fatty acids.

The first 5 caudal vertebrae from tetracycline marked fish were dissected, fixed in 4% formalin and then histologically processed by glycol-methacrylate embedding followed by sectioning at 7 μm. The extent of mineral deposition was determined by measuring the distance between the two bands deposited tetracycline labeling under a fluorescence-microscope (Olympus IX-81). Points of active bone resorption were identified by observing interruptions in the marked tetracycline bands.

2.4. Statistical analysis

Data are presented as mean of three replicates ± standard deviation. Data were subjected to a one-way analysis of variance, and when appropriate, means were compared by the Student-Newman-Keuls test. Parameters expressed as percentages were subjected to arcsin square root transformation. A correlation matrix between parameters of bone fatty acid composition or enzyme activities and individual fatty acid intakes (calculated per unit body weight) was also computed. Statistical significance was tested at 0.05 probability level. All statistical tests were performed using the SPSS V18 software.

3. Results

As expected, analysis of the fatty acid composition of the experimental diets reflected that of dietary oil sources, confirming that FO diet had the highest level of n-3 LC-PUFA, SBO diet the highest level of n-6 fatty acids, in particular linoleic acid (LA, 18:2n-6), LSO diet a high level of n-3 non LC-PUFA (mainly α-linolenic acid, 18:3n-3) and PO diet the highest level of saturated fatty acids (Table 2).

Overall growth performance criteria were not significantly affected by the various dietary treatments (P>0.05). Over a period of 46 days, fish more than doubled their initial body weight, reaching a final body weight ranging from 85 to 90 g (Table 3). Specific growth rate varied between 1.68 and 1.80. Feed efficiency ranged from 0.72 to 0.79. Whole-body composition of fish was not affected by dietary treatments (data not shown). Given this relative constancy of weight gain, feed intake and whole-body composition of fish, values for protein and fat retention were also unaffected (P<0.05) by dietary changes (data not shown).

Table 3. Growth performance and proximate composition of vertebral bone of seabream (IBW: 39.3 ± 1.4 g) fed the experimental diets.

	FO	SBO	LSO	PO
FBW, g	85.08 ± 1.10	88.55 ± 3.43	86.03 ± 4.89	90.16 ± 5.50
Wt. Gain, %IBW/day	2.33 ± 0.05	2.50 ± 0.16	2.39 ± 0.24	2.58 ± 0.29
SGR, %/day	1.68 ± 0.03	1.76 ± 0.09	1.70 ± 0.12	1.80 ± 0.14
FE	0.72 ± 0.00	0.76 ± 0.03	0.76 ± 0.06	0.79 ± 0.10
VFI, %IBW/day	3.26 ± 0.07	3.26 ± 0.07	3.15 ± 0.07	3.27 ± 0.06
PER	1.72 ± 0.00	1.82 ± 0.08	1.82 ± 0.14	1.89 ± 0.24
Bone composition				
Dry matter, %	55.55 ± 0.35	56.13 ± 0.64	55.22 ± 0.76	54.29 ± 0.89
Protein, % DM	46.27 ± 1.07	46.55 ± 1.89	44.65 ± 1.14	43.61 ± 2.26
Lipids, % DM	26.72 ± 1.67	25.82 ± 2.35	26.73 ± 2.44	28.89 ± 1.24
Ash, % DM	26.59 ± 0.58	26.92 ± 0.61	26.44 ± 0.54	26.30 ± 0.31
Bone minerals				
Phosphorus (μg/mg ash)	2.97 ± 0.36 [b]	2.08 ± 0.27 [a]	1.94 ± 0.26 [a]	2.22 ± 0.35 [a]
Calcium (μg/mg ash)	108.95 ± 2.40	103.16 ± 1.24	107.20 ± 3.07	104.09 ± 2.01

Data is presented as mean and standard deviation (n=3).
Values in a row with different superscripts are significantly different (P<0.05).
IBW (g): Initial mean body weight.
FBW (g): Final mean body weight.
Specific growth rate, SGR (%/day): (Ln FBW – Ln IBW) x 100/days.
Feed efficiency, FE: weight gain / crude feed intake.
Voluntary feed intake, VFI (%BW/day): (crude feed intake/IBW/days) x 100.
Protein efficiency ratio, PER: wet weight gain/crude protein intake.

The proximate composition of vertebral bone was not affected (P>0.5) by dietary treatments (Table 3). Similarly, the calcium content on vertebral bone was little affected (P>0.05) by the various dietary oil sources. However, bone phosphorus content in fish fed the fish oil rich diet (FO) was significantly higher (P<0.05) than those found in seabream fed the diets with the inclusion of vegetable oils (Table 3). Microscopic observation of tetracycline-marked vertebrae from fish fed the various experimental diets allowed us to identify active points of bone remodeling. In comparison to fish fed diets FO and SBO, mineral deposition in vertebrae of fish fed the LSO diet was significantly reduced (P<0.05) (Figure 1).

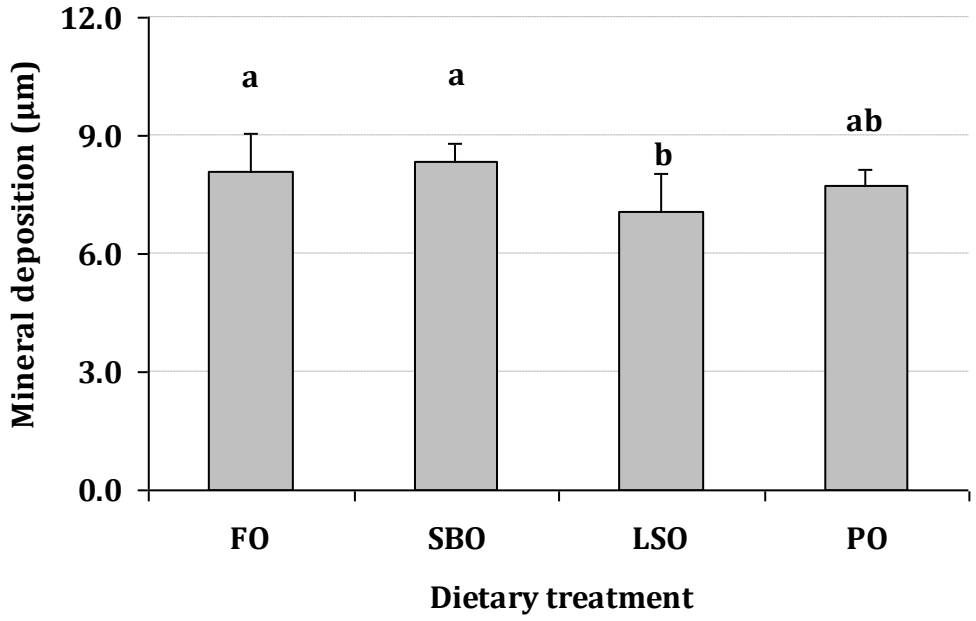

Figure 1. Mineral deposition in tetracycline-marked vertebrae at the end of the feeding period of 46 days with the various dietary treatments.

Data is presented as mean and standard deviation (n=20).
Bars with different superscript letters are significantly different (P<0.05).

Table 4. Fatty acid composition of lipids extracted from seabream vertebral bone (% of total FAME).

Fatty acid, %	FO	SBO	LSO	PO
Saturated (SFA)				
14:0	4.83 ± 0.38	4.57 ± 0.30	4.57 ± 0.42	4.36 ± 0.80
16:0	19.0 ± 1.07	19.7 ± 0.94	18.03 ± 0.79	20.50 ± 2.11
18:0	3.32 ± 0.06 [a]	3.39 ± 0.05 [a]	3.56 ± 0.09 [b]	3.63 ± 0.10 [b]
Other SFA	2.46 ± 0.03	2.54 ± 0.17	2.48 ± 0.19	2.37 ± 0.11
Monoenes				
16:1	8.98 ± 0.57	7.98 ± 0.65	7.43 ± 0.20	7.89 ± 0.60
18:1	27.40 ± 0.24 [a]	26.61 ± 0.56 [a]	26.82 ± 0.24 [a]	31.20 ± 0.81 [b]
20:1	4.01 ± 0.12 [d]	2.70 ± 0.22 [c]	2.11 ± 0.20 [b]	1.56 ± 0.20 [a]
Other MUFA	2.71 ± 0.26 [c]	1.77 ± 0.31 [b]	1.62 ± 0.26 [b]	1.29 ± 0.21 [a]
Polyenes				
18:2n6 – LA	5.91 ± 0.20 [a]	14.20 ± 0.68 [c]	8.55 ± 0.49 [b]	7.81 ± 0.34 [b]
18:3n3 - ALA	1.14 ± 0.13 [a]	2.02 ± 0.13 [b]	7.88 ± 0.70 [c]	1.30 ± 0.16 [a]
18:4n3	1.50 ± 0.04 [b]	1.24 ± 0.04 [a]	1.59 ± 0.05 [b]	1.17 ± 0.07 [a]
20:4n6 - ARA	0.42 ± 0.03	0.32 ± 0.01	0.39 ± 0.02	0.48 ± 0.07
20:4n3	0.62 ± 0.03	0.46 ± 0.04	0.59 ± 0.01	0.57 ± 0.09
20:5n3 - EPA	4.57 ± 0.15 [b]	3.30 ± 0.12 [a]	3.95 ± 0.17 [b]	4.74 ± 0.79 [b]
22:5n3	1.12 ± 0.11	0.75 ± 0.33	1.11 ± 0.09	1.39 ± 0.27
22:6n3 - DHA	7.00 ± 0.70 [b]	5.04 ± 1.07 [a]	5.07 ± 0.57 [a]	5.43 ± 0.97 [a]
Other PUFA	2.08 ± 0.06 [b]	1.64 ± 0.14 [a]	2.29 ± 0.06 [b]	2.43 ± 0.14 [b]
Non ID	2.88 ± 0.45	1.72 ± 0.30	2.06 ± 0.35	1.92 ± 0.34
Total SFA	29.70 ± 1.42	30.20 ± 1.38	28.61 ± 1.18	30.81 ± 2.91
Total MUFA	43.10 ± 0.23 [c]	39.00 ± 0.63 [b]	37.90 ± 0.24 [a]	42.00 ± 0.66 [c]
Total PUFA	24.40 ± 1.16 [a]	29.00 ± 1.84 [b]	31.40 ± 1.01 [b]	25.30 ± 2.63 [a]
DHA/EPA	1.52 ± 0.10	1.48 ± 0.26	1.26 ± 0.08	1.14 ± 0.03
EPA/ARA	10.90 ± 0.33	10.30 ± 0.15	10.00 ± 0.09	9.82 ± 0.24

Data is presented as mean and standard deviation (n=3).
Values in a row with different superscripts are significantly different (P<0.05).

Data on the individual fatty acid profile of bone (Table 4) shows that its composition is altered by the dietary fatty acid profile. Total saturated fatty acids were not affected by dietary treatments, but vertebral bone of fish fed the FO and SBO diets showed significantly lower (P<0.05) levels of stearic acid (C18:0) than fish fed the LSO and PO diets. Total monounsaturated fatty acids were affected (P<0.05) by the dietary lipid sources, being higher in fish fed FO and PO, intermediate in those fed SBO and lowest in LSO fed fish. These changes were mainly due to relevant changes on oleic acid (C18:1n-9) and gondoic acid (C20:1n-9) and in close association to dietary profiles. Total levels of polyunsaturated fatty acids (PUFA) in vertebral bone were significantly higher (P<0.05) in seabream fed SBO and LSO than in fish fed FO and PO diets. This increase was mainly associated to a significantly increase (P<0.05) of LA and ALA in fish fed diets SBO and LSO, respectively. Bone ARA levels were not affected (P>0.05) by dietary treatments. EPA was significantly reduced

(P<0.05) in bone of fish fed the SBO, while DHA was significantly higher (P<0.05) in fish fed the FO diet. The association between dietary and vertebral bone PUFA levels are presented in Figure 2.

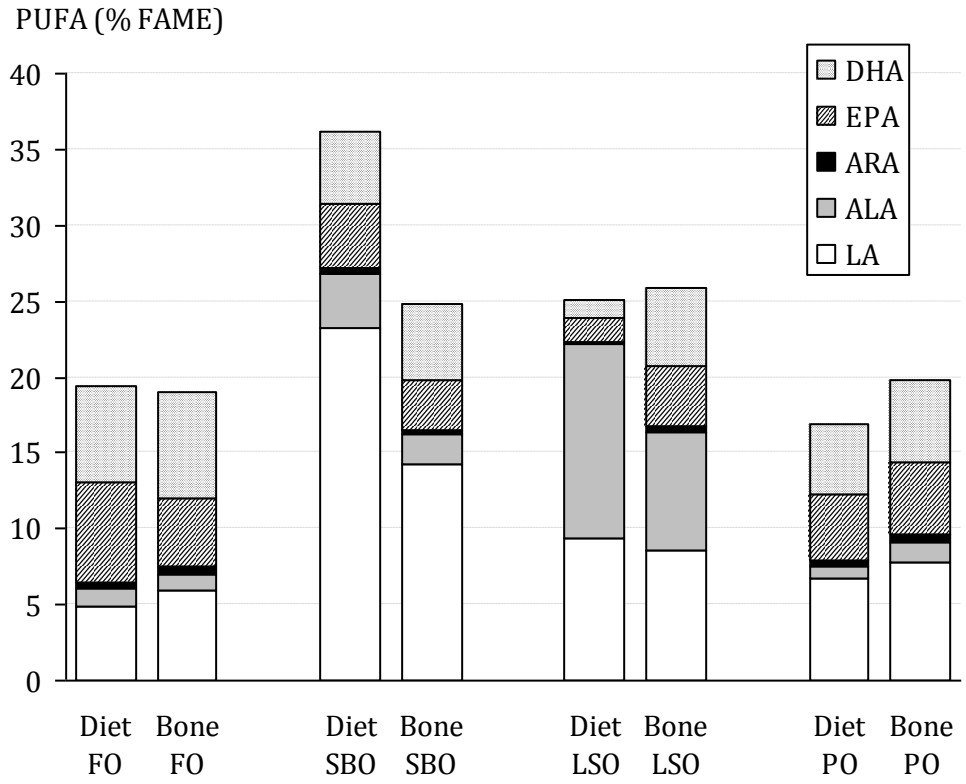

Figure 2. Association between experimental diets and vertebral bone polyunsaturated fatty acid (PUFA) profile.

LA: linoleic acid (C18:2n-6); ALA: α-linolenic acid (C18:3n-3); ARA: arachidonic acid (C20:4n-6); EPA: eicosapentaenoic acid (C20:5n-3); DHA: docosahexaenoic acid (C22:6n-3).

Activities of alkaline phosphatase (ALP) and tartrate-acid resistant phoshatase (TRAP) in bone homogenates are presented in Figure 3. In comparison to fish fed the FO diet, those fed the LSO diet showed a significant reduction (P<0.05) of ALP activity. Moreover, the activity of ALP in bone homogenates was positively correlated with intake of EPA (r=0.763) and DHA (r=0.793). Highest value of TRAP activity was found in fish fed the SBO diet, being significantly different (P<0.05) from those found in fish fed the LSO diet. TRAP activity was positively correlated (r = 0.658) with LA intake, but an increased intake of EPA (r = -0.478) and DHA (r = -0.536) tended to reduce TRAP activity.

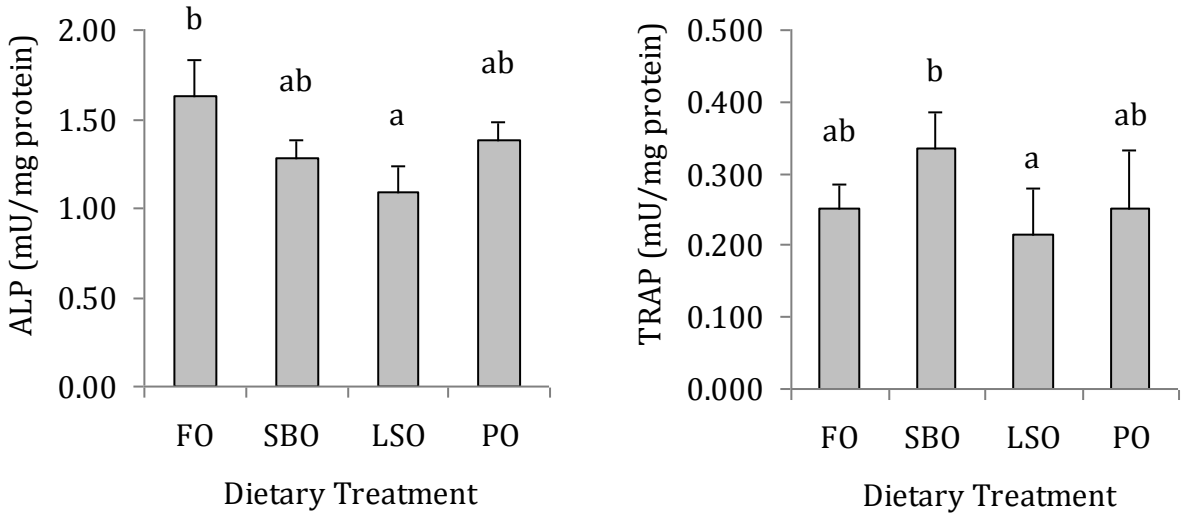

Figure 3. Activity of ALP and TRAP in seabream vertebral bone homogenates.

Data is presented as mean and standard deviation (n=6).
Bars with different superscript letters are significantly different (P<0.05).

Activities of total phospholipase A_2 in fish bone homogenates were significantly affected (P<0.05) by dietary lipid sources, being higher in fish fed FO and LSO than in those fed SBO and PO diets. However, such chances can not be clearly associated to the altered levels of the intracellular Ca^{2+}-dependent cytosolic family ($cPLA_2$), which were little affected by dietary treatments. The activity of the secretory PLA2 fraction in seabream bone homogenates was below the detection limit of the analytical kit.

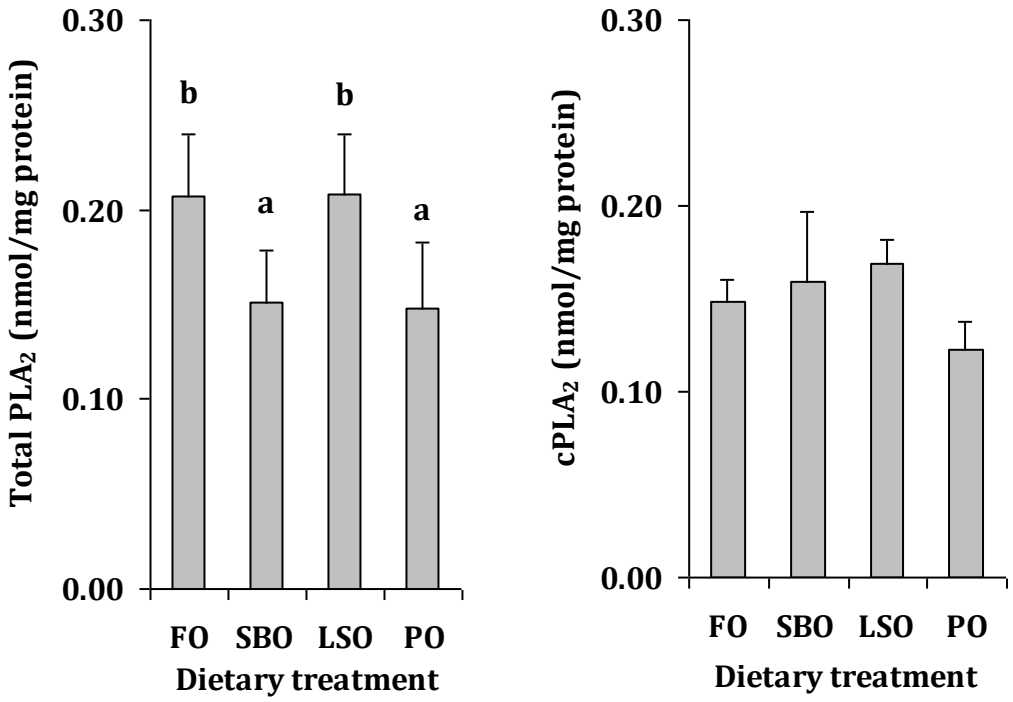

Figure 4. Activity of total PLA_2 and $cPLA_2$ in seabream vertebral bone.

Data is presented as mean and standard deviation (n=3).
Bars with different superscript letters are significantly different ($P<0.05$).

4. Discussion

The aquaculture industry has, in recent times, experienced dramatic growth and currently provides approximately 50% of the global fish and seafood supply (FAO, 2010). However, such growth poses great challenges in accommodating the sustainable use of natural resources. About 35% of the global aquaculture production relies on the use of industrial compound feeds containing marine harvested resources in the form of fish meal and fish oil (Tacon and Metian, 2008). Therefore the replacement of fish oil with renewable land-based products such as vegetable oils has been extensively researched and the results incorporated into currently used commercial aquafeeds. Overall available knowledge supports that the partial replacement of fish oil by vegetable oils in diets for marine species, such as gilthead seabream, is possible without affecting good feed utilization and growth performance (Izquierdo et al., 2005; Benedito-Palos et al., 2008). However, care as to be taken, to guarantee that the supply of such alternative dietary lipids is balanced for optimal growth, but also preserve the nutritional quality of fish (Fountoulaki et al., 2009; Benedito-Palos et al., 2009; 2010), and do not compromise the immune functions and broader welfare status of fish

(Montero et al., 2010; Montero and Izquierdo, 2010). The growth performance data of our trial, confirms that provided that the requirements for the essential fatty acids (EPA, DHA and ARA) are covered, the replacement of 40% of fish oil by soybean oil, palm oil or linseed oil does not impair growth or feed utilization in juvenile seabream. Similar results have been reported by Izquierdo et al. (2005) and Fountoulaki et al. (2009). Another relevant aspect of our work is the fact that by covering the essential fatty acid requirements of gilthead seabream juveniles (Kalogeropoulos et al., 1992; Ibeas et al., 1994; NRC, 2011) the use of purified diets sustained a good growth performance of fish, which more than doubled their weight in 46 days and in agreement with performance data from Couto et al. (2008) for a similar weight class and rearing temperature.

Most studies involving FO replacement by VO sources show clear changes in the pattern and ratios of fatty acids circulating in plasma and deposited in various tissues of fish (Menoyo et al., 2004; Caballero et al., 2006). Dietary lipids were shown to alter the fatty acid composition of bone compartments in rodents (Watkins et al., 2000; Weiler et al., 2002; Lau et al., 2010). Data on the composition of bone and its modulation by dietary factors is limited in fish. The lipid content of fish bone is highly variable, ranging from 23 $g \cdot kg^{-1}$ in cod to 509 $g \cdot kg^{-1}$ in mackerel, and seems to be associated to the general classification of fatty and lean fish (Toppe et al., 2007). Our data suggests that the vertebral bone of gilthead seabream is positioned in the mid-range, with a total lipid content of 260 $g \cdot kg^{-1}$, a value lower than that reported in a more fatty fish such as Atlantic salmon by Berge et al. (2009). But more important in the scope of the study, the various dietary lipid profiles had no marked effect on the total fat content or overall proximate composition of seabream bone. Similarly, Atlantic salmon fed fish oil or soybean oil diets showed no changes on the total fat content of bone (Berge et al., 2009). While the calcium content in the vertebral bone of seabream was little affected by the various dietary lipid sources, we found that bone phosphorus levels in seabream fed the fish oil rich diet was significantly higher than in fish fed the diets with the inclusion of vegetable oils. Berge et al. (2009) reported that the content of phosphorus in Atlantic salmon vertebrae tended to be higher ($P=0.06$), in fish fed a fish oil based diet in relation to those fed a soybean oil diet. In humans and other terrestrial vertebrates high intake of saturated fats is generally related to detrimental effects on skeletal mineralization (Corwin et al., 2006). Additionally, palm oil and palm olein (its low melting fraction) generally used on infant milk formulas have been associated to lower calcium absorption and bone mineralization, due to the formation of unabsorbable calcium-fatty acid-soap complexes (Koo et al., 2006).

Our data regarding the calcium content in vertebral bone does not support an impairment of calcium absorption by high levels of palmitic acid in fish fed the palm oil rich diet (PO). But it is also known, that significant changes on tissue calcium levels are particularly difficult to demonstrate in fish, given their ability to absorb waterborne calcium (NRC, 2011).

As expected, our data in seabream juveniles supports the general findings that the fatty acid profile of vertebral bone reflects that of dietary intake (Gil-Martens et al., 2010; Berge et al., 2009). For instance, the experimental feed incorporating soybean oil contained a high level of linoleic acid (18:2n-6) and consequently, this fatty acid was markedly increased in bone lipids of seabream fed the SBO diet. Similar associations can be found for α-linolenic acid (18:3n-3) in fish fed the linseed oil diet (LSO) and DHA in fish oil rich diet (FO). However, it should be noted that the fatty acid composition of seabream vertebrae did not exactly mirrored that of the diets. It is the case of ARA and EPA, which were provided at significantly lower levels in the LSO diet, but were found in the vertebrae at similar levels to those found with the other vegetable oil based diets. It seems clear that dietary fatty acids are not simply deposited in fish bone, but as in other tissues, undergo different metabolic processes, involving the utilization for energy production (beta-oxidation), bio-conversion (chain elongation and desaturation) and lipogenesis (Tocher, 2003). Thus, similarly to what is found in fish fillets (Benedito-Palos et al., 2008; Turchini et al., 2011), there seems to exist a biological ‘‘buffering” on the overall fatty acid composition of fish bone. In future studies, it would be interesting to characterize the dietary modulation of bone fatty acid profile in respect to the neutral and phospholipid classes, since it is known that the extent of such conservative mechanism is variable among this lipid classes.

In terrestrial vertebrates, several studies showed that dietary lipids, by altering the fatty acid composition of bone compartments, impact the local production of factors influencing bone modelling in animals (Watkins et al., 2001). The mechanism for such effects has been largely attributed to reduced prostaglandin E_2 (PGE_2) synthesis in bone (Poulsen et al. 2007; Kruger et al., 2010). PGE_2 is a primary product of arachidonic acid metabolism and is synthesized via the cyclooxygenase (COX) and prostaglandin synthase pathways. Although not clearly established for fish, PGE_2 is a potent regulator of bone metabolism in mammals, since at low concentrations they can increase in vitro both bone resorption and bone formation, but at high concentrations may have the opposite effect leading to inhibition of collagen synthesis and bone formation (Poulsen et al., 2007). Only few studies have attempted to relate the dietary modulation of PGE_2 levels and bone remodeling in fish. Atlantic salmon fed a soybean oil

based diet and subjected to lyposaccharide injection had a significantly elevation of plasma PGE_2 levels compared to those fed a fish oil based diet (Gil-Martens et al., 2010). Additionally, by means of an in vitro approach, Ytteborg et al. (2010) reported that the addition of DHA to the osteoblast media resulted in a reduction of PGE_2 production and increased expression of osteocalcin in Atlantic salmon cells. However, Petropoulos et al. (2009) found no dietary effects and high individual variation in plasma ARA-derived PGE_2 levels after the substitution of dietary fish oil with a blend of vegetable oils in three strains of Atlantic salmon. Phospholipase A_2 (PLA_2) constitutes a superfamily of enzymes that catalyze the hydrolysis of the phospholipid sn-2 ester bond, generating a free fatty acid and a lysophospholipid. The PLA_2 reaction is the primary pathway through which ARA is liberated from phospholipids. By measuring the activities of total PLA_2 and intracellular Ca^{2+}-dependent cytosolic fraction ($cPLA_2$) in seabream bone homogenates, we intended to get an approximate indication of the utilization of ARA in bone. Despite the relatively constant ARA levels found in bone, we observed lower activities of total PLA_2 in fish fed the soybean oil and palm oil rich diets. However, a clear explanation of the mechanisms behind this modulation is relatively difficult, since the levels of free arachidonic acid available for eicosanoid production are controlled by the rate of deacylation by PLA_2 and by competing processes such as reacylation into phospholipid and efflux from cells (Balsinde et al., 2002). Furthermore, a series of negative feedback regulation loops (e.g. via cAMP, cellular calcium levels, lipid oxidation metabolites) have been associated to the modulation of PLA_2 activity (Balsinde et al., 2002; Leslie, 2004; Burke and Dennis, 2009). In this same context, despite the absence of statistical significance, Berge et al (2009) reported that Atlantic salmon fed a fish oil diet presented consistently higher levels of COX-2 mRNA expression than those fed the soybean oil diet. This occurrence was possibly explained by a PGE_2-mediated negative feedback mechanism in relation to the dietary EPA:ARA ratios.

Alkaline phosphatase (ALP) is a by-product of osteoblast activity essential for calcification and elevated levels indicate an active bone formation status (Goto and Tsakumoto, 2003). The results of the current experiment show that in comparison to fish fed the fish oil diet (FO), those fed the linseed oil diet (LSO) showed a significant reduction of ALP activity. Moreover, the activity of ALP in seabream bone was found to be positively correlated with both EPA and DHA intake. This trend is also in agreement with our bone mineralization data, in which the microscopic observation of tetracycline-marked vertebrae showed us that in comparison to fish fed diets FO and SBO, the mineral deposition in vertebrae of fish fed the linseed oil diet

was significantly reduced. Taken together, these results suggest that attention should be taken when assessing the role of n-3 PUFA in bone metabolism, since effects seem to be dependent with the carbon chain length. On the other hand, we found that the activity of tartrate acid-resistant phosphate (TRAP), which is secreted by osteoclasts during bone resorption, was positively correlated with LA intake and negatively correlated to EPA and DHA intake. Again, this observation suggests that diets with lower levels of highly unsaturated fatty acids of the n-3 series (EPA and DHA) tend to promote bone a resorption status in fish.

The results of the current experiment suggest that vegetable oil as a replacement for fish oil influence the bone metabolic status of seabream juveniles and cause a tendency towards reduced vertebrae mineralization. However given the short duration of the trial, future efforts should encompass long-term feeding periods, a detailed assessment of any detrimental effects in terms of skeletal deformities and a broader range of deposited minerals. Additionally, these results should be viewed in more global perspective of practical feed formulation, in which both fish meal and fish oil are being simultaneously replaced by vegetable-based ingredients. Such formulation practices comprise not only changes on the dietary fatty acid supply, but also inherent changes on selected minerals, vitamins, phytosterols, phytoestrogens and cholesterol levels, all substances with a clear association to bone remodeling processes.

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