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Michael Nogueira Viegas

Masters in Molecular Biology and Microbiology

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Declaração de autoria de trabalho

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.

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Michael Nogueira Viegas

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Abstract

The aquaculture industry aims at replacing significant amounts of marine fish oil by vegetable oils in fish diet. Dietary lipids have been shown to alter the fatty acid composition of bone compartments, which would impact the local production of factors controlling bone formation. Knowledge on the mechanisms underlying the nutritional regulation of bone metabolism is however scarce in fish. Two *in vitro* bone-derived cell systems developed from seabream (an important species for aquaculture in the Mediterranean region) vertebra, capable of *in vitro* mineralization and exhibiting pre-chondrocyte (VSa13) and pre-osteoblast (VSa16) phenotype, were used to assess the effect of certain polyunsaturated fatty acids (PUFAs; arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids) on cell proliferation, extracellular matrix (ECM) mineralization and gene expression. While all PUFAs promoted morphological changes in both cell lines, VSa16 cell proliferation appeared to be stimulated by PUFAs in a dose dependent manner until 100μM, whereas proliferation of VSa13 cells was impaired at concentrations above 10μM. AA, EPA and DHA inhibited VSa13 ECM mineralization, alone and in combination, while VSa16 ECM mineralization was only inhibited by AA and EPA. DHA had the opposite effect, increasing mineralization almost by 2 fold. When EFAs were combined, DHA apparently compensated for the inhibitory effect of AA and EPA. Expression of marker genes for bone and lipid metabolisms has been investigated by qPCR and shown to be regulated in pre-osteoblasts exposed to individual PUFAs. Our results show that PUFAs are effectors of fish bone cell lines, altering cell morphology, proliferation and mineralization when added to culture medium. This work also demonstrates the suitability of our *in vitro* cell systems to get insights into mineralization-related effects of PUFAs *in vivo* and to evaluate the replacement of fish oils by vegetable oil sources in fish feeds.

Keywords: Polyunsaturated fatty acids; cell culture; bone; mineralization

Resumo

Um dos objetivos da indústria de aquacultura é a substituição, na dieta dos peixes, de óleos de peixe por óleos vegetais. Sendo que os lípidos, presentes na dieta, alteram a composição de ácidos gordos nos compartimentos de osso, este facto afectaria a produção local de factores que controlam a formação óssea. O conhecimento de mecanismos subjacentes à regulação nutricional do metabolismo ósseo é, no entanto, escasso em peixes. Neste estudo, foram utilizadas duas linhas celulares, derivadas de vértebra da dourada, com capacidade de mineralização *in vitro*, quando submetidas a determinadas condições. Estas exibem um fenótipo de pré-condrócitos (VSa13) e pré-osteoblástos (VSa16) e foram utilizadas para avaliar o efeito de ácidos gordos polinsaturados, selecionados (PUFAs; araquidónico (AA), eicosapentaenóico (EPA) e docosahexaenóico (DHA)) sobre a proliferação celular, mineralização da matriz extra celular (ECM) e expressão genética. Embora se tenham observado alterações morfológicas em ambas as linhas celulares, devido ao tratamento com PUFAs, houve, também, a estimulação da proliferação nas células VSa16, dependendo da dose utilizada e até uma concentração de 100μM. Por outro lado, a proliferação das células VSa13 é inibida em concentrações acima de 10μM. Os tratamentos com AA, EPA e DHA mostraram uma inibição da mineralização nas células VSa13, isoladamente e em combinação. No caso das células VSa16 a mineralização foi afectada, negativamente pelo uso de AA e EPA e, positivamente pelos tratamentos com DHA. Aquando da combinação dos PUFAs, o DHA aparentemente compensou o efeito inibitório dos AA e EPA. A expressão de genes envolvidos no metabolismo do osso e de lípidos foi investigada por qPCR e mostra a existência de regulação em pré-osteoblastos, expostos a PUFAs individuais. Os nossos resultados mostram que os PUFAs alteram a morfologia celular, proliferação e mineralização em linhas de células de peixes derivadas de osso. Este trabalho demonstra, também, a aptidão dos nossos sistemas celulares *in vitro* na obtenção de conhecimento relacionado com o efeito que a utilização de PUFAs tem na mineralização *in vivo*, bem como permite avaliar os efeitos da substituição de óleos de peixe por fontes de óleos vegetais na alimentação de peixes.

Palavras-chave: Acidos gordos polinsaturados; cultura celular; osso; mineralização

Abbreviation list

AA	Arachidonic acid
ALP	Alkaline phosphatase
AR-S	Alizarin red S
BMP	Bone morphogenetic protein
COL1	Type I collagen
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FBS	Fetal bovine serum
LOX	Lipoxygenase
MGP	Matrix Gla protein
MSCs	Mesenchymal stem cells
OC	Osteocalcin
ON	Osteonectin
OP	Osteopontin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE	Prostaglandin E
PLA2	Phospholipase A2
p-NP	p-nitrophenol
p-NPP	p-nitrophenyl phosphate
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
qPCR	Quantitive polymerase chain reaction
RNA	Ribonucleic acid
RUNX2	Runt-related transcription factor 2
TNAP	Tissue non-specific alkaline phosphatase
UV	Ultra violet
ω-3	omega-3 fatty acid
ω-6	omega-6 fatty acid

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I.   Introduction

I.1.   Vertebrate skeleton: Function and structure

Vertebrate skeleton has a wide range of functions. It provides protection for vital organs and mechanical support for the body; it also allows for movement through space through its attachment to muscles. In mammals, it serves as a vital body reservoir of minerals (such as calcium) and houses the bone marrow, source of the hematopoietic cells in adults (Rodan, 2003). Fish skeleton plays similar roles, although it is not a hematopoietic tissue (it does not contain bone marrow) and does not serve as a major calcium reservoir in normal conditions, as fish can retrieve this mineral from the aquatic environment (Lall, 2002).

Vertebrate skeleton is made up of two main types of connective tissue: bone and cartilage. Of particular interest for this work, bone is a dynamic connective tissue with the ability to adapt its mass and morphology to functional demands, to auto repair itself and to rapidly mobilize mineral reserves according to metabolic needs, making this particular type of tissue an example of "structure follows function" (Sommerfeldt *et al.*, 2001). Bone is made up of an organic matrix (25%) an inorganic matrix (calcium-phosphate mineral hydroxyapatite, 70%) and water (5%). This organic matrix, prior to its mineralization, is called osteoid and is mainly consisted of collagen and other proteins that play a role during the mineralization process (*e.g.* osteopontin (OP), osteonectin (ON), osteocalcin (OC) and matrix Gla protein (MGP)) or have important signalling functions (*e.g.* bone morphogenetic proteins (BMPs), growth factors, cytokines, adhesion molecules) (Sommerfeldt *et al.*, 2001).

Bone is formed through two main processes (*n.b.* other modes of ossification exist (Hall, 2005) but will not be discussed here). Endochondral ossification (**Figure 1A**) needs a cartilaginous structure to serve as a mold that will later be degraded and substituted by bone, whereas intramembranous ossification (**Figure 1B**) occurs when mesenchymal cells aggregate and differentiate into osteoblasts that in turn initiate the mineralization process. Bone is consisted of three major cell types: osteoblasts, osteoclasts and osteocytes (**Figure 2**). In mammals, as in fish, osteoblasts originate from the mesenchymal cell lineage and are involved in the synthesis and mineralization of the bone matrix. Osteoblasts are characterized by a large, spherical nucleus and a high content of rough endoplasmic reticulum, Golgi apparatus and mitochondria, allowing the synthesis and secretion of high quantities of bone matrix proteins, *e.g.* type I

collagen (Jayakumar *et al.*, 2010). Osteoclasts are macrophage-like cells that resorb the bone matrix and participate in bone remodelling. Mammals typically possess giant multinucleated osteoclasts exhibiting a strong resorbing activity, whereas in advanced teleost fish osteoclasts are small mononucleated cells with a reduced resorbing activity (**Figure 3**; Witten *et al.*, 2010). In both mammals and fish, osteoclasts secrete tartrate-resistant acid phosphatase, collagenase and other hydrolases that act locally, digesting bone organic matrix (Junqueira *et al.*, 2004). Osteoblasts that become entrapped in the bone matrix differentiate into osteocytes. The matrix collects around osteocyte body and extensions, thus forming gaps and canaliculi in bone. Osteocytes are characterized by reduced endoplasmic reticulum and Golgi apparatus, and a nucleus with condensed chromatin. Although these ultra-structural characteristics indicate low synthetic activity, osteocytes are essential for maintaining the bone matrix (Junqueira *et al.*, 2004).
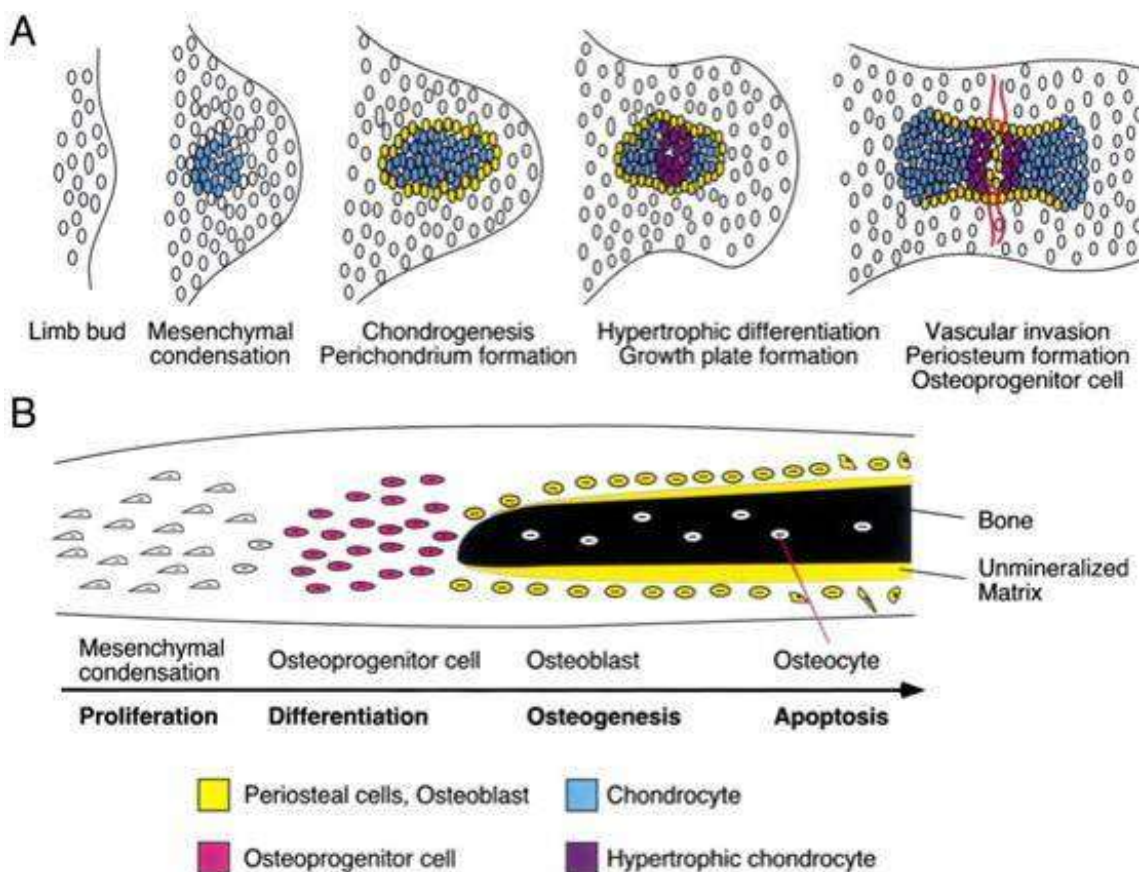
Figure 1. Endochondral (A) and intramembranous (B) ossification. Endochondral ossification begins with the condensation of mesenchymal cells and the differentiation of central cells into chondrocytes, which hypertrophy (purple). Progression to the mature growth plate accompanies the development of the perichondrium (yellow), vascular invasion, and the formation of a center of ossification containing osteoblasts (yellow). During intramembranous ossification, undifferentiated mesenchymal cells condense and differentiate into osteoprogenitor cells (pink), which progress to mature osteoblasts (yellow) responsible for the deposition and mineralization of bone matrix. Osteoblasts either die by apoptosis or are entrapped in the matrix, becoming osteocytes. Adapted from Ornitz (2002).

While basal teleost fish (*e.g.* the zebrafish) have a cellular/osteocytic bone, *i.e.* osteoblast are entrapped into newly synthesized bone matrix and become osteocytes (**Figure 3**), the majority of advanced teleosts (*e.g.* the gilthead seabream) have an acellular/ anosteocytic bone, *i.e.* bone matrix is devoid of osteocytes since osteoblasts receding from the mineralization front never become entrapped (Parenti, 1986; Witten *et al.*, 2004).
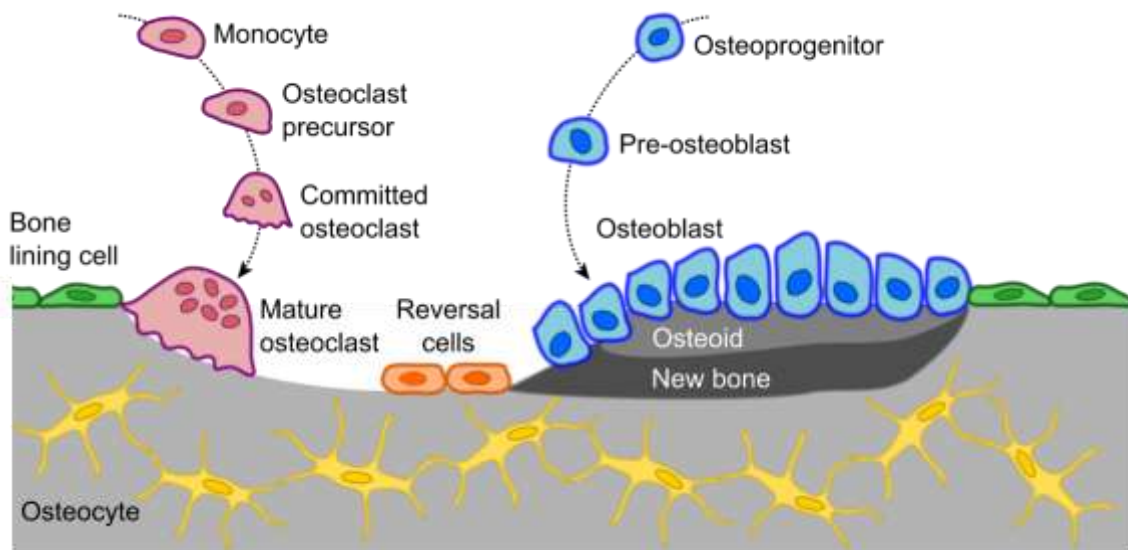

Figure 2. Schematic representation of bone remodeling. Osteocytes (yellow cells), which are embedded within mineralized bone, communicate via ramifications of dendritic processes and orchestrate the spatial and temporal recruitment of the cells that form (osteoblasts) and resorb (osteoclasts) bone. Haemopoietic cells of the monocyte/macrophage lineage differentiate to mature osteoclasts (pink cells) and resorb bone. During the reversal phase (orange cells), osteoblastic progenitors are recruited to the site of resorption, differentiate and synthesize osteoid, and mineralize the new bone matrix (blue cells). Adapted from Nicholls (2012).

Because both osteocytic and anocteocytic bone contain similar cell types (osteoblasts and osteoclasts), because regulatory mechanism, chemical composition, development as well as homology and expression patterns of bone-related have been mostly conserved throughout vertebrate evolution, mammalian and fish bones are considered to perform the same function (Alexander *et al.*, 2007; Renn *et al.*, 2006; Witten *et al.*, 2004). Therefore, most of the results obtained in one system may be extrapolated to the other system.

Of particular interest for this work, piscine bone metabolism is regulated by a variety of factors: parathyroid hormone and parathyroid hormone related protein (Guerreiro *et al.*, 2007); stanniocalcin, a predominant hormone that regulates calcium and phosphate

homeostasis in salmon and several freshwater fish (Wagner *et al.*, 1998); autocrine and paracrine factors, including prostaglandins, cytokines, and growth factors (Lall *et al.*, 2007), and <u>dietary factors and nutrients</u>, including lipids (such as fatty acids) and vitamins.
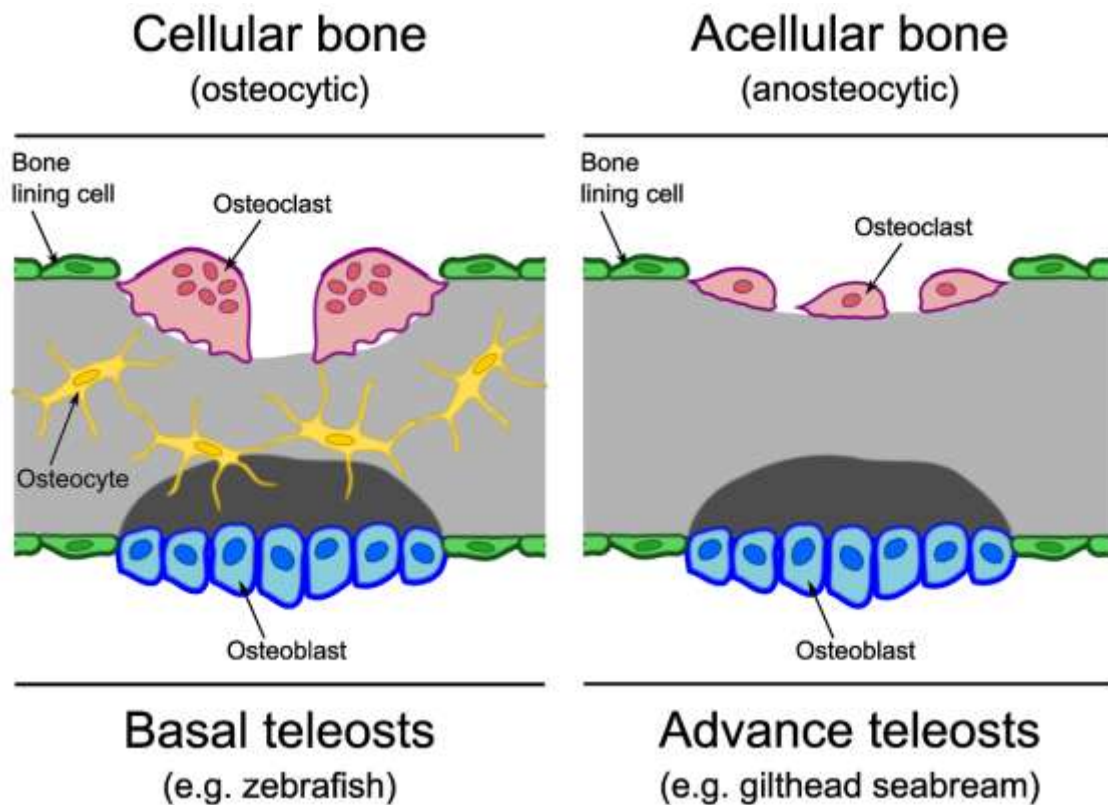
Figure 3. <u>Main differences between cellular and acellular bone.</u> Osteocytes (yellow cells), embedded in bone matrix, osteoclasts (pink cells) resorbing bone, osteoblasts (blue cells) laying down osteoid and bone lining cells (green cells). Adapted from (Witten *et al.*, 2010).

I.2. Dietary lipids and bone metabolism
I.2.1. Lipids

Given their complexity and heterogeneity, lipids are difficult to define. The most common definition is based on their solubility: ''Those substances which are (a) insoluble in water; (b) soluble in organic solvents such as chloroform, ether or benzene; (c) contain long-chain hydrocarbon groups in their molecules; and (d) are present in or derived from living organisms'' (O'Keefe (2008) and references there in). Lipids may be broadly defined as <u>hydrophobic</u> or <u>amphiphilic</u> small molecules. It is possible to divide biological lipids into eight categories: <u>fatty acyls</u> (generic name for fatty acids and their derivatives), <u>glycerolipids</u>, <u>glycerophospholipids</u>, <u>sphingolipids</u>, <u>saccharolipids</u> and <u>polyketides</u> (derived from condensation of ketoacyl subunits); and

sterol and prenol lipids (derived from condensation of isoprene subunits) (Fahy *et al.*, 2009).

The biological functions and structures of lipids are diverse. Fats provide animals with thermal insulation and padding. Waxes in cell walls, exoskeletons, and skins protect the surfaces of some organisms. Biological membranes contain a variety of amphipathic lipids, including glycerophospholipids and sphingolipids. Gangliosides and other glycosphingolipids are located at the cell surface and can participate in cellular recognition. Metabolic energy can be derived from triacylglycerols (fats and oils), that function as intracellular storage molecules. Lipids also have highly specialized functions, *e.g.* steroid hormones regulate and integrate a host of metabolic activities in animals, and eicosanoids exert complex control over many physiological processes, mainly in inflammation or immunity, and as messengers in the central nervous system.

Of particular interest for this work, fatty acids are aliphatic monocarboxylic acids present in animal or vegetable fat, oil or wax. Natural fatty acids commonly have a chain of 4 to 28 carbons (usually unbranched and even-numbered), which may be saturated (do not contain any carbon-carbon double bonds) or unsaturated (contain at least one carbon-carbon double bond). Polyunsaturated fatty acids (PUFAs) play an essential role in the cellular membrane, significantly influencing its fluidity and function. Within the plasma lipoprotein particles, PUFAs serve as the major constituents of phospholipids, triglycerides, and cholesterol esters; they are organized in 3 major families: omega-3 (ω-3), omega-6 (ω-6) and omega-9 (ω-9), all of which are metabolized using the same group of enzymes (Le *et al.*, 2009). Among these PUFAs, arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids have been shown to have significant effects on bone metabolism (Coetzee *et al.*, 2007; Coetzee *et al.*, 2009; Das, 2006; Grey, 2009; Kelly *et al.*, 2003; Kruger *et al.*, 2010; Lall *et al.*, 2007; Watkins *et al.*, 2001b).

I.2.2. Effects of PUFAs on bone metabolism

Emerging evidence from human and terrestrial vertebrate studies supports the hypothesis that dietary lipids play an important role in skeletal biology and bone health (Sun *et al.*, 2003; Watkins *et al.*, 2001a). Several studies have shown that dietary lipids alter the fatty acid composition of bone compartments (Watkins *et al.*, 2000), which would impact the local production of factors influencing bone modelling in animals. In

general, high levels of ω-3 long-chain PUFA enhances bone formation rate. In humans, the mechanism has been largely attributed to reduced <u>prostaglandin E_2</u> (PGE_2) synthesis in bone (Burke *et al.*, 2002; Watkins *et al.*, 2003). The PGE_2 is a potent stimulator of both bone formation and bone resorption and is formed through the metabolism of ω-6 PUFAs. The extent of stimulation appears to be affected in part by PGE_2 concentration, with moderate concentrations elevating bone formation, and high concentrations elevating bone resorption. In terrestrial vertebrates, the ω-3 PUFAs are precursors to PGE_3, which is equally potent to PGE_2 in bone. However, conversion is less effective than for PGE_2 from ω-6 PUFA resulting in lower total PGE levels. In other words, an excessive consumption of ω-6 PUFA could have a negative effect on bone metabolism by increasing bone resorptive activity through elevated endogenous production of PGE_2 (Watkins *et al.*, 2001b).

I.3.   Why study the action of dietary fatty acids on fish bone metabolism?

The aquaculture industry is highly dependent on fish oil as a source of nutrients in fish feeds but the strain on marine fisheries and the increasing costs of fish meal and fish oil (Naylor *et al.*, 2000; Tacon *et al.*, 2008) may jeopardize the future of fish farming. A new trend has recently emerged in fish feeds: the <u>replacement of fish oil by vegetable oil</u>, a resource with a higher availability, a better sustainability and more cost effective than fish oil (Turchini *et al.*, 2009). The use of vegetable oils as the sole lipid source is however unsuitable to the farming of marine fish species due to the low conversion of linoleic and linolenic acids, abundant in vegetable oils, into AA, EPA and DHA which are PUFAs abundant in fish oils (Sargent *et al.*, 1997; Turchini *et al.*, 2009) and essential for marine fish species (Sargent *et al.*, 1993; Sargent *et al.*, 1997). For that reason, successful replacement of fish oil by vegetable oils will only be possible when these fatty acids are present in marine fish feeds in sufficient quantities to meet the essential requirements. Substitution of up to 60% fish oil by vegetable oils did not compromise growth, survival, feed utilisation or major fillet organoleptic properties in gilthead seabream, although it altered the fatty acid composition of flesh (Izquierdo *et al.*, 2005). Dietary lipids were also shown to alter the fatty acid composition of bone compartments in rodents (Watkins *et al.*, 2000), which would impact the local production of factors influencing bone mineralization and possibly trigger skeletal

deformities. In this sense, AA, EPA and DHA have been shown to modulate bone metabolism in terrestrial vertebrates (Poulsen *et al.*, 2007; Watkins *et al.*, 2000; Watkins *et al.*, 2006; Weiler *et al.*, 2002) and further research using fish models is needed.

II. Objectives and experimental set up

To better understand the action of dietary fatty acids on fish bone mineralization, we propose to evaluate the mineralogenic potential of selected PUFAs using a fish cell system capable of *in vitro* mineralization.

II.1. Experimental system: gilthead seabream mineralogenic cell lines

Over 200 fish cell lines (see FICEL database at www.fcma.ualg.pt/edge) have been established since the first report by Wolf and colleagues 50 years ago (Wolf *et al.*, 1962) but only few of them have the *in vitro* capacity to mineralize their extracellular matrix. In fact, most (if not all) of these <u>mineralogenic cell lines</u> have been developed at the EDGE-CCMAR cell culture facilities from calcified tissues of the <u>gilthead seabream</u> <u>*Sparus aurata*</u> (Marques *et al.*, 2007; Pombinho *et al.*, 2004; Rafael *et al.*, 2010), a marine teleost living along the Atlantic coasts of Europe and the Mediterranean Sea (Froese *et al.*, 2003) and one of the most important species in the aquaculture industry in Southern Portugal and the Mediterranean region (Stephanis, 1996).

Among these cell lines, the <u>chondro/osteo-progenitor cell lines</u> VSa13 and VSa16, both capable of extracellular matrix (ECM) mineralization (**Figure 4**), were derived from vertebrae of the gilthead seabream (Pombinho *et al.*, 2004) and have been already used in a variety of studies aiming at investigating bone and cartilage marker gene expression (Conceição *et al.*, 2008; Fonseca *et al.*, 2007; Laizé *et al.*, 2005; Rafael *et al.*, 2006; Tiago *et al.*, 2011b); signalling pathways (Tiago *et al.*, 2008a), transfectability and promoter activity analysis (Braga *et al.*, 2006; Conceição *et al.*, 2008) and proliferative and mineralizing effect of vanadate (Tiago *et al.*, 2008b; Tiago *et al.*, 2011a; Tiago *et al.*, 2011b). Such studies have shown the suitability of these cells to study the effect of certain molecules, such as fatty acids, on proliferation, mineralization and gene expression.
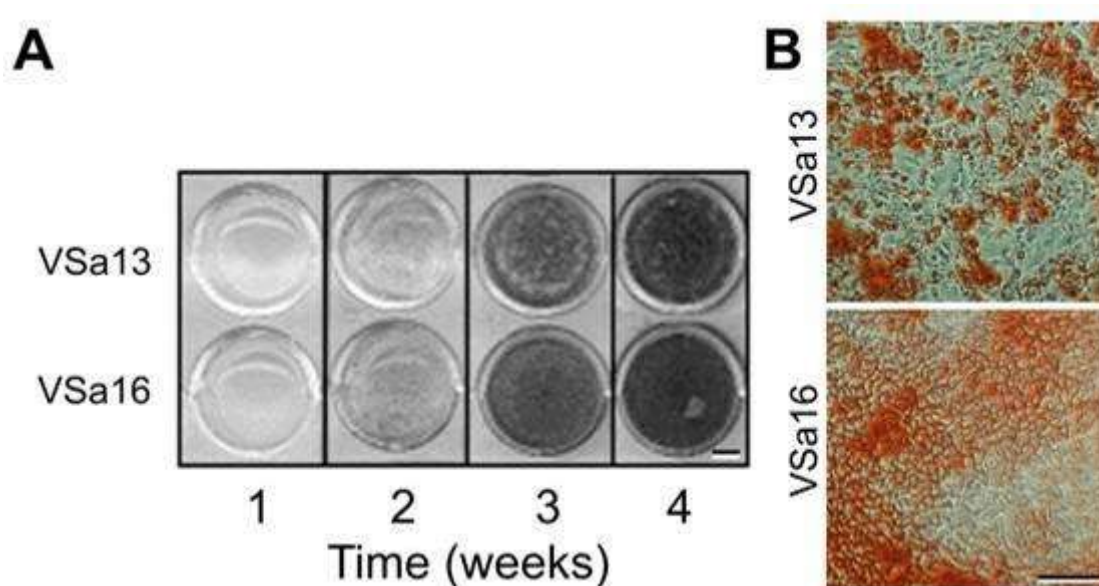
Figure 4. _In vitro_ mineralization of the extracellular matrix of fish skeletal cell lines. Micrographs of von Kossa-stained (A) and Alizarin red-stained (B) VSa13 and VSa16 cell lines undergoing ECM mineralization. Mineral nodules appear in black (A) or in red (B). Bar is 5 mm in A and 50 μm in B.

II.2.   Experimental PUFAs: AA, EPA and DHA

As mentioned above AA, EPA, DHA (**Figure 5A**) and their derivatives (**Figure 5B**) are important modulators of bone metabolism and essential, in dietary terms, for marine fish species. Arachidonic acid (_all-cis_-5,8,11,14-eicosatetraenoic acid; 20:4ω-6), is present in most phospholipids of cellular membranes and a precursor in the production of eicosanoids (_i.e._ prostaglandins, thromboxanes, prostacyclin and leukotrienes) through enzymes including cyclooxygenase, lipoxygenase and peroxidase (Jump, 2002). Eicosapentaenoic acid (5,8,11,14,17-icosapentaenoic acid; 20:5ω-3) is an important constituent of the phospholipids in animal tissues and is the precursor of the series 3 prostaglandins and thromboxanes and series 5 leukotrienes, which have anti-inflammatory effects (Calder, 2009). EPA is the primary fatty acid of fish oil (~20–25% in weight; (Watkins _et al._, 2008) and has been investigated extensively for its action as a competitive inhibitor of arachidonic acid metabolism (James _et al._, 2000). Docosahexaenoic acid (_all-cis_-docosa-4,7,10,13,16,19-hexaenoic acid; 22:6ω-3) contributes to aminophospholipids, phosphatidylserine and phosphatidylethanolamine of cell membranes (Kim, 2007; Salem _et al._, 2001). DHA is known to be the major PUFA in the outer segments of the retina rods and cones, where it can constitute as much as 80% of all the PUFAs (Giusto _et al._, 2000), as well as influence signalling events that are vital to neuronal survival and differentiation (Kim, 2007). DHA is also a primary component of fish oil (~8–20% in weight; Watkins _et al.,_ 2008).
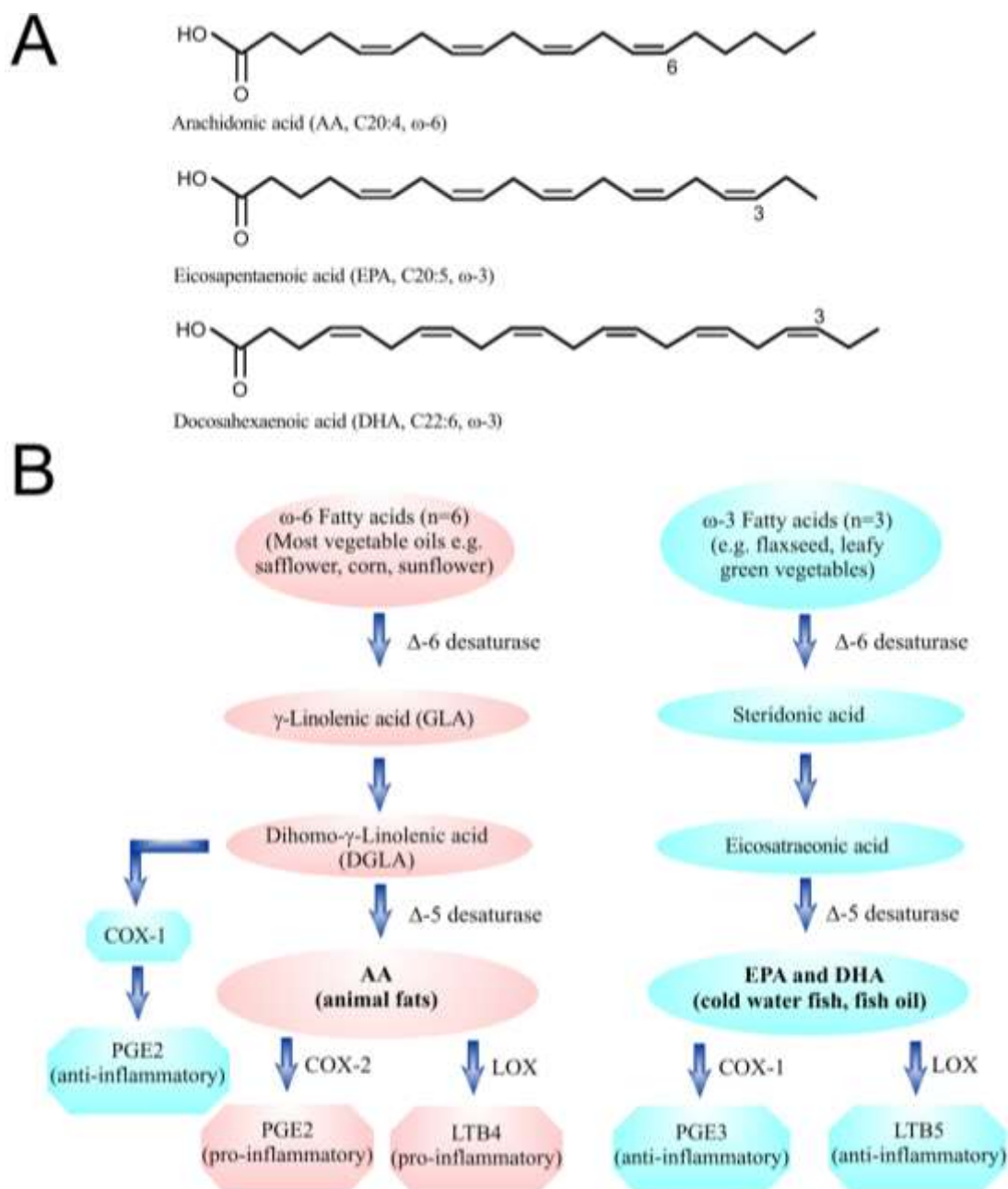
Figure 5. Selected PUFAs (A) and associated metabolism (B). AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; COX, cyclooxygenase; LOX, lipoxygenase; LTB, leukotriene B; PGE, prostaglandin E. Adapted from www.beatcoloncancer.com/fatty_acid_ metabolism.html.

II.3. Endpoints: cell proliferation, ECM mineralization and gene expression

Proliferative and mineralogenic potentials of the above mentioned PUFAs will be determined by direct comparison of untreated versus PUFA-treated cells. While actively dividing cell cultures will be used to test proliferative effect (endpoint: cell number at 10 days), growth-arrested cell cultures will be used to test their mineralogenic effect (endpoints: ECM mineralization, Alkaline phosphatase (ALP) activity, collagen content

at 2.5 weeks). The potential of selected PUFAs in underlineregulating the expression of genes involved in ECM mineralization and fatty acid metabolism will also be tested by quantitative real-time PCR (endpoint: relative gene expression at 2.5 weeks of ECM mineralization). The following marker genes will be investigated: Bone morphogenetic protein 2 (BMP2, essential for bone formation; (Hassan *et al.*, 2006); Matrix Gla protein (MGP, a vitamin K-dependent protein of the extracellular matrix with an inhibitory effect on mineralization and a marker of chondrogenesis; (Boström *et al.*, 2001); Runt-related transcription factor 2 (RUNX2, an osteoblast-specific transcription factor with a crucial role in bone development: (Komori *et al.*, 1997); Type I collagen (COL1, a major structural element in bones, tendons, ligaments, skin, and sclerae, essential for normal bone function; (Jin *et al.*, 2009); Osteopontin (OP or SPP1, a secreted phosphoprotein abundant in newly formed bone and thought to control the sizes and shapes of mineral crystals; (Morinobu *et al.*, 2003; Sodek *et al.*, 2000); Osteonectin (ON or SPARC, a secreted protein, acidic and rich in cysteine and a calcium-binding matricellular glycoprotein secreted by many different types of cells; (Yan *et al.*, 1999); Tissue non-specific alkaline phosphatase (TNAP, an enzyme responsible for the cleavage of the pyrophosphate – a mineralization inhibitor – and a biochemical marker of osteoblast activity; (Addison *et al.*, 2007; Christenson, 1997; Singer *et al.*, 2008); Cyclooxygenase 2 (COX2, a prostaglandin synthase responsible for the initial rate-limiting conversion of arachidonic acid to prostaglandin E_2; (Watkins *et al.*, 2003); 5-Lipoxygenase (5-LOX, an enzyme that generates lipid mediators, such as leukotrienes, promoting bone resorption and inducing osteoclastogenesis; Poulsen *et al.* (2007) and references there in); Phospholipase A2 (PLA2, responsible for the liberation of PUFAs from the phosphoglycerides present in cellular membranes; Tassoni *et al.* (2008) and references there in).

III. Materials and methods

III.1. Materials

Arachidonic, eicosapentaenoic and docosahexaenoic acids were purchased from Cayman Chemical at a purity ≥98%. Stock solutions were prepared under nitrogen vapours in absolute ethanol (Merck) at a concentration of 100 mM then stored at -80ºC. Cell culture reagents and dishes were from Invitrogen and Sarstedt, respectively. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

III.2. Cell maintenance

Cell lines VSa16 and VSa13, derived from the vertebra of the gilthead seabream *Sparus aurata*, were maintained as described previously (Pombinho *et al.*, 2004). Briefly, cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% fungizone, and 2 mM L-glutamine, and incubated at 33ºC in a 10% CO_2 humidified atmosphere. Confluent cultures were sub-divided 1:3 every 3-4 days using 0.2% trypsin-EDTA solution (in PBS. 1× PBS is 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). Cultures between passage 130 and 150 were used in this work.

III.3. Cell counting

Cells were counted microscopically before each seeding from 10 μl of trypsinized-cell suspension using a double Neubauer counting chamber (Brand), as described in Phelan (2007).

III.4. Cell proliferation assay

Cells were seeded in 96-well plates at 1.5×10^3 cells per well. After 16 h, culture medium was replaced with fresh medium containing ethanol (vehicle) or PUFAs, and was renewed every second day. Cell proliferation was determined after 10 days of treatment using the CellTiter 96 non-radioactive proliferation assay kit (Promega). This assay is based on the ability of dehydrogenases found in metabolically active cells to convert the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophen-yl)-2H-tetraz-olium salt into a formazan product soluble in culture medium. Formazan production, directly proportional to the number of living cells in culture, was measured by spectrophotometry at 490 nm using a Synergy 4 microplate reader (BioTek).

III.5. Oil red O staining

Adipocyte-like differentiation was evaluated through Oil red O staining in VSa13 and VSa16 cell cultures exposed to PUFAs for 10 days. Oil red O stock solution (0.5% (w/v) in isopropanol) was first diluted 6:4 in distilled water, allowed to stand for 10 min, and then filtered through Whatman #1 paper. Cells were washed with PBS, fixed in 10% (v/v) formalin (in PBS) for 10 min at room temperature, stained for 1 h at room temperature then rinsed with distilled water and allowed to air dry. The coloration was observed using an Axiovert 25 inverted microscope (Zeiss).

III.6. Extracellular matrix mineralization

ECM mineralization was induced in confluent cell cultures by supplementing medium with 50 μg/mL of L-ascorbic acid, 10 mM β-glycerophosphate and 4 mM calcium chloride. Ethanol (vehicle) or PUFAs were added at the time of medium renewal, twice a week. At appropriate times, mineral deposition was revealed through Alizarin red S (AR-S) staining (Puchtler *et al.*, 1969). Cells were washed 3 times with ice-cold PBS, fixed with 4% (v/v) formalin (in PBS) for 1 h at 4°C, washed 3 times with distilled water and finally incubated with 40 mM AR-S at pH 4.2 for 15 min. Excess of dye was removed by washing cells 5 times with distilled water and calcium-bound dye was solubilized in 10% (w/v) cetylpyridinium chloride (in 10 mM sodium phosphate buffer at pH 7) and quantified by spectrophotometry at 550 nm using a microplate reader.

III.7. Sirius red staining

Collagen fibres were detected using Sirius red according to the method described by Tullberg-Reinert *et al* (1999). Cells were washed 3 times with PBS and fixed using Bouin's fluid (prepared by mixing 15 mL of saturated aqueous picric acid with 5 mL of 35% formalin and 1 mL of glacial acetic acid). After fixation, cell layer was washed with running tap water during 15 min, air dried, then incubated for 1 h with Sirius red staining solution. Excess of dye was removed by extensive washing with 0.01 N HCl and collagen-bound dye was solubilized in 0.1 N NaOH and quantified by spectrophotometry at 550 nm using a microplate reader.

III.8. Alkaline phosphatase activity

Enzymatic activity of ALP was determined according to Tiago *et al* (2008a). Cells were washed 3 times with PBS, scrapped in the presence of 200 µl of 0.1% Triton X-100 then centrifuged for 1 min at 16,000×*g*. ALP activity was determined at 33°C from initial rates of *p*-nitrophenyl phosphate (*p*-NPP) hydrolysis into *p*-nitrophenol (*p*-NP). Cell extract was added to a reaction buffer (0.5 mM p-NPP, 55 mM glycine and 0.55 mM MgCl$_2$, pH 10.5) and the production of *p*-NP was monitored by spectrophotometry at 405 nm using a microplate reader. ALP activity was normalized with total protein content and expressed in pmol/min/µg of protein. Total protein content was determined from cell extract by spectrophotometry at 595 nm using Bio-Rad Protein assay.

III.9. RNA extraction and quantitative real-time PCR

Total RNA was extracted from cell cultures using Ambion TRI-Reagent and purified using QIAGEN RNeasy Mini kit. RNA integrity and concentration were assessed on 1% formaldehyde/agarose gels and by UV spectrophotometry, respectively. Total RNA (1 µg) was submitted to DNase I treatment (Promega) for 30 min at 37°C and reverse-transcribed for 1 h at 37°C using M-MLV reverse transcriptase (Invitrogen), oligo-d(T) universal primer [5'-ACGCGTCGACCTCGAGATCGATG(T)$_{13}$-3'] and RNaseOUT (Invitrogen). Quantitative real-time PCR (qPCR) assays were performed using the StepOnePlus Real-Time PCR system (Applied Biosystems). Reaction mixture containing 1× SsoFast EvaGreen supermix (Bio-Rad), 0.2 µM of forward and reverse gene-specific primers and 1:50 dilution of reverse-transcribed RNA was submitted to the following PCR conditions: 2 min at 95°C then 50 × [20 s at 95°C, 20 s at 68°C]. Gene expression was normalized using ribosomal protein L27a housekeeping gene and relative quantification was determined using the ΔΔCt method (Pfaffl, 2006). Primers used in this study are listed in **Table 1**.

III.1. Statistical analysis

Data was expressed as mean ± standard deviation of at least 2 independent experiments, each performed in quadruplicate, and was analyzed with one-way ANOVA (analysis of variance) for cell proliferation, Alizarin red staining and gene expression and student's t-test for Sirius red staining and ALP activity. Differences were considered to be statistically significant for *p*<0.05.

Table 1. <u>Primers used for qPCR analysis of gilthead seabream gene expression</u>

Gene name (acronym)	GenBank accession No.	Sequence (5'-3')
5-Lipoxygenase (5-LOX)	FP334124	FW:GCACGCCGCCGTCAACTTCG RV:ACTCGTTCTCCTGATACTGGCTGAGGG
β-actin (bACT)	X89920	FW:CTTCCTCGGTATGGAGTCCTGCGG RV:TCCTGCTTGCTGATCCACATCTGCT
Bone morphogenetic protein 2b (BMP2b)	AY500244	FW:GCGAAGGGCATGGGCTGTCTTTGGT RV:AGCAGTACCACGAGAGAGCGGACCAC
Collagen type 1 alpha 1 (COL1A1)	DQ324363	FW:GAGATGGCGGTGATGTGGCGGAGTC RV:GCCTGGTTTGGCTGGATGAAGAGGG
Cyclooxygenase- 2 (COX2)	(*)	FW:AGGGAAAACGCCATCTTTGGGGAA RV:AAGGATGCCACGGGACAGGGTC
Matrix Gla protein (MGP)	AY065652	FW:GTGAGGACTACTCGCCCTGCCGCTTC RV:CGGGAGATGCCACAGAACAACTACA
Osteonectin (ON)	AY239014	FW:AGGAGGAGGTCATCGTGGAAGAGCC RV:GTGGTGGTTCAGGCAGGGATTCTCA
Osteopontin (OP)	AY651247	FW:AAAACCCAGGAGATAAACTCAAGACAACCCA RV:AGAACCGTGGCAAAGAGCAGAACGAA
Phospholipase A2 (PLA2)	(**)	FW:CGGGAACTCCTCTGGACGACTTGG RV:TGGTTCTGAGCGAAGCAGTGAGCC
Ribosomal protein L27a (RPL27a)	AY188520	FW:AAGAGGAACACAACTCACTGCCCCAC RV:GCTTGCCTTTGCCCAGAACTTTGTAG
Runt-related transcription factor 2 (RUNX2)	AJ619023	FW:CGGACCGACAGCCCCAACTTTCT RV:TAGTTCTCGTCGTTGCCCGCCATA
Tissue non-specific alkaline phosphatase (TNAP)	AY266359	FW:CATCGCAACCCTTTTCACAGTCACCCG RV:AACAGTGCCCAAACAGTGGTCCCATTAGC

* reconstructed from expressed sequence tag (EST) sequences AM296029 and HS983334

** reconstructed from EST sequences FG263263, FM151852, FM151574, FP333360 and FM153485

IV. Results and discussion

IV.1. PUFAs stimulate the proliferation of VSa13 and VSa16 cells and change their morphology

Initial tests were performed to determine PUFA concentrations that would not adversely affect the proliferation of VSa13 and VSa16 cells upon chronic exposure. Proliferation/survival rates were determined after 10 days of exposure of sub-confluent cell cultures and for PUFA concentrations of up to 100 µM.
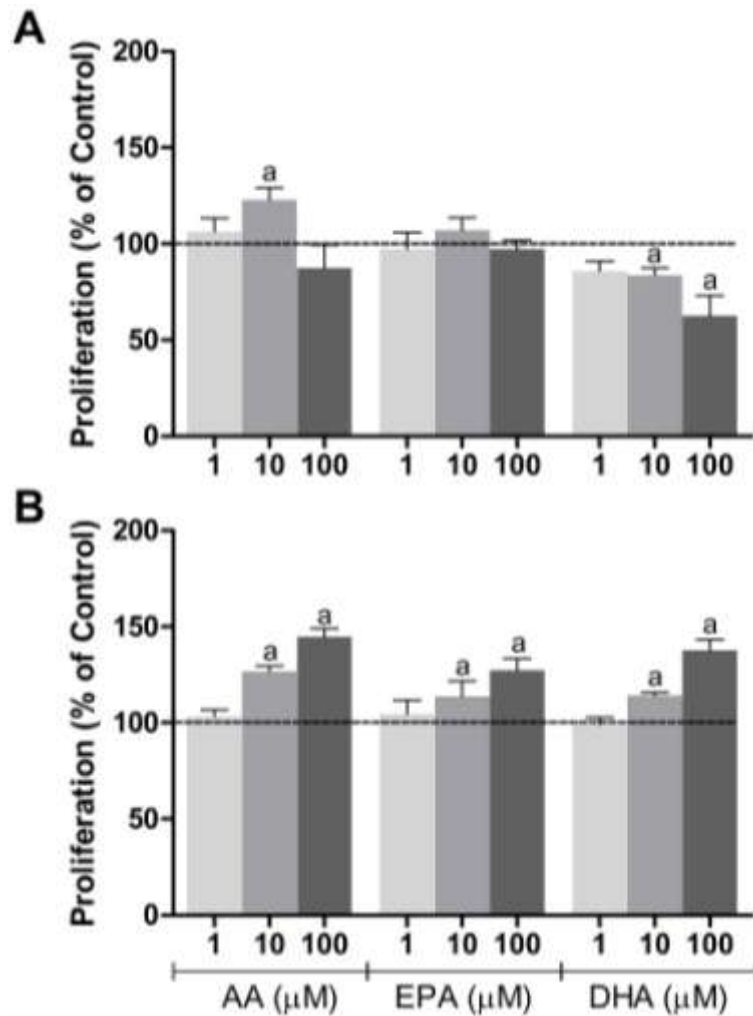

Figure 6. <u>Effect of polyunsaturated fatty acids (1-100 µM) on VSa13 (A) and VSa16 (B) cell proliferation/survival</u>. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Value of vehicle-treated samples was set to 100% and is indicated with a dotted line. Values are the average of at least 8 biological replicates and are presented ± standard deviation. a indicates values statistically different (one-way ANOVA $p<0.05$) from control.

While AA, EPA and DHA showed a dose-dependent stimulatory effect on VSa16 cell proliferation, AA and DHA concentrations higher than 10 µM exhibited an adverse effect on the proliferation of VSa13 cells (**Figure 6**) as well as an increase in cell death

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observed microscopically (results not shown). Although not toxic in the time frame used for proliferation assays, all PUFAs at 100 μM induced transient changes in both VSa13 and VSa16 cell morphology in the first days of treatment – increased size and spherical shape – and the brief appearance of vesicles in few cells, indicative of an adipocyte-like phenotype (**Figure 7**). Osteoblasts and adipocytes both originate from mesenchymal stem cells (MSCs) and osteoblasts have been reported to be able to transdifferentiate into adipocytes upon exposure to long chain fatty acids (Diascro *et al.*, 1998).
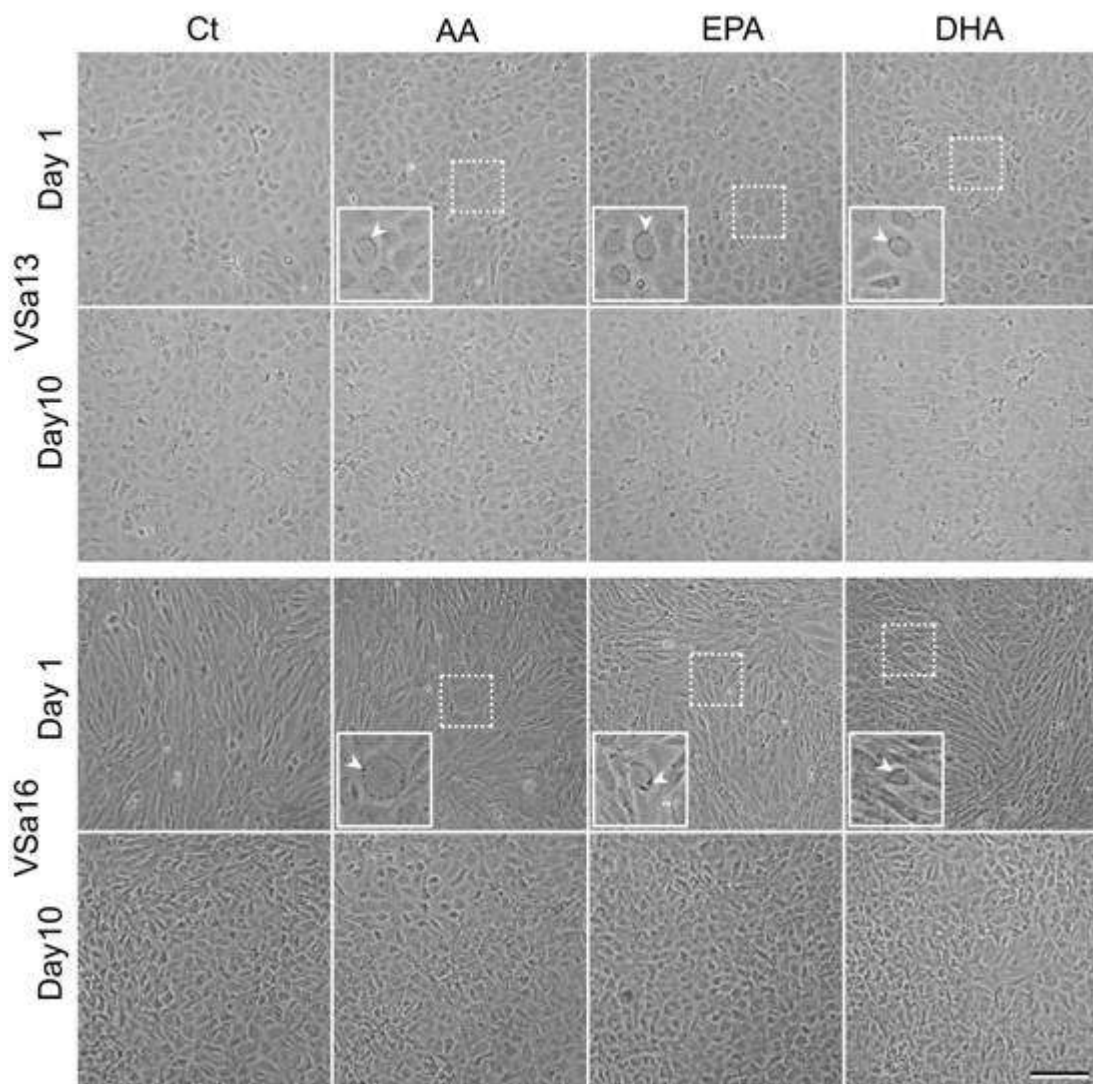
Figure 7. <u>Phase-contrast micrographs of VSa13 and VSa16 cells</u> at day 1 and 10 of being cultured in regular medium (Ct) or in medium supplemented with 100 μM of arachidonic acid (AA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Bar is 50 μm. Inserts are ×2 magnifications of selected areas. Arrowheads indicate intracellular vesicles.

VSa13 and VSa16 chondro/osteo progenitor cells treated with 100 μM of PUFAs for 10 days were stained with Oil red O, a dye specific for triglycerides and cholesteryl oleate

(Ramírez-Zacarías *et al.*, 1992). Vesicles were negative for Oil red O staining but also transient (not visible at the end of the treatment) indicating that they are most likely not related to adipocitic trans-differentiation of chondro/osteo progenitor cells and probably represent an adaptive stage of the cells to the high amounts of fatty acids.
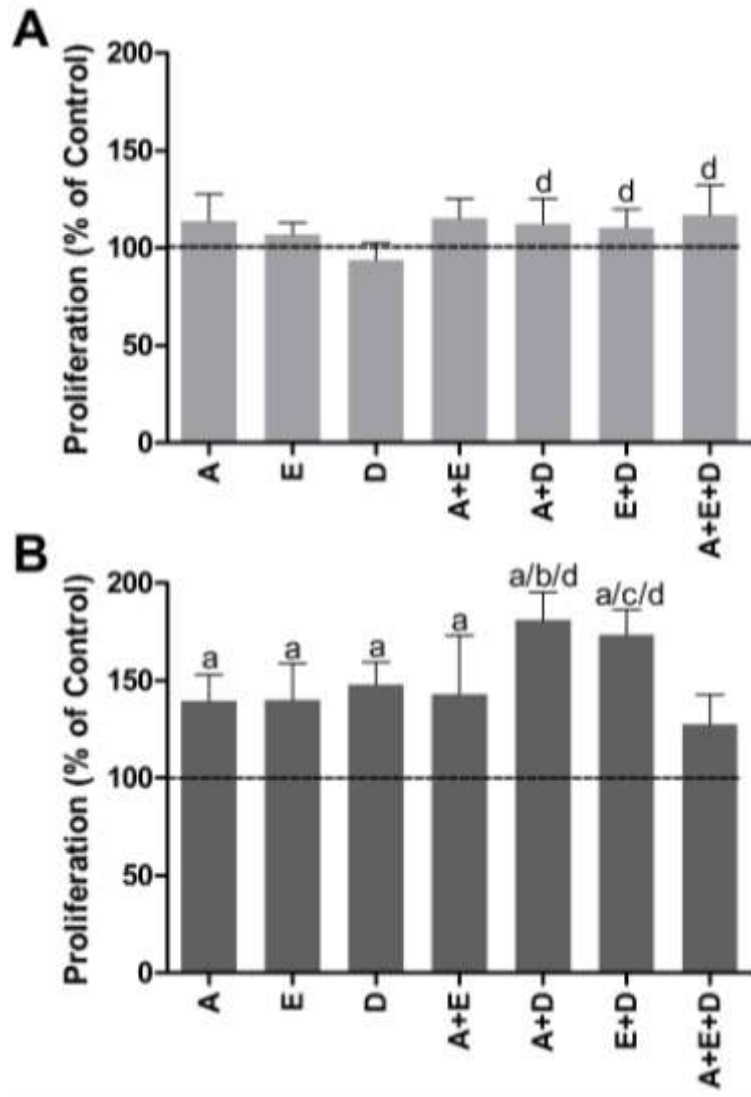

Figure 8. Effect of polyunsaturated fatty acids on VSa13 (10 μM, **A**) and VSa16 (100 μM, **B**) cell proliferation. A, arachidonic acid; E, eicosapentaenoic acid; D, docosahexaenoic acid. Proliferation of vehicle-treated cells was set to 100% and is indicated with a dotted line. Values are the average of at least 8 biological replicates and are presented ± standard deviation. a, b, c and d indicate values statistically different (one-way ANOVA $p<0.05$) from control, A, E and D values, respectively.

Single PUFAs (10 μM) did not alter VSa13 cell proliferation significantly, although when used in combination the treatments containing DHA had a slight increase of proliferation when compared to DHA alone (**Figure 8A**). PUFAs (100 μM) significantly stimulated VSa16 cell proliferation (**Figure 8B**) by 35-45% for single PUFA and 60-80% for 2-by-2 combinations. The combination of all three PUFAs did

not result in a stronger stimulation, possibly because the high PUFA load (100 μM for each PUFA, 300 μM in total) may induce a chemical strain affecting cell proliferation. Few studies have reported an effect of PUFAs on the morphology and proliferation of bone-derived cell lines. While Fujimori *et al.* (1989) showed that AA (100 μM) stimulated the growth of mouse osteoblast-like MC3T3-E1 cells and rat osteogenic sarcoma UMR106 cells, Coetzee *et al* (2007) showed that AA (66 μM) and DHA (61 μM) both inhibited the proliferation of human osteosarcoma MG-63 cells and MC3T3-E1 cells. In both studies, PUFAs had no effect on cell morphology. It is clear from available data that AA, EPA and DHA modulate bone cell proliferation but the question remains whether they have a pro- or an anti-proliferative effect. Differences observed may be related to differences in cell types, in the degree of cell differentiation or in cell culture conditions (*e.g.* the composition of the serum, which brings growth factors, varies from batch to batch). They may also depend on the species at the origin of each cell line. In this sense, the high levels of PUFAs that are usually present in fish tissues (Lall *et al.*, 2007) may trigger a different response of fish bone cells to PUFA treatment.

IV.2. Anti-mineralogenic (AA and EPA) and pro-mineralogenic (DHA) effects of PUFAs

Mineralogenic effects of PUFAs were assessed by supplementing culture medium of mineralizing VSa13 and VSa16 cells with AA, EPA and/or DHA. In VSa13 cell cultures, single PUFAs and 2-by-2 combinations inhibited ECM mineralization by approximately 80% (**Figure 9A**), while the combination of all PUFAs inhibited ECM mineralization to a lesser extent (66%). Similarly, AA and EPA strongly inhibited VSa16 ECM mineralization by 87% but DHA had the opposite effect, stimulating ECM mineralization by almost 100% (**Figure 9B**). When PUFAs were combined 2-by-2, ECM mineralization in cell cultures exposed to AA + EPA was decreased to levels similar to those in cultures exposed to AA or EPA. When combined with DHA, AA and EPA only inhibited ECM mineralization by 18% and 30%, respectively. When all PUFAs were combined, levels of mineralization were similar to those of control cell cultures. From these results, we concluded that DHA has a pro-mineralogenic effect and can apparently compensate, to some extent, the anti-mineralogenic effect of AA and EPA in osteoblast-like cells.
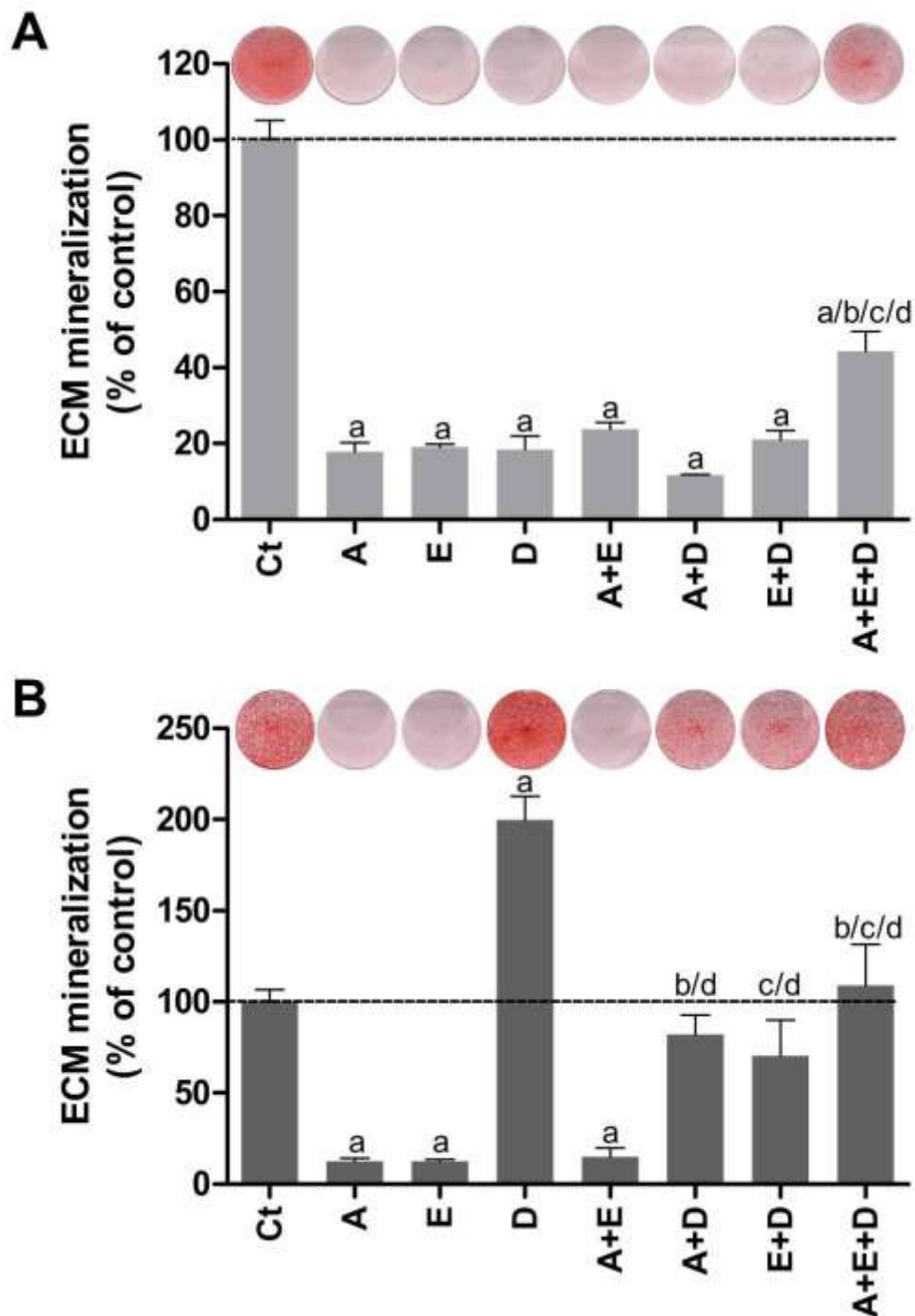
Figure 9. Effect of polyunsaturated fatty acids on VSa13 (10 μM, **A**) and VSa16 (100 μM, **B**) extracellular matrix mineralization. Ct, control; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. ECM mineralization of vehicle-treated cells was set to 100% and is indicated with a dotted line. Values are the average of at least 4 biological replicates and are presented ± standard deviation. a, b, c, and d indicate values statistically different (one-way ANOVA $p<0.05$) from Ct, A, E and D respectively. Representative pictures of Alizarin red-stained cells are presented above each treatment.

Anti-mineralogenic effect of AA and EPA may be related to an increase in prostaglandins, a downstream product of AA and EPA, known to inhibit the synthesis of collagen fibres and ECM mineralization in osteoblasts (Fall *et al.*, 1994; Harrison *et al.*,

1994; Kajii *et al.*, 1999; Ogiso *et al.*, 1992). Similarly, pro-mineralogenic effect of DHA in VSa16 cells may be related to a decrease in prostaglandin levels. It has been shown that pre-incubation of cultured human decidual cells with DHA decreased prostaglandin production by up to 80% (Roman *et al.*, 2006) and that DHA improves bone mass by increasing the accretion of calcium in bone of growing male rats (Kruger *et al.*, 2005). Our *in vitro* results in fish are consistent with those reported in mammals, *i.e.* pro- and anti-mineralogenic effect of DHA and AA/EPA, respectively, and we propose that prostaglandins could also play a similar role in fish, although this will need to be further tested.

To better understand the pro-mineralogenic effect of DHA on VSa16 cells, assays were performed to determine whether this effect resulted from altered collagen synthesis or alkaline phosphatase activity, two parameters essential to bone formation and matrix mineralization. VSa16 cells were mineralized in the presence of DHA (100 μM) and stained with Sirius red, a method that has been described as highly specific and sensitive in the assessment of collagen synthesis by cells (Tullberg-Reinert *et al.*, 1999), or cellular extracts were recovered for enzymatic analysis. In agreement with its pro-mineralogenic activity, DHA slightly stimulated total collagen production (**Figure 10A**), a finding that is in agreement with previous reports indicating a positive effect of ω-3 PUFAs on collagen synthesis in avian epiphyseal chondrocytes (Seifert *et al.*, 1997). This effect may be due to the inhibition of PGE_2 production (known to decrease collagen synthesis in a osteoblasic cell line; (Fall *et al.*, 1994) by ω-3 PUFAs (Bagga *et al.*, 2003). On the contrary, ALP activity was reduced by almost 50% (**Figure 10B**) upon DHA treatment mineralizing conditions. ALP is mostly needed in the initial stages of mineralization to supply cells with organic phosphate and remove pyrophosphate inhibitory strain (Addison *et al.*, 2007); its production decreases as mineralization progresses (Beck *et al.*, 2000). At the time of ALP activity measurement, extracellular matrix of control cells were still undergoing mineralization, while cell cultures supplemented with DHA were at a later stage of mineralization when ALP is needed to a lesser extent.
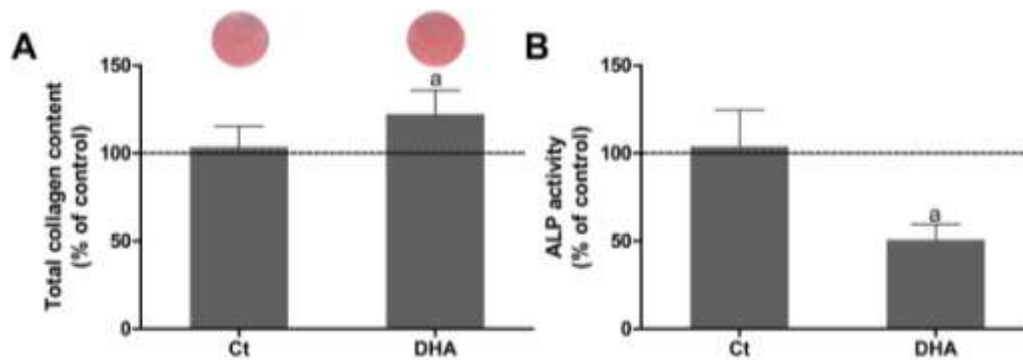
Figure 10. Effect of DHA (100 μM) on collagen synthesis (**A**) and alkaline phosphatase activity (**B**) in mineralizing VSa16 cell cultures. Ct, control; DHA, docosahexaenoic acid. Levels of Sirius red staining and ALP activity in vehicle-treated cells was set to 100% and is indicated with a dashed line. Values are the average of at least 4 biological replicates and are presented ± standard deviation. a, indicates values statistically different (Student's t-test $p<0.05$) from Ct. Representative pictures of Sirius red-stained cells are presented above each treatment.

IV.3. Gene expression regulation by PUFAs in VSa16 cells

In order to further study the pro- and anti-mineralogenic action of specific PUFAs, expression of various genes related to mineralization and fatty acid metabolism was analyzed by qPCR in PUFA-treated VSa16 cells (**Figure 11**). Expression of PLA2 and 5-LOX genes could not be detected in VSa16 cells, transcript levels being very low (i.e. lower than qPCR detection limit) or gene-specific primers being not suitable for qPCR; both genes were therefore excluded from expression analysis.

Type I collagen is the major structural component of osteoblast ECM (Bou-Gharios *et al.*, 2008) and the gene coding for α1 chain (COL1A1) has been shown to be highly expressed in pre-osteoblastic VSa16 cells (V. Laizé personal communication). Although COL1A1 gene expression was slightly higher in DHA-treated cells than in control cells, no significant change in gene expression was observed among PUFA-treated cells. Osteonectin (ON) is a matricellular glycoprotein with calcium binding domains and it has been associated with the mineralization of osteoblast ECM (Yan *et al.*, 1999). Down-regulation of the seabream gene has been observed during *in vitro* mineralization of VSa16 cells (Laizé *et al.*, 2005); however none of the PUFAs significantly affected ON gene expression. Matrix Gla protein (MGP) is a vitamin K-dependent protein functioning as an inhibitor of ECM mineralization (Luo *et al.*, 1997); it is poorly expressed in VSa16 cells (V. Laizé personal communication). All PUFAs down-regulated the expression of MGP gene to 35-40% of control value but no significant change in gene expression was observed among PUFA-treated cells. Osteopontin (OP) is a highly phosphorylated sialoprotein and a prominent component of bone and teeth mineralized ECM (Sodek *et al.*, 2000); a strong up-regulation of seabream gene has

been observed during the *in vitro* mineralization of VSa16 cells (Tiago *et al.*, 2011b). While OP gene expression was down-regulated upon exposure to AA (40%) and EPA (70%), it remained unaltered in DHA-treated cells. In agreement with expression and mineralization data presented here, the down regulation of OP gene expression by EPA has been previously reported and associated with a reduced vascular calcification in rats (Kanai *et al.*, 2011). Runt-related transcription factor 2 (RUNX2) is a key transcription factor in osteoblast differentiation (Ducy, 2000). While levels of gene expression were apparently lower in EPA- and DHA-treated cells than in control cells, no significant change in gene expression was observed among PUFA-treated cells. Similar results were reported during vascular calcification in rats that were administered EPA (Kanai *et al.*, 2011). Bone morphogenetic protein 2 (BMP2) is a glycosylated protein involved in the induction of osteoblast differentiation and ECM mineralization through BMP signalling pathway (Takuwa *et al.*, 1991; Wang *et al.*, 1988); seabream gene is expressed in VSa16 cells but is not regulated during *in vitro* mineralization (Rafael *et al.*, 2006). While BMP2 gene expression was unaltered in AA-treated cells, it was down-regulated by EPA (60%) and up-regulated by DHA (200%). Cyclooxygenase 2 (COX-2) is a membrane-associated heme-containing bifunctional enzyme responsible for prostaglandin production from fatty acids such as AA and EPA (Rouzer *et al.*, 2003). While COX2 gene expression was unaltered in AA-treated cells, it was up-regulated by both EPA (40%) and DHA (170%). An increase of COX2 gene expression upon EPA treatment has been previously reported in mammalian cell systems (Maldve *et al.*, 2000; Nieves *et al.*, 2006), but there is no report, to the best of our knowledge, of a similar stimulation by DHA. On the contrary, available data indicate a decrease of COX2 gene expression upon DHA treatment in human colon cancer cells (Swamy *et al.*, 2004) and saphenous vein endothelial cells (Massaro *et al.*, 2006). Tissue non-specific alkaline phosphatase (TNAP) is a membrane bound glycosylated enzyme involved in the hydrolysis of pyrophosphate, an inhibitor of tissue mineralization (Addison *et al.*, 2007) and a biochemical marker of bone turnover (Balcerzak *et al.*, 2003; Orimo, 2010). While TNAP gene expression was unaltered in AA-treated cells, it was up-regulated by both EPA (46%) and DHA (180%); this up-regulation is in agreement with the stimulation of alkaline phosphatase activity by ω-3 PUFAs reported in various studies (Kruger *et al.*, 2010) but contradicts the observed decrease in enzyme activity stated previously. Although it should be tested in future experiments, we propose that this discrepancy may be related to the fact that we measured total ALP

activity, *i.e.* activity of all alkaline phosphatases expressed by VSa16 cells, while gene expression was specifically targeted for the tissue non-specific ALP (TNAP).
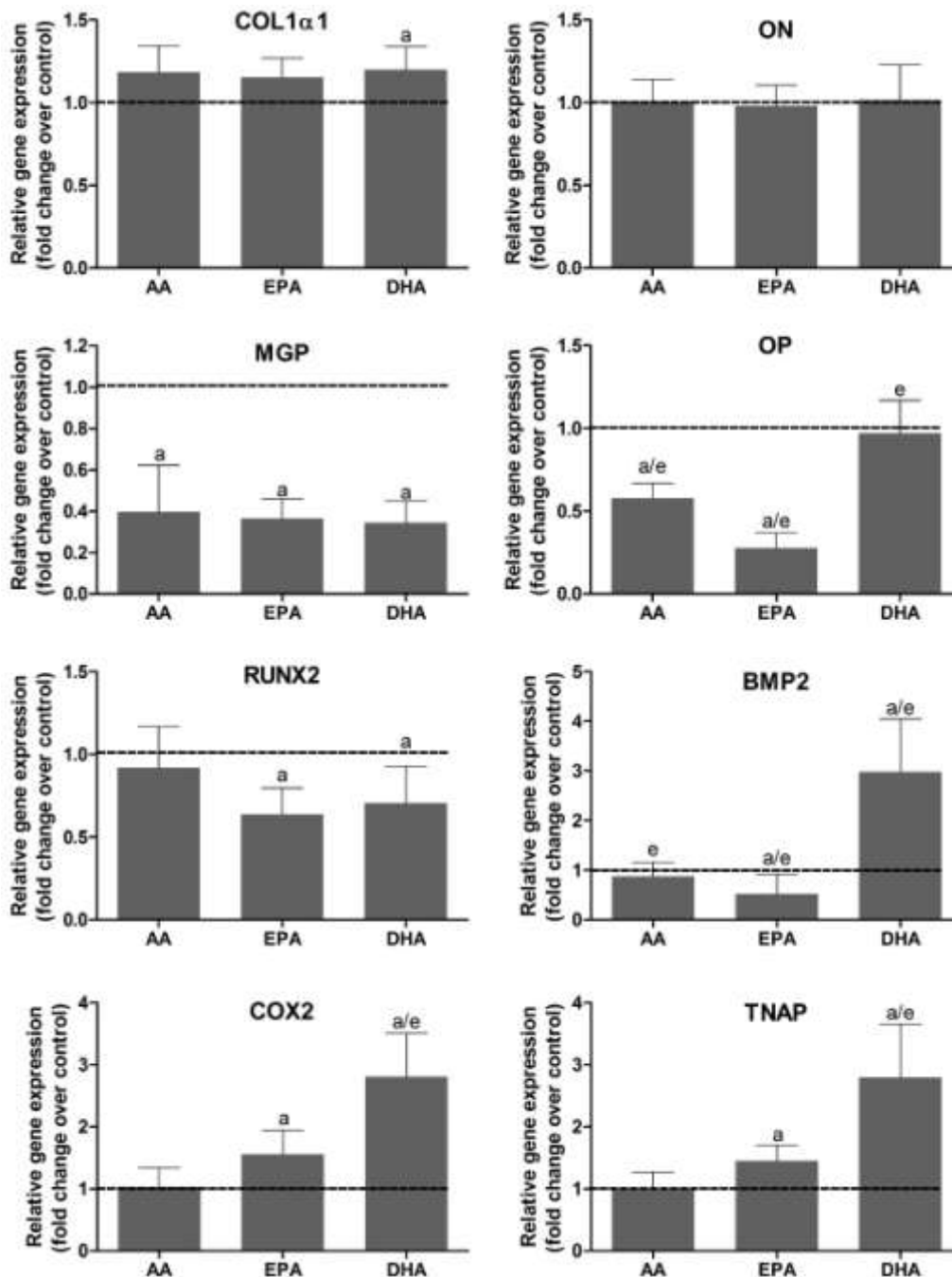

Figure 11. Effect of PUFAs (100 μM) on mRNA levels in 2.5-week mineralizing VSa16 cells. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; COL1A1, type I collagen α1; OP, osteopontin; TNAP, tissue non-specific alkaline phosphatase; ON, osteonectin; RUNX2, runt-related transcription factor 2; COX2, cyclooxygenase 2; BMP2, bone morphogenetic protein 2; MGP, matrix Gla protein. Levels of mRNA from vehicle-treated cells were set to 1 and are indicated with a dashed line. Values are presented as mean ± standard deviation calculated from 3 biological replicates (the value of each biological replicate is the mean of at least 4 technical replicates). a and e indicate values statistically different (one-way ANOVA $p<0.05$) from control and PUFA-treated cells, respectively. PCR efficiency (EFF%) for each of the genes assayed in this study is 101 (COL1A1), 103 (OP), 96 (TNAP), 110 (ON), 85 (RUNX2), 106 (COX2), 84 (BMP2), 100 (MGP) and 103 (RPL27a).

The next step in the analysis of these expression data was to try to make sense of the different and sometime opposite effects observed upon fatty acid treatment in relation to their mineralogenic effect. Data for COL1A1, ON, MGP and RUNX2 genes were not considered since changes in gene expression upon PUFA treatment were not significant or inexistent; they will therefore likely not play a role in the mineralogenic effects of PUFAs. Data for OP, BMP2, TNAP and COX2 genes were further analyzed. Concomitant increase in BMP2 and TNAP gene expression in DHA-treated cells may indicate a stimulation of VSa16 cell differentiation towards osteoblast lineage and a consequent positive effect on ECM mineralization. However, opposite effects in EPA-treated cells or no effect in AA-treated cells are not sufficient to explain anti-mineralogenic effect of these PUFAs. In this sense, the strong decrease in OP transcript may trigger a decrease in ECM mineralization, which is not seen in DHA-treated cells. Increase in COX2 gene expression in EPA- and DHA-treated cells may indicate the stimulation of prostaglandin production. According to available data (Kajii *et al.*, 1999; Kruger *et al.*, 2010), an increase in prostaglandin synthesis would negatively affect the mineralization of VSa16 cells; while it seems to be the case in EPA-treated cells, results from AR-S staining for DHA-treated cells show the opposite. The possible competition between AA and DHA for COX2 cyclooxygenase site and the consequent impairment of prostaglandin biosynthesis (Ringbom *et al.*, 2001) may trigger, in our cell system, a compensatory mechanism resulting in an up-regulation of COX2 gene expression. The apparent contradiction between COX2 expression data and ECM mineralization data upon DHA treatment will need to be further studied and additional data, *e.g.* prostaglandin levels will need to be collected.

V. Conclusions and future perspectives

Our results show that essential fatty acids (*i.e.* AA, EPA and DHA) are effectors of fish bone cell lines, altering cell morphology, proliferation, mineralization and gene expression patterns when added to culture medium. PUFA effects were dose dependent (100 μM inhibited VSa13 proliferation while VSa16 proliferation was stimulated), molecule and cell type-specific (All PUFAs decreased VSa13 mineralization while on the other hand DHA and AA/EPA have opposite effects on VSa16 mineralization), process-specific (additive effect on VSa16 proliferation and compensatory effect during mineralization) and in some cases, target gene-specific (apparent opposite regulation of

TNAP, BMP2 and COX2 gene expression by AA/EPA and DHA). This work also demonstrates the suitability of these *in vitro* cell systems to get insights into mineralization-related effects of PUFAs *in vivo* and eventually to evaluate the effects in bone of the replacement of fish oils by vegetable oil sources in fish feeds. Some of the results presented here support previous findings in established mammalian cell systems, some are contradictory; cellular response to fatty acids may depend on the maturation of the cell but also on the species at the origin of the cell lines.

Future studies should aim at (i) determining whether proliferative and mineralogenic activity of selected PUFAs in gilthead seabream VSa cells can be generalized to other mineralogenic cell systems/ fish species (*e.g.* using mineralogenic cell lines derived from calcified tissues of the Atlantic salmon (*Salmo salar*) or the Senegalese sole (*Solea senegalensis*) or other gilthead seabream mineralogenic cell lines derived from branchial arches and lower jaw, all developed and available at EDGE-CCMAR laboratory) and also (ii) measuring precisely the amount of PUFAs that will be up taken and metabolized and participate in proliferative or mineralogenic activity (*e.g.* measuring PUFAs in cell extracts by gas chromatography–mass spectrometry).

Identification of the metabolic pathways affected by PUFAs could help understand how PUFAs exert their effect on cellular proliferation, ECM mineralization and gene expression. For this, the supplementation of agonist and antagonists for known PUFA metabolic pathways, such as (i) indomethacin, a non-selective inhibitor of cyclooxygenases, (ii) nordihydroguaiaretic acid, an inhibitor of lipoxygenases, (iii) aristolochic acid I, an inhibitor of the phospholipase A2, (iv) fenofibrate, a fibric acid derivative affecting the peroxisome proliferator-activated receptor type alpha (PPARα, PPARs modulate lipid homeostasis; (Lee *et al.*, 2003) and (v) pioglitazone, an enhancer of the PPARγ and, to a lesser extent, the PPARα, should be tested during the processes above mentioned. A complementary approach would be the use of a novel commercial system, the Cignal 45-Pathway Reporter Array (QIAGEN) which allows for the comprehensive analysis of 45 signalling pathways simultaneously, pinpointing the pathways that contribute to PUFA effects. Finally, a global analysis of gene expression through a microarray approach or RNA-Seq (whole transcriptome shotgun sequencing) could help identify genes differentially expressed upon exposure to PUFAs and putatively involved in their proliferative and mineralogenic effect, therefore improving general knowledge regarding fatty acid action on tissue mineralization and bone formation.

VI. References

Addison WN, Azari F, Sørensen ES, Kaartinen MT, McKee MD (2007) Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. J. Biol. Chem. **282**:15872-15883

Alexander PG, Boyce AT, Tuan RS (2007) Skeletal development. In: Moody SA (Ed.), Principles of developmental genetics, Elsevier Academic Press, Amsterdam; Boston, pp866-907

Bagga D, Wang L, Farias-Eisner R, Glaspy Ja, Reddy ST (2003) Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. Proc. Natl. Acad. Sci. U. S. A. **100**:1751-1756

Balcerzak M, Hamade E, Zhang L, Pikula S, Azzar G, Radisson J, Bandorowicz-Pikula J, Buchet R (2003) The roles of annexins and alkaline phosphatase in mineralization process. Acta Biochim. Pol. **50**:1019-1038

Beck GR, Zerler B, Moran E (2000) Phosphate is a specific signal for induction of osteopontin gene expression. Proc. Natl. Acad. Sci. U. S. A. **97**:8352-8357

Boström K, Tsao D, Shen S, Wang Y, Demer LL (2001) Matrix Gla protein modulates differentiation induced by bone morphogenetic protein-2 in C3H10T1/2 cells. J. Biol. Chem. **276**:14044-14052

Bou-Gharios G, De Crombrugghe B (2008) Type I collagen: Structure, synthesis and regulation. In: Bilezikian JP, Raisz LG, Martin TJ (Eds.) Principles of bone biology, Elsevier, Amsterdam, pp285-318

Braga D, Laizé V, Tiago D, Cancela M (2006) Enhanced DNA transfer into fish bone cells using polyethylenimine. Mol. Biotechnol. **34**:51-54

Burke A, Weiler H (2002) The effect of prostaglandin E_2 (PGE_2) and long-chain polyunsaturated fatty acids (LC PUFA) on bone formation in piglets: a model for bone growth in nutritional investigation. Prostaglandins Leukot. Essent. Fatty Acids **67**:229-235

Calder PC (2009) Polyunsaturated fatty acids and inflammatory processes: New twists in an old tale. Biochimie **91**:791-795

Christenson RH (1997) Biochemical markers of bone metabolism: An overview. Clin. Biochem. **30**:573-593

Coetzee M, Haag M, Joubert AM, Kruger MC (2007) Effects of arachidonic acid, docosahexaenoic acid and prostaglandin E_2 on cell proliferation and morphology of MG-63 and MC3T3-E1 osteoblast-like cells. Prostaglandins Leukot. Essent. Fatty Acids **76**:35-45

Coetzee M, Haag M, Kruger MC (2009) Effects of arachidonic acid and docosahexaenoic acid on differentiation and mineralization of MC3T3-E1 osteoblast-like cells. Cell Biochem. Funct. **27**:3-11

Conceição N, Laizé V, Simões B, Pombinho AR, Cancela ML (2008) Retinoic acid is a negative regulator of matrix Gla protein gene expression in teleost fish *Sparus aurata*. Biochim. Biophys. Acta **1779**:28-39

Das UN (2006) Essential fatty acids: Biochemistry, physiology and pathology. Biotechnol J. **1**:420-439

Diascro DDJ, Vogel RL, Johnson TE, Witherup KM, Pitzenberger SM, Rutledge SJ, Prescott DJ, Rodan GA, Schmidt A (1998) High fatty acid content in rabbit serum is responsible for the differentiation of osteoblasts into adipocyte-like cells. J. Bone Miner. Res. **13**:96-106

Ducy P (2000) Cbfa1: A molecular switch in osteoblast biology. Dev. Dyn. **219**:461-471

Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CRH, Shimizu T, Spener F, van Meer G, Wakelam MJO, Dennis EA (2009) Update of the LIPID MAPS comprehensive classification system for lipids. J. Lipid Res. **50**:S9-S14

Fall PM, Breault DT, Raisz LG (1994) Inhibition of collagen synthesis by prostaglandins in the immortalized rat osteoblastic cell line Py1a: Structure-activity relations and signal transduction mechanisms. J. Bone Miner. Res. **9**:1935-1943

Fonseca V, Laizé V, Valente M, Cancela M (2007) Identification of an osteopontin-like protein in fish associated with mineral formation. FEBS J. **274**:4428-4439

Froese R, Pauly D (2003) FishBase, World Wide Web electronic publication. http://www.fishbase.org [cited 2012 24 September]

Fujimori A, Tsutsumi M, Yamada H, Fukase M, Fujita T (1989) Arachidonic acid stimulates cell growth in an osteoblastic cell line, MC3T3-E1, by noneicosanoid mechanism. Calcif. Tissue Int. **44**:186-191

Giusto NM, Pasquaré SJ, Salvador GA, Castagnet PI, Roque ME, Ilincheta de Boschero MG (2000) Lipid metabolism in vertebrate retinal rod outer segments. Prog. Lipid Res. **39**:315-391

Grey A (2009) Fatty acids and bone. Clin. Rev. Bone. Miner. Metab. **7**:210-215

Guerreiro PM, Renfro JL, Power DM, Canario AVM (2007) The parathyroid hormone family of peptides: structure, tissue distribution, regulation, and potential functional roles in calcium and phosphate balance in fish. Am. J. Physiol. Regul. Integr. Comp. Physiol. **292**:679-696

Hall BK (2005) Bones and cartilage developmental and evolutionary skeletal biology. San Diego, Calif.; London, Elsevier Academic Press. pp13-30

Harrison JR, Lorenzo JA, Kawaguchi H, Raisz LG, Pilbeam C (1994) Stimulation of prostaglandin e_2 production by interleukin-1α and transforming growth factor α in osteoblastic MC3T3-E1 cells. J. Bone Miner. Res. **9**:817-823

Hassan MQ, Tare RS, Lee SH, Mandeville M, Morasso MI, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2006) BMP2 commitment to the osteogenic lineage involves activation of Runx2 by DLX3 and a homeodomain transcriptional network. J. Biol. Chem. **281**:40515-40526

Izquierdo MS, Montero D, Robaina L, Caballero MJ, Rosenlund G, Ginés R (2005) Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. Aquaculture **250**:431-444

James MJ, Gibson RA, Cleland LG (2000) Dietary polyunsaturated fatty acids and inflammatory mediator production. Am. J. Clin. Nutr. **71**:343S-348S

Jayakumar P, Di Silvio L (2010) Osteoblasts in bone tissue engineering. Proc. Inst. Mech. Eng. H. **224**:1415-1440

Jin H, Stewart TL, Van't Hof R, Reid DM, Aspden RM, Ralston S (2009) A rare haplotype in the upstream regulatory region of COL1A1 is associated with reduced bone quality and hip fracture. J. Bone Miner. Res. **24**:448-454

Jump DB (2002) The biochemistry of n-3 polyunsaturated fatty acids. J. Biol. Chem. **277**:8755-8758

Junqueira LCU, Carneiro J, Abrahamsohn PA, Zorn TMT, Santos MFD (2004) Histologia básica: texto e atlas. 10 ed. Rio de Janeiro, Guanabara-Koogan

Kajii T, Suzuki K, Yoshikawa M, Imai T, Matsumoto A, Nakamura S (1999) Long-term effects of prostaglandin E_2 on the mineralization of a clonal osteoblastic cell line (MC3T3-E1). Arch. Oral Biol. **44**:233-241

Kanai S, Uto K, Honda K, Hagiwara N, Oda H (2011) Eicosapentaenoic acid reduces warfarin-induced arterial calcification in rats. Atherosclerosis **215**:43-51

Kelly O, Cusack S, Jewell C, Cashman KD (2003) The effect of polyunsaturated fatty acids, including conjugated linoleic acid, on calcium absorption and bone metabolism and composition in young growing rats. Br. J. Nutr. **90**:743-750

Kim H-Y (2007) Novel metabolism of docosahexaenoic acid in neural cells. J. Biol. Chem. **282**:18661-18665

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell **89**:755-764

Kruger MC, Schollum LM (2005) Is docosahexaenoic acid more effective than eicosapentaenoic acid for increasing calcium bioavailability? Prostaglandins Leukot. Essent. Fatty Acids **73**:327-334

Kruger MC, Coetzee M, Haag M, Weiler H (2010) Long-chain polyunsaturated fatty acids: Selected mechanisms of action on bone. Prog. Lipid Res. **49**:438-449

Laizé V, Pombinho AR, Cancela ML (2005) Characterization of *Sparus aurata* osteonectin cDNA and *in silico* analysis of protein conserved features: Evidence for more than one osteonectin in Salmonidae. Biochimie **87**:411-420

Lall SP (2002) The Minerals. In: Halver JE, Hardy RW (Eds.) Fish nutrition, Academic Press, San Diego, Calif., pp259-308

Lall SP, Lewis-McCrea LM (2007) Role of nutrients in skeletal metabolism and pathology in fish - An overview. Aquaculture **267**:3-19

Le HD, Meisel JA, de Meijer VE, Gura KM, Puder M (2009) The essentiality of arachidonic acid and docosahexaenoic acid. Prostaglandins Leukot. Essent. Fatty Acids **81**:165-170

Lee C-H, Olson P, Evans RM (2003) Minireview: Lipid Metabolism, Metabolic Diseases, and Peroxisome Proliferator-Activated Receptors. Endocrinology **144**:2201-2207

Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G (1997) Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature **386**:78-81

Maldve RE, Kim Y, Muga SJ, Fischer SM (2000) Prostaglandin E_2 regulation of cyclooxygenase expression in keratinocytes is mediated via cyclic nucleotide-linked prostaglandin receptors. J. Lipid Res. **41**:873-881

Marques C, Rafael M, Cancela M, Laizé V (2007) Establishment of primary cell cultures from fish calcified tissues. Cytotechnology **55**:9-13

Massaro M, Habib A, Lubrano L, Turco SD, Lazzerini G, Bourcier T, Weksler BB, De Caterina R (2006) The omega-3 fatty acid docosahexaenoate attenuates endothelial cyclooxygenase-2 induction through both NADP(H) oxidase and PKCε inhibition. Proc. Natl. Acad. Sci. U. S. A. **103**:15184-15189

Morinobu M, Ishijima M, Rittling SR, Tsuji K, Yamamoto H, Nifuji A, Denhardt DT, Noda M (2003) Osteopontin expression in osteoblasts and osteocytes during bone formation under mechanical stress in the calvarial suture *in vivo*. J. Bone Miner. Res. **18**:1706-1715

Naylor RL, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MC, Clay J, Folke C, Lubchenco J, Mooney H, Troell M (2000) Effect of aquaculture on world fish supplies. Nature **405**:1017-1024

Nicholls JJ, Brassill MJ, Williams GR, Bassett JHD (2012) The skeletal consequences of thyrotoxicosis. J. Endocrinol. **213**:209-221

Nieves D, Moreno JJ (2006) Effect of arachidonic and eicosapentaenoic acid metabolism on RAW 264.7 macrophage proliferation. J. Cell. Physiol. **208**:428-434

O'Keefe SF (2008) Nomenclature and classification of lipids. In: Akoh CC, Min DB (Eds.) Food lipids chemistry, nutrition, and biotechnology, CRC Press/Taylor & Francis Group, Boca Raton, pp3-39

Ogiso B, Hughes FJ, Davies JE, McCulloch CA (1992) Fibroblastic regulation of osteoblast function by prostaglandins. Cell. Signal. **4**:627-639

Orimo H (2010) The mechanism of mineralization and the role of alkaline phosphatase in health and disease. J. Nippon Med. Sch. **77**:4-12

Ornitz DM, Marie PJ (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. Genes Dev. **16**:1446-1465

Parenti LR (1986) The phylogenetic significance of bone types in euteleost fishes. Zool. J. Linn. Soc. **87**:37-51

Pfaffl MW (2006) Relative quantification. In: Dorak T (Ed.), Real-time PCR, International University Line, pp63-82

Phelan MC (2007) Basic techniques in mammalian cell tissue culture. Curr Protoc Cell Biol **36**:1.1.1-1.1.18

Pombinho AR, Laizé V, Molha DM, Marques SM, Cancela ML (2004) Development of two bone-derived cell lines from the marine teleost *Sparus aurata*; evidence for extracellular matrix mineralization and cell-type-specific expression of matrix Gla protein and osteocalcin. Cell Tissue Res. **315**:393-406

Poulsen RC, Moughan PJ, Kruger MC (2007) Long-chain polyunsaturated fatty acids and the regulation of bone metabolism. Exp. Biol. Med. (Maywood) **232**:1275-1288

Puchtler H, Meloan SN, Terry MS (1969) On the history and mechanism of alizarin and alizarin red s stains for calcium. J. Histochem. Cytochem. **17**:110-124

Rafael M, Laizé V, Cancela M (2006) Identification of *Sparus aurata* bone morphogenetic protein 2: Molecular cloning, gene expression and *in silico* analysis of protein conserved features in vertebrates. Bone **39**:1373-1381

Rafael M, Marques C, Parameswaran V, Cancela M, Laizé V (2010) Fish bone-derived cell lines: an alternative *in vitro* cell system to study bone biology. J. Appl. Ichthyol. **26**:230-234

Ramírez-Zacarías JL, Castro-Muñozledo F, Kuri-Haruch W (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. Histochem. Cell Biol. **97**:493-497

Renn J, Winkler C, Schartl M, Fischer R, Goerlich R (2006) Zebrafish and medaka as models for bone research including implications regarding space-related issues. Protoplasma **229**:209-214

Ringbom T, Huss U, Stenholm Å, Flock S, Skattebøl L, Perera P, Bohlin L (2001) COX-2 inhibitory effects of naturally occurring and modified fatty acids. J. Nat. Prod. **64**:745-749

Rodan GA (2003) The development and function of the skeleton and bone metastases. Cancer **97**:726-732

Roman AS, Schreher J, Mackenzie AP, Nathanielsz PW (2006) Omega-3 fatty acids and decidual cell prostaglandin production in response to the inflammatory cytokine IL-1β. Am. J. Obstet. Gynecol. **195**:1693-1699

Rouzer CA, Marnett LJ (2003) Mechanism of free radical oxygenation of polyunsaturated fatty acids by cyclooxygenases. Chem. Rev. **103**:2239-2304

Salem N, Litman B, Kim H-Y, Gawrisch K (2001) Mechanisms of action of docosahexaenoic acid in the nervous system. Lipids **36**:945-959

Sargent JR, Bell JG, Bell MV, Henderson RJ, Tocher DR (1993) The metabolism of phospholipids and polyunsaturated fatty acids in fish. In: Lahlou B, Vitiello P (Eds.) Aquaculture: Fundamental and applied research, American Geophysical Union, Washington, DC, pp103-124

Sargent JR, McEvoy LA, Bell JG (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture **155**:117-127

Seifert MF, Watkins BA (1997) Role of dietary lipid and antioxidants in bone metabolism. Nutr. Res. **17**:1209-1228

Singer FR, Eyre DR (2008) Using biochemical markers of bone turnover in clinical practice. Clevel. Clin. J. Med. **75**:739-750

Sodek J, Ganss B, McKee MD (2000) Osteopontin. Crit. Rev. Oral Biol. Med. **11**:279-303

Sommerfeldt DW, Rubin CT (2001) Biology of bone and how it orchestrates the form and function of the skeleton. Eur. Spine J. **10 Suppl 2**:S86-S95

Stephanis J (1996) Mediterranean aquaculture industry trends in production, markets and marketing In: Handbook of contributions and short communications at the International Workshop on Seabass and Seabream Culture: Problems and Prospects

Sun D, Krishnan A, Zaman K, Lawrence R, Bhattacharya A, Fernandes G (2003) Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. J. Bone Miner. Res. **18**:1206-1216

Swamy MV, Cooma I, Patlolla JM, Simi B, Reddy BS, Rao CV (2004) Modulation of cyclooxygenase-2 activities by the combined action of celecoxib and decosahexaenoic acid: novel strategies for colon cancer prevention and treatment. Mol. Cancer Ther. **3**:215-221

Tacon AGJ, Metian M (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture **285**:146-158

Takuwa Y, Ohse C, Wang EA, Wozney JM, Yamashita K (1991) Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells, MC3T3-E1. Biochem. Biophys. Res. Commun. **174**:96-101

Tassoni D, Kaur G, Weisinger RS, Sinclair AJ (2008) The role of eicosanoids in the brain. Asia Pac J Clin Nutr. **17**:220-228

Tiago D, Cancela M, Aureliano M, Laizé V (2008a) Vanadate proliferative and anti-mineralogenic effects are mediated by MAPK and PI-3K/Ras/Erk pathways in a fish chondrocyte cell line. FEBS Lett. **582**:1381-1385

Tiago D, Laizé V, Cancela M, Aureliano M (2008b) Impairment of mineralization by metavanadate and decavanadate solutions in a fish bone-derived cell line. Cell Biol. Toxicol. **24**:253-263

Tiago D, Cancela M, Laizé V (2011a) Proliferative and mineralogenic effects of insulin, IGF-1, and vanadate in fish osteoblast-like cells. J. Bone Miner. Metab. **29**:377-382

Tiago D, Laizé V, Bargelloni L, Ferraresso S, Romualdi C, Cancela M (2011b) Global analysis of gene expression in mineralizing fish vertebra-derived cell lines: New insights into anti-mineralogenic effect of vanadate. BMC Genomics **12**:310

Tullberg-Reinert H, Jundt G (1999) In situ measurement of collagen synthesis by human bone cells with a Sirius Red-based colorimetric microassay: effects of transforming growth factor β2 and ascorbic acid 2-phosphate. Histochem. Cell Biol. **112**:271-276

Turchini GM, Torstensen BE, Ng W-K (2009) Fish oil replacement in finfish nutrition. Rev. Aquaculture **1**:10-57

Wagner GF, Haddad M, Fargher RC, Milliken C, Copp DH (1998) Calcium is an equipotent stimulator of stanniocalcin secretion in freshwater and seawater salmon. Gen. Comp. Endocrinol. **109**:186-191

Wang EA, Rosen V, Cordes P, Hewick RM, Kriz MJ, Luxenberg DP, Sibley BS, Wozney JM (1988) Purification and characterization of other distinct bone-inducing factors. Proc. Natl. Acad. Sci. U. S. A. **85**:9484-9488

Watkins BA, Li Y, Allen KGD, Hoffmann WE, Seifert MF (2000) Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats. J. Nutr. **130**:2274-2284

Watkins BA, Li Y, Seifert MF (2001a) Nutraceutical fatty acids as biochemical and molecular modulators of skeletal biology. J. Am. Coll. Nutr. **20**:410S-416S

Watkins BA, Lippman HE, Le Bouteiller L, Li Y, Seifert MF (2001b) Bioactive fatty acids: role in bone biology and bone cell function. Prog. Lipid Res. **40**:125-148

Watkins BA, Li Y, Lippman HE, Feng S (2003) Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast function and bone metabolism. Prostaglandins Leukot. Essent. Fatty Acids **68**:387-398

Watkins BA, Li Y, Seifert MF (2006) Dietary ratio of n-6/n-3 PUFAs and docosahexaenoic acid: actions on bone mineral and serum biomarkers in ovariectomized rats. J. Nutr. Biochem. **17**:282-289

Watkins SM, German JB (2008) Unsaturated Fatty Acids. In: Akoh CC, Min DB (Eds.) Food lipids chemistry, nutrition, and biotechnology, CRC Press/Taylor & Francis Group, Boca Raton, pp529-530

Weiler HA, Fitzpatrick-Wong SC (2002) Modulation of essential (n-6):(n-3) fatty acid ratios alters fatty acid status but not bone mass in piglets. J. Nutr. **132**:2667-2672

Witten P, Huysseune A, Franz-Odendaal T, Fedak T, Vickaryous M, Cole A, Hall B (2004) Acellular teleost bone: Dead or alive, primitive or derived? The Palaeontology Newsletter **55**:37-41

Witten PE, Huysseune A (2010) The unobtrusive majority: mononucleated bone resorbing cells in teleost fish and mammals. J. Appl. Ichthyol. **26**:225-229

Wolf K, Quimby MC (1962) Established eurythermic line of fish cells *in vitro*. Science **135**:1065-1066

Yan Q, Sage EH (1999) SPARC, a matricellular glycoprotein with important biological functions. J. Histochem. Cytochem. **47**:1495-1506