# Fatty acids and temperature effects in fish adipogenesis and osteogenesis: *in vitro* and *in vivo* approaches

## Efecte dels àcids grassos i la temperatura sobre l'adipogènesi i l'osteogènesi en peixos: enfocament *in vitro* i *in vivo*

Natalia Riera Heredia

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Department of Cell Biology, Physiology and Immunology, University of Barcelona

# Fatty acids and temperature effects in fish adipogenesis and osteogenesis: *in vitro* and *in vivo* approaches.

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A thesis submitted by **Natàlia Riera Heredia** for the degree of

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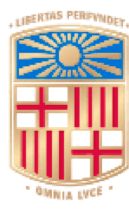


UNIVERSITAT DE BARCELONA

Departament de Biologia Cel·lular, Fisiologia i Immunologia, Universitat de Barcelona

Programa de doctorat d'Aqüicultura

# Efecte dels àcids grassos i la temperatura sobre l'adipogènesi i l'osteogènesi en peixos: enfocament *in vitro* i *in vivo*

Memòria presentada per **Natàlia Riera Heredia** per optar al títol de

**Doctor per la Universitat de Barcelona**

**Directora:**

**Dra. Isabel Navarro**

**Directora i Tutora:**

**Dra. Encarnación Capilla**

**Doctoranda:**

**Natàlia Riera Heredia**

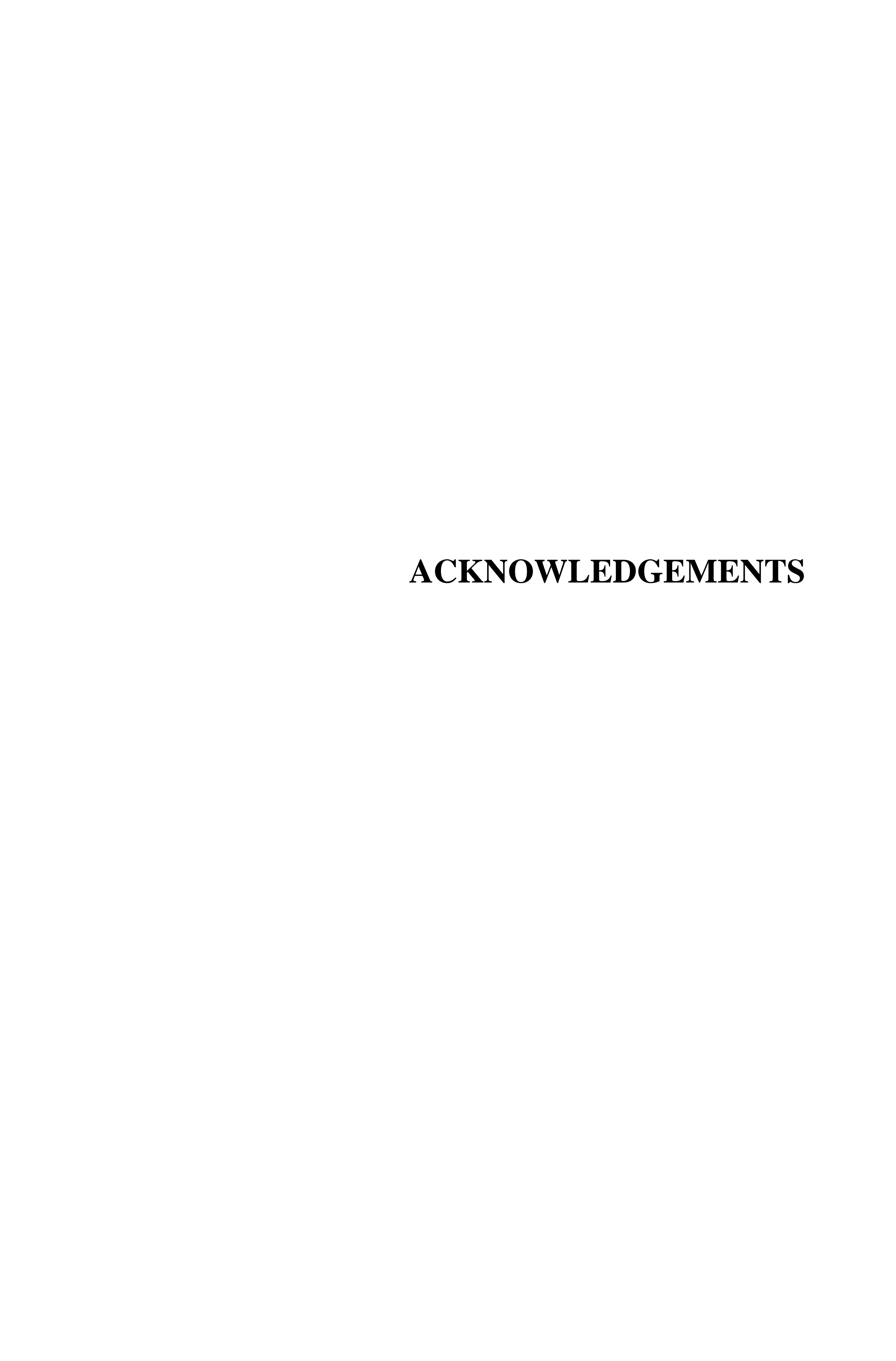
Barcelona, Setembre 2019

El cel blau per teulada
parets de penya-segats
les gavines fan niuada
en un racó arrecerat.

Veuen que passen les barques
plenes de braus mariners,
veuen la mar sense onades
les barques aparellades
sempre de dos en dos.

Cala Montgó
racó de pau i de llum.
racó de pau i perfum.
racó de més bell encís.
Cala Montgó
un bocí de paradís.

*Cala Montgó - Els pescadors de l'Escala*



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Del doctorat sobretot em quedo amb l'estada a França, que tot i que hi vaig anar espantada, va ser una experiència inoblidable i vaig tenir el plaer de conèixer molt bona gent.

Je suis extrêmement reconnaissant de mon accueil dans l'équipe de recherche de l'INRA St. Pée sur Nivelle. Je tiens à remercier Karine et Florian pour leur aide dans les Western blots, Marienne pour son enseignement de l'histologie, sa gentillesse et son amitié. Aussi à Sarah, pour sa prédisposition à m'aider dans tout ce dont j'avais besoin et enfin à Maroussia pour sa joie, son amitié et pour être une personne adorable. Enfin, je voudrais remercier Iban pour son attention et son dévouement à mon travail ainsi que pour son attitude louable envers la recherche et son perfectionnisme. Je termine mon paragraphe en français en remerciant Paula pour sa patience et pour les après-midis amusantes au cours desquelles vous m'avez appris un peu de français. Merci à tous.

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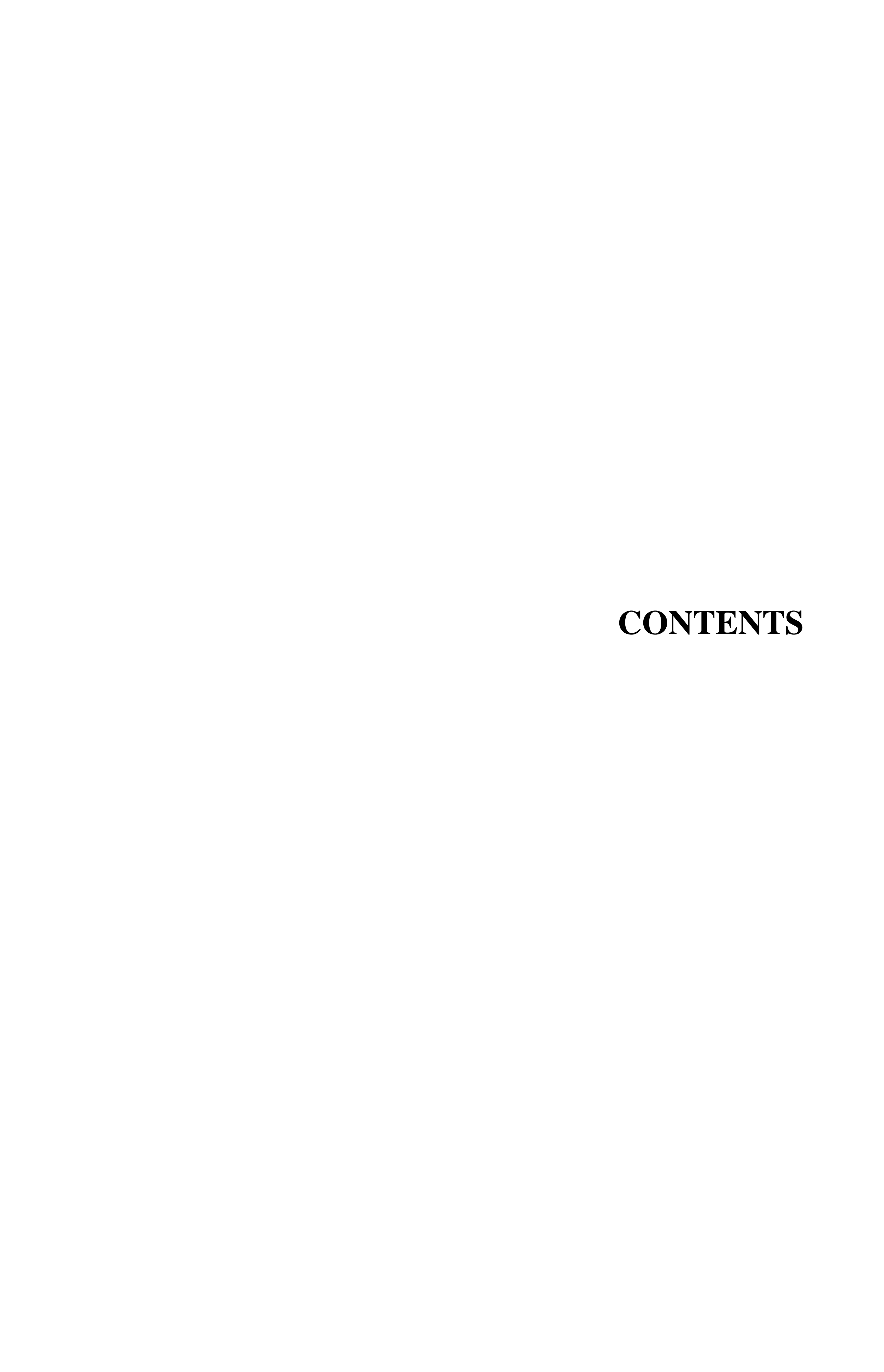
# ABSTRACT

Aquaculture needs to develop new strategies and tools to face challenges as the reduction of fish oil use in fish feeds and climate change, to assure the obtention of a valuable high-quality product while preserving fish health, welfare and proper growth. This thesis includes the characterization of the gene expression pattern of preadipocytes during differentiation into mature adipocytes in rainbow trout, as well as during the process of osteogenesis in gilthead sea bream both, *in vitro* and *in vivo*. Moreover, it has been characterized the *in vitro* plasticity of the bone-derived cells to differentiate into the adipogenic linage. In addition, the effect of fatty acids derived from fish or vegetable oils has been tested *in vitro* on rainbow trout preadipocytes and gilthead sea bream bone-derived cells and *in vivo*, by oral administration in rainbow trout. In addition, it has been evaluated the effect of temperature over bone-derived cells and the *in vivo* skeletal development of gilthead sea bream, as well as the effect on lipid metabolism in this species when challenged with partially substituted fish oil diets by different vegetable oils. Finally, this study presents a characterization of the expression pattern of osteogenic genes in malformed skeletal structures of gilthead sea bream. Regarding the first block of studies, results demonstrate that adipogenesis and osteogenesis in fish are similar than in mammals, and *in vitro* gene expression patterns have reflected clear similarities with *in vivo* models thus, highlighting the potential tool of these cells as model systems to study their behaviour under new conditions. Furthermore, it has been demonstrated that the bone-derived cells, as seen in mammals, have the ability to differentiate into other linages, concretely into adipocyte-like cells not only with a differentiation media but also by the presence of a single fatty acid. In this context, these fatty acids showed their potential to act as inductors of adipogenesis by upregulating *pparg* gene expression or inducing higher lipid accumulation through transporters depending on the fatty acid oil source. The same fatty acids increased intracellular lipid accumulation and the activation of the adipogenic process in preadipocytes from rainbow trout. Moreover, the oral administration of fatty acids in the same species produced an activation of β-oxidation, lipogenesis and lipid transport in adipose tissue, whereas in liver a decrease in these processes was observed. Concerning temperature, both

*in vitro* and *in vivo* affectation of the normal gene expression during differentiation and development respectively was shown, with the addition in the latter of the demonstration of a thermal imprinting existence after a temperature challenge. In this sense, an elevated temperature has been also shown to increase in gilthead sea bream somatic growth and adipogenesis as well as fatty acid oxidation in the liver. In these fish fed diets with different vegetable oil sources, it was shown that the combination of palm and rapeseed oils is the most equilibrated formulation to assure growth and balanced lipid accumulation at this warm condition. Finally, the vertebral column deformities (lordosis and LSK syndrome) have revealed a modified expression pattern for the genes related to extracellular matrix formation and mineralization and bone resorption, pointing to key genes to be used as molecular markers, whereas for the operculum, dental and jaw deformities gene markers have not been identified. Overall, the present thesis describes adipogenic factors that can be the base to found potential links with whole body energy regulation and demonstrates that nutrition and temperature influence adipose tissue and bone development and lipid metabolism in fish, providing different valuable models to evaluate new challenges for improving a sustainable aquaculture.



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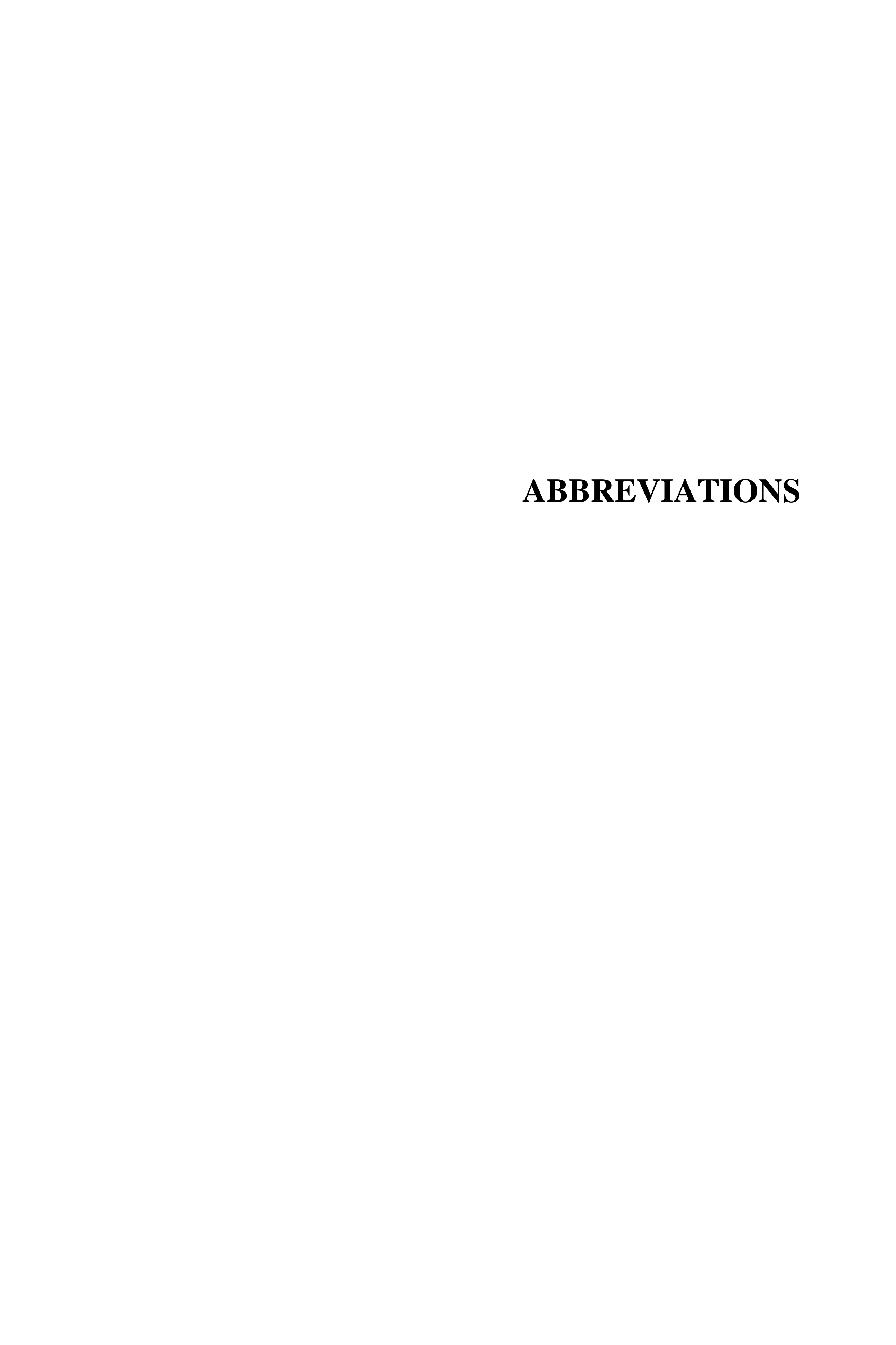


# ABBREVIATIONS

List of the most important abbreviations used in the present thesis:

**ALA:** α-linolenic acid

**ATGL**: adipose triglyceride lipase

**CD36**: cluster of differentiation 36

**C/EBP:** CCAAT/enhancer-binding protein factor

**COL1:** collagen type 1

**DHA:** docosahexaenoic acid

**ECM:** extracellular matrix

**EFA:** essential fatty acids

**EPA:** eicosapentaenoic acid

**FA:** fatty acid

**FABP:** fatty acid binding protein

**FAS:** fatty acid synthase

**FATP:** fatty acid transport protein

**Fib1a:** fibronectin

**FM:** fish meal

**FO:** fish oil

**HDL:** high density lipoproteins

**HSL:** hormone-sensitive lipase

**HUFA:** highly unsaturated fatty acid

**LA:** linoleic acid

**LDL:** low-density lipoproteins

**LO:** linseed oil

**LPL:** lipoprotein lipase

**LXR:** liver x receptor

**MGL:** monoacylglycerol lipase

**MSCs:** mesenchymal stem cells

**MUFA:** monounsaturated fatty acids

**OA:** oleic acid

**OCN:** osteocalcin

**ON:** osteonectin

**OP:** osteopontin

**PLIN:** perilipin

**PO:** palm oil

**PPAR:** peroxisome proliferator-activated receptor

**PUFA:** polyunsaturated fatty acid

**RO:** rapeseed oil

**Runx2:** runt-related transcription factor 2

**RXR:** retinoid x receptor

**SFA:** saturated fatty acids

**SO:** soya oil

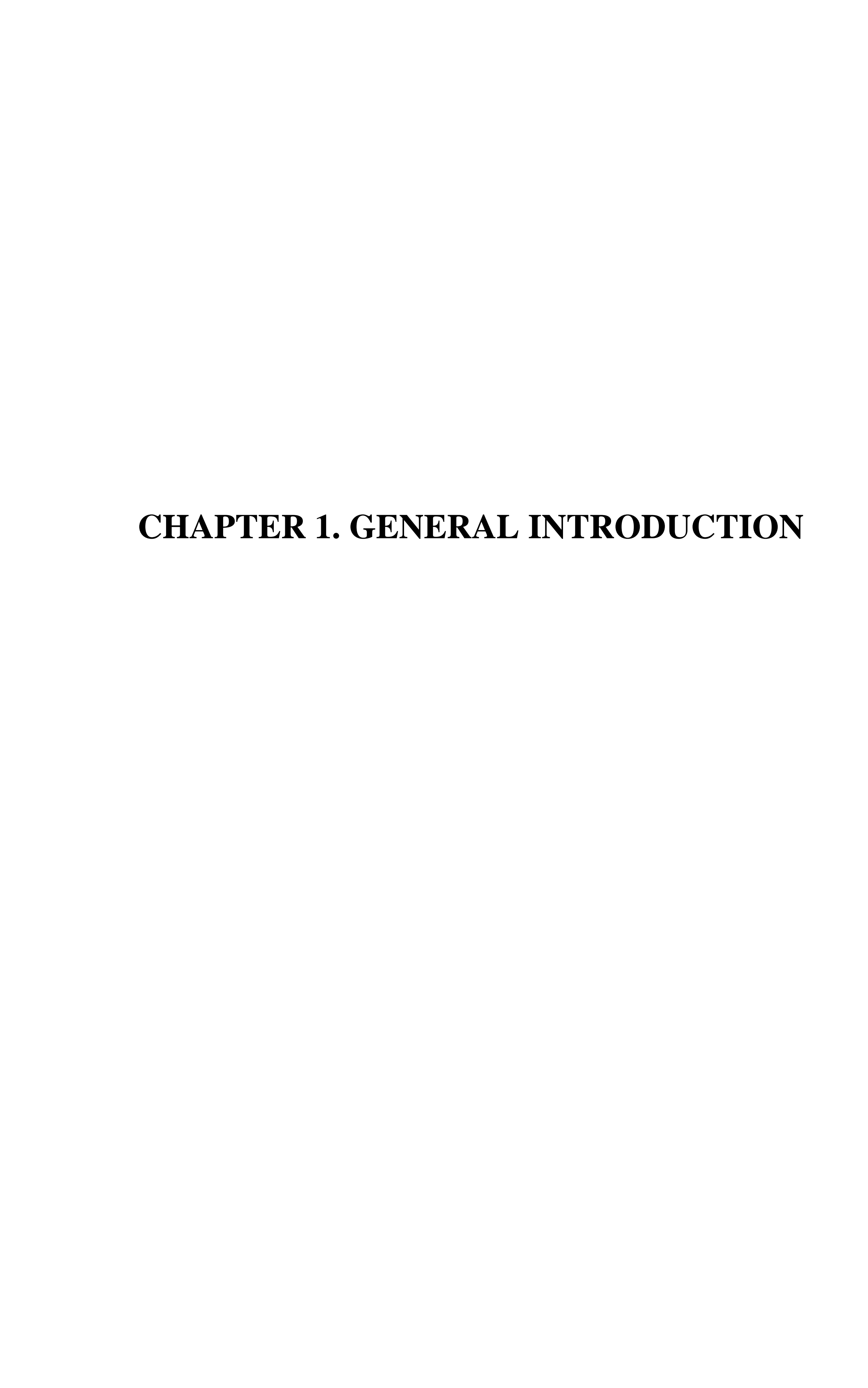
**TG:** triglycerides

**TNAP:** tissue non-specific alkaline phosphatase

**UFA:** unsaturated fatty acids

**VLDL:** very low-density lipoproteins

**VO:** vegetable oil



# CHAPTER 1. GENERAL INTRODUCTION

## 1.1.Present status and prospects in aquaculture

The total world fish production in 2017 reached the top of 205.6 million tonnes, and aquaculture was responsible of over 50 percent without considering production for non-food uses (Figure 1) (APROMAR, 2019). According to the Food and Agriculture Organization of the United Nations (in SOFIA, 2018), over the last years, due to the challenge of an increasing population, aquaculture has had to counteract the stagnation in which capture fishing has been immersed since 1980s, thus being necessary an increase of fish production in order to feed humans. Since 1962 up to 2016 the rates of fish ingestion had risen over the rate of population expansion; in particular the rate per capita of fish consumption has increased in 11.2 Kg in this period of time, and the estimation of rising rates are predicted to follow this 1.5 percent increment per year. In 2016, the global capture fisheries production has reached the total amount of 90.9 million tonnes of fish, with 12.8 percent produced in inland waters and 87.2 percent in marine waters. On the contrary, all marine catch decreased about 2 million tonnes in 2016, a reduction that is consequence of the stock overfishing. Despite the efforts of the government agents to regulate fishing and restoring stocks, aquaculture has only been able to reconstitute 33.1 percent of these reserves, consequently, more time is needed since 2 or 3 generations of the species would be required to rebuild stocks. In 2017, the 88 percent of the total fish from capture and aquaculture were used for human consumption whereas the remaining 12 percent (coming from capture) was mostly used for non-food purpose and converted to fishmeal (FM) or fish oil (FO). Nowadays, FM and FO, both considered optimal for fish farmed nutritional requirements, are largely produced with products that were usually wasted in the past, however the utilization of FM and FO is being reduced and now used more selectively (in SOFIA, 2018).

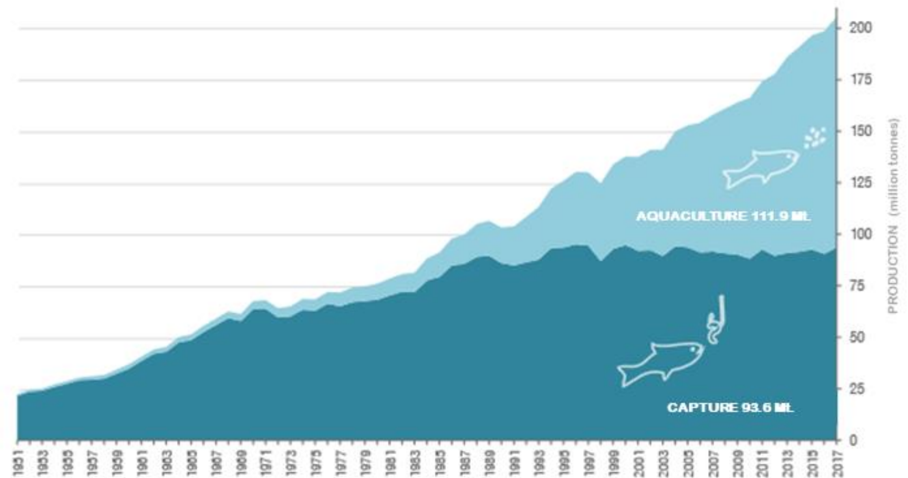



**Figure 1.** World capture fisheries and aquaculture production (excluding aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants). Adapted from APROMAR (2019).

Asian countries were leaders of the world's marine capture fisheries (54%) being China the major producer, followed by North and South America countries' (20%) and Europe (17%) in 2016 (FAO, 2018). According to APROMAR (2019), in the aquaculture sector, Asia was the major producer with the 92% of the global production, followed by North and South America countries' (3.2%) and Europe (2.7%). In this sense, the European Union total production (aquaculture and capture) of fisheries products in 2017 was estimated in 7 million tonnes and the 51.7% came from aquaculture sector in three Member States; including Spain (23%), the United Kingdom (16.4%), and France (10.9%). More concretely, in Spain 348.395 tonnes of fish, molluscs and crustaceans were produced in aquaculture, being the blue mussel (*Mytilus edulis*) the most produced with 273.600 tonnes, followed by the European sea bass (*Dicentrarchus labrax*) with 22.460 tonnes, rainbow trout (*Oncorhynchus mykiss*) 18.856 tonnes and gilthead sea bream (*Sparus aurata*) 14.930 tonnes. According to FAO (2018), due to the high quality of aquatic resources in Spain, this sector has been focused in the farming of rainbow trout. The first steps of inland aquaculture in Spain started in 1129 in Galicia but the industrialization was developed in 1961. Since then, the production of trout has decreased from 25.000 tonnes in 1964 to 16.000 tonnes in

2015 because of the reduction of demand. Despite that rainbow trout supposes the 99 percent of the continental production in Spain, other species are produced including tilapia (*Oreochromis niloticus*), sturgeon (*Acipenser nacarii*) and European eel (*Anguilla anguilla*) among others. On the other hand, marine aquaculture has experienced an enhanced growth in the last 20 years, specifically as a result of increased production of gilthead sea bream, turbot (*Psetta maxima*) and sea bass. Paradoxically, industrialization in marine production started about 30 years ago although the first enterprises date from 1973, almost all focused initially in research and development. In 2018, the most common cultivated marine species have been the blue mussel , sea bass, gilthead sea bream, turbot, red tuna (*Thunnus thynnus*), carpet shell spp., corvine (*Argyrosomus regius*), European eel, Senegalese sole (*Solea senegalensis*), Blackspot sea bream (*Pagellus bogaraveo*), mullet (*Mugil spp*.), Atlantic salmon (*Salmo salar*), and flat oysters (*Ostrea edulis*) among others (Figure 2).

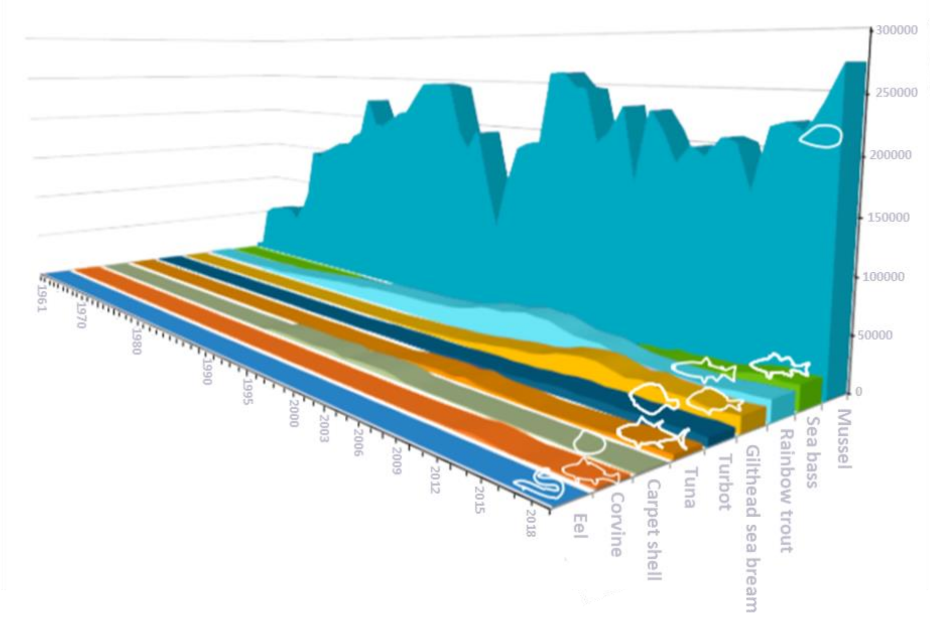



**Figure 2.** Evolution of aquaculture production in Spain represented in tonnes and by species during the 1960-2018 period. Adapted from APROMAR (2019).

In 2018, the estimated value of Spanish aquaculture production was about 470 million euros (APROMAR, 2019), therefore, the relevance of marine and inland fisheries production in economic terms has been noteworthy. Moreover, fish and derivates are known to contribute to human nutrition (Allison, Delaporte & Hellebrandt da Silva, 2013; Roos, 2016) providing a high-quality and bioavailable protein, essential amino acids, essential minerals, vitamins and omega-3 long-chain polyunsaturated fatty acids (LC-PUFA) especially in fatty fish. Aquaculture is considered of a high economical value and a source of employment; moreover, its products can contribute to a healthier human nutrition. So, to preserve the valuable properties of this industry, aquaculture must improve its methods to face challenges as the reduction of FO use in fish feeds and other newest as climate change, to always assure the obtention of a valuable product.

#### 1.1.1. Raw materials for fish feeds production

The preservation of cultured fish health and welfare is one of the major challenges in aquaculture (Jennings et al, 2016), demonstrating the need to develop new strategies and tools to ensure compliance with these conditions as well as fish proper growth. Feeding is an important aspect in aquaculture as a strategy to improve animal welfare and health (Hasan, 2001). In this sense, new approaches are being implemented in the formulation of diets to face new challenges as climate change effects, stress conditions, infectious agents, among others, with the ultimate goal of lowering production costs in industry but without compromising fish development and growth (Hixson, 2014).

The sustainability of raw materials used in aquaculture feeds is being questioned due to the increased production of aquatic species depending on these feeds, since farmed species have increased from 12 to 51 million tonnes in 10 years (Hasan, 2017a). Therefore, it is not surprising, that the production of aquaculture feeds in the same period has increased from 8 to 48 million tonnes (Hasan, 2017b; Tacon, Hasan & Metian, 2011). Aquaculture feeds contain key ingredients as FM and FO, produced from highly nutritious wild fish. The main species used in the production

of FM and FO are the Peruvian anchovy (*Engraulis ringens*), mackerel (*Trachurus/Scomber spp*.), sand eel (*Ammodyte spp*.), capelin (*Mallotus spp*.), menhaden (*Brevoortia spp*.), and to some extent herring (*Clupea harrengus*) and pollock (*Pollachius spp*.), being the Peruvian anchovy the species most used in the industry. These species are concretely pelagic and are considered “fatty fish” for having a fat content of 8% or more, presenting high amount of n-3 LC-PUFA, although specific fatty acid profiles in fish vary influenced by age, size, species, geographic location among other factors (Turchini, Ng & Tocher, 2010). FO is originally a derived product of the reduction process that converts raw fish to FM and the procedure permits to manufacture 20 and 5 kg of FM and FO respectively, from 100 kg of fresh-fish raw material. In the last years, 25% of the global world capture fishing has been used as raw material for FM and FO production, generating an increasing controversy because of the fact that these captures could be for human consumption (Tacon & Metian, 2009). Furthermore, this raw material has been also used in feeds for pet and animal productions’ sector (De Silva & Turchini, 2008). As a result of the declining of captures provoked by the limiting stocks of these species, the situation has led to the reduced usage of FM and FO in feeds formulation, thus replaced by plant-based ingredients, especially proteins and oils (Hasan & New, 2013; Little, Newton & Beveridge, 2016; Tacon et al, 2011). In this context, according to Turchini et al (2010), the most commonly used vegetable oils (VO) are palm (PO), soybean (SO), rapeseed (RO), and sunflower that are composed by different fatty acids, most of them having n-6 and n-9 PUFA in abundance. VO do not have n-3 highly unsaturated fatty acids (HUFA, $C \geq 20$) therefore, a reduction in the nutritional qualities of the final product has been observed in fish fed diets with an elevated replacement of FO with VO (Rosenlund et al, 2010). Thus, the healthy benefits on human of the n-3 HUFA present in fish could be lost with the reduction of these fatty acids in fish fed VO-based diets. According to Thanuthong et al (2011), the use of VO is an opportunity to elucidate the different fatty acid classes within these oils and their impact on lipid utilization and metabolism in farmed fish species.

In fact, it is known that most VO contain LC-PUFA that freshwater species have the ability to bioconvert into HUFA and this aspect could minimize the negative impact of FO replacement in freshwater species. In this sense, in Atlantic salmon diets, the proportion of FM has been reduced from 65 to 24 percent and FO from 19 to 11 percent in approximately 25 years (Ytrestøyl, Aas & Åsgård, 2015) and in rainbow trout, FM has been substituted up to 47% and completely the FO without negative effects in digestibility (Lund et al, 2012). Regarding gilthead sea bream, changes in the fatty acid profile of fish fed with partially substituted diets and the possible usage of a finishing phase with a wash-out diet to revert the effects of FO substitution has been reported (Benedito-Palos et al, 2009; Fountoulaki et al, 2009). These achievements have been possible through better formulation, manufacturing and management methods in feeds production. Although for now, the use of these plant-derived ingredients continues to be optimized, aquaculture is still closely linked to terrestrial sources for protein and therefore, alternatives such as insects and microbial crops are now being evaluated as future candidate materials, though their application will still take some years.

From the human point of view, Western society diet presents a nutritional imbalanced ratio of n-3/n-6 PUFA, which is characterized by increased consumption of n-6 PUFA commonly found in modern processed diets. To combat this unhealthy habit and its pathological effect, an increased demand of products containing elevated proportions of n-3 HUFA has put in check the aquaculture industry, which has the great responsibility to feed humans with a high valuable protein and with species rich in the healthy n-3 HUFA. Due to the limited stock of captures and considering that ocean productivity is estimated to decrease a 20 percent worldwide due to climate change (Schmittner, 2005) consequently, less raw material will be available for reduction process in feeds production. Therefore, the challenge in aquaculture will suppose the finding of new strategies to obtain fish rich in these valuable marine-based lipid sources.

### 1.1.2. Impact of climate change in aquaculture

Aquaculture and fisheries production, in addition to the handicap of limited stocks, have to face the major problems of climate change that are affecting distribution, growth and development of fish (Mazumder et al, 2015). Climate impact has forced certain marine species to move from their habitat to other regions in a search for optimal conditions, therefore changing their distribution, majorly in deeper waters. Furthermore, water acidity is fomenting calcification of organisms in natural environments as in marine aquaculture. Finally, the increased number of storms, the changes in the cycle of water and the rising sea level are known to affect either inland fisheries or aquaculture industries (Seggel, De Young & Soto, 2016).

These variations in the environment have being studied for long (Barange et al, 2014; Gattuso et al, 2015) and in the last reports is revealed that in almost all coastal countries either marine and terrestrial aquaculture and fisheries production will experience a decrease, which will vary depending on the potential of the country to adapt to the new conditions (Blanchard et al, 2017). Thus, a global response is necessary to mitigate climate change effects in all food production sectors investing in new opportunities to minimize the negative impacts, assuring secure food and livelihood provision.

One of the worrying aspects of climate change causing severe effects on fish production is the increased temperature of water. According to IPCC (2014), global mean temperature could increase 4ºC by 2100 causing changes in the ecosystem performance (Woodward, Perkins & Brown, 2010). Changes in ocean surface temperature can impact on fish distribution, growth and metabolic demands. Some studies conducted in Portugal have demonstrated that the predictions of global change effects on water temperature, salinity, etc, will suppose a compromised development in the early life stages of fish such as *Sparus aurata* (Pimentel et al, 2016) or *Sardina pilchardus* (Faleiro et al, 2016) among others, fish that are usually commercialized. The effects will vary depending on the species ability to adapt or respond to the changing conditions thus, conditioned

by the physiological tolerance and genetic plasticity of the individuals to resist under multiple pressures and stressors. In this sense, owning that fish have specific temperature requirements that differ between different life-stages and species (Souchon & Tissot, 2012), growth rates and reproduction are predicted to be decreased once the maximum temperature tolerated is overpassed (Potts, Götz & James, 2015). In a study in brown trout (*Salmo trutta*), a range between 4 and 12ºC was tested in initial developmental stages, observing that those eggs reared at 6 and 8ºC presented the maximum survival rate, whereas at 12ºC survival was dramatically reduced to 0.9% (Réalis-Doyelle et al, 2016). Furthermore, at 4ºC even though the survival rate was high (76.4%) the appearance of skeletal deformities was higher (22%). In fact, water increases of 1 or 2 degrees can provoke sub-lethal effects in the physiology of tropical fish (Ficke et al, 2007) as well as water temperature over 17ºC has been demonstrated to be damaging in farmed salmon as a consequence of the lower efficacy of the feed (De Silva & Soto, 2009). In this context, Bendiksen, Jobling and Arnesen (2002), showed that for the same species at higher temperatures, low-fat feeds result in a better performance. Therefore, future research is focusing on the development of adaptive measures, to mitigate the influence of temperature on the efficacy of feed utilization as well as in protein and lipid usage for growth thus, assuring that at elevated temperatures body functions are preserved (De Silva & Soto, 2009).

Overall, intending to keep these promising benefits (employment, economic value of the sector and properties for human health), new strategies must be developed in this industry to face the new challenges, such as environmental effects, as well as the reduction of FM and FO in the formulation of diets as described in this section, among others. All these actions must be done to obtain a good and valuable product assuring at the same time a rentable and sustainable aquaculture production.

## 1.2. Biology of the fish species studied

### 1.2.1. Rainbow trout (*Oncorhynchus mykiss*)

According to FAO (2018), rainbow trout (*O. mykiss*) belongs to the salmonid family, and the subfamily of *Salmoninae,* and it is characterized by olive green to blue coloration with a pink and silver band along the lateral line besides, it presents small black or dark dots in the loin, head and fins (Figure 3). This coloration suffers variations depending of the sexual condition, habitat and size that is commonly 60 cm length although the maximum is 120 cm. This species is normally found in cold waters of lakes and rivers from Europe, to North America or northern Asia, specifically, its anadromous nature implies that during its life cycle the trout, which lives in the sea in a range of depths between 0 and 200 m, needs to go up the river to spawn.

Fingerlings of trout feed on zooplankton whereas, terrestrial or aquatic insects, molluscs, crustaceans, small fish or fish eggs configure the diet of adult rainbow trout in nature, as well as freshwater shrimp, source of the carotenoid that gives the orange pigmentation in the meat.

**Figure 3.** Rainbow trout (*Oncorhynchus mykiss*).

Rainbow trout is a medium-fat fish (5-10% lipid in fillet) (Taşbozan & Gökçe, 2017) and it is nutritionally relevant for its input of high biological value protein, n-3 fatty acids as well as vitamins (B12) and minerals (selenium and phosphorus). Despite the beneficial properties known for this species, consumption decreased at the same time that the Atlantic salmon culture was expanded (decreasing the price

of all salmon species) thus not being a luxury, of which trout had been a cheaper substitute (Asche & Bjørndal, 2011; Asche et al, 2005).

The rainbow trout comes from the continental aquaculture and it is the species sold in the market throughout all year, despite its meat is white usually it can be found with the orange pigmentation due to synthetic pigments (astaxanthin and canthaxanthin) included in the feeds.

The global rainbow trout aquaculture production decreased 0.6 percent when compared to 2016 with the total amount of 811.590 tonnes produced, and Iran, Turkey, Chile, Norway and Perú were the principal producers. This species is majorly produced in fresh water (70%) although an important part of its production is finished in marine water as in Chile and Norway. In Spanish aquaculture in 2018, the production increased 5% when compared to 2017 with the total estimated amount of 18.856 tonnes, far away from the 35.000 tonnes obtained in 2001, but the recent tendency is showing slightly a recovery (APROMAR, 2019). With all that, rainbow trout is an interesting product, nutritious, that tolerates different environments and is easy to maintain and to reproduce in culture.

### 1.2.2. Gilthead sea bream (*Sparus aurata*)

According to FAO (2018), gilthead sea bream belongs to the family of the *Sparids* and it is commonly known for its silver-grey colour with a golden band between the eyes that is bordered by two dark areas also with a large black spot at the origin of the lateral line (Figure 4). This species presents an oval body, with a curved head profile, small eyes and a low mouth with thick lips. With powerful teeth, this carnivorous fish breaks the shell to be fed with molluscs or crustaceans, fish and punctually algae. It can reach sizes of 70 cm or weight up to 6 kg although the most frequent sizes are between 30 and 40 cm or 500 g. Bizarrely, this type of fish presents sexual investment according to the population requirements, being males in the first years and then becoming females depending on the needs.

**Figure 4.** Gilthead sea bream (*Sparus aurata*).

This white fish contains low muscle lipid levels thus, the energetic contribution to humans is moderate when compared to other species and offers a modest amount of protein of high biological value (Reviewed in Khora, 2013). Furthermore, it presents selenium, phosphorus and potassium at interesting rates and B12 vitamin valuable values, contrarily to the low presence of iron and magnesium. These characteristics have made gilthead sea bream a proper ingredient in low lipidic and caloric human diets.

In the wild, gilthead sea bream is present in sandy shorelines with algae and sometimes in more rocky areas, and in winter it descends to deeper (30 m) waters. It can be found living in small groups either alone and is commonly found in the Mediterranean Sea and in the Atlantic coast from United Kingdom to Senegal. Because they are euryhaline and eurythermic fish, they support a wide range of salinities and temperatures, being the optimal range between 16 and 22ºC in larvae stages and 25ºC the temperature for optimal growth of farmed juvenile specimens (Reviewed in Volstorf, 2019). Therefore, gilthead sea bream that is born in the open sea, in the juvenile state migrate to coastal waters to find resources and milder temperatures. Finally, when they become adults, they migrate to the deeper waters to reproduce.

In aquaculture, gilthead sea bream rearing is extended from most of the Mediterranean countries to some of the Atlantic coast and in the Black Sea. Hatcheries promote controlled spawning from females that can reach two million eggs for each kilogram of weight, being the spawning period from two to three months. The commercial size generally ranges between 200 and 1.500 g, and

approximately, between 18 and 24 months are necessary to reach an individual of 400 g.

European and Mediterranean gilthead sea bream total production was over 285.000 tonnes in 2018, being Spain in the fifth position of Mediterranean producers. In concordance with the tendency observed in capture statistics, the aquaculture production of this species in Spain is estimated to decrease 11.6 percent in 2019. Overall, gilthead sea bream is known to be a high value product for human diet and for the Spanish economy (APROMAR, 2019).

## 1.3. Adipose tissue and adipogenesis

In mammals, two types of adipose tissue exist: white and brown (Lee, Mottillo & Granneman, 2014); while only the white type has been described in teleost fish, since no evidence of the existence of brown or beige adipocytes has been found in this group of vertebrates up to date (Salmerón, 2018). White adipose is a type of connective tissue mainly formed by adipocytes that are cells filled with neutral lipids. Adipose tissue functions involve control of energy homeostasis of the organism by the regulation of lipid turnover and the secretion of adipokines, which endocrine functions regulate lipid metabolism among many other processes. (Esteve Ràfols, 2014; Konige, Wang & Sztalryd, 2014; Rutkowski, Stern & Scherer, 2015; Salmerón et al, 2015a, 2015b; Saltiel, 2001; Sánchez-Gurmaches et al, 2012). Teleost fish accumulate fat mainly as triglycerides (TG) in different body parts depending on the species, but the preferential sites for lipid storage are perivisceral and subcutaneous fat, muscle and liver (Flynn, Trent & Rawls, 2009; He et al, 2015; Weil, Lefèvre & Bugeon, 2012). In mammals and fish, adipose tissue is known to regulate its lipid content depending on the energy requirements by two main processes, lipogenesis that consists in the production by mature adipocytes of TG from different substrates and their storage, and lipolysis that consist in breaking these TG into fatty acids and glycerol to be released into the blood. Finally, lipids can also be metabolized by adipocytes and their oxidation

is a source of energy within the mitochondria (Salmerón, 2018). In Figure 5 are schematically represented the processes involved in lipid metabolism in the adipocyte.

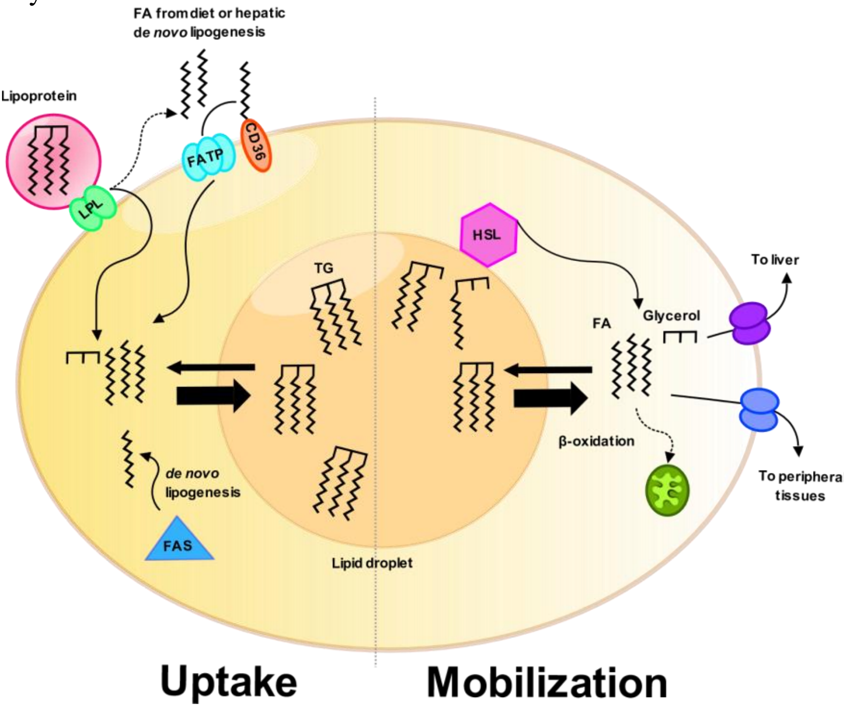



**Figure 5.** Schematic representation of uptake (lipogenesis) and mobilization (lipolysis) of lipids in a mature adipocyte. Fatty acids (FA) from hepatic *de novo* lipogenesis or cleaved from triaglycerides (TG) from lipoproteins are internalised by lipoprotein lipase (LPL), fatty acid transporter protein (FATP) or the fatty acid transporter cluster of differentiation 36 (CD36). These FA or those synthetized *de novo* by fatty acid synthase (FAS) and together with glycerol stored as TG in lipid droplets. Lipolysis or mobilization consists in the action of hormone-sensitive lipase (HSL) among other lipases to obtain FA and glycerol from TG. FA can then be used for β-oxidation or sent to peripheral tissues while glycerol is sent to the liver. Adapted from Rutkowski et al (2015).

Adipose tissue grows by hypertrophy that favours increased lipid accumulation in the existing adipocytes when nutrients in the diet excess requirements (Muir et al, 2016) and/or hyperplasia (a.k.a. adipogenesis) that consists in the formation of new adipocytes from precursor cells (Hausman et al, 2001). Hyperplasia in fish as in mammals has been suggested to occur during early life and throughout life

(Fauconneau et al, 1997; Spalding et al, 2008; Umino, Nakagawa & Arai, 1996). Moreover, it has been observed that some ingredients in the diet can increase fish adipocyte cell size (Cruz-García et al, 2011), thus inducing cell hypertrophy and in consequence obtaining a less functional/responsiveness adipocyte, suggesting as in mammals the apparition of metabolic disorders (Salmerón, 2018).

Adipogenesis, the process necessary to obtain new adipocytes from precursor cells (i.e. hyperplasic growth), requires a complex and coordinated transcriptional organization to finally express genes associated with different functions characteristic of the mature adipocyte such as lipid uptake, transport and synthesis among others (Den Broeder et al, 2015; Rosen & MacDougald, 2006). Adipogenesis in mammals comprise a determination phase where it is required the commitment of multipotent cells to the adipocyte linage, producing the preadipocytes, which express transcription factors' genes as peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT-enhancer-binding proteins alpha and beta (C/EBPα and C/EBPβ); and a differentiation phase, consisting in the conversion of these preadipocytes into mature adipocytes. At this later stage, adipocytes express lipogenic enzymes such as lipoprotein lipase (LPL) and adipose triglyceride lipase (ATGL), fatty acid binding proteins (FABP), and high levels of the lipid-droplet-associated protein perilipin 1 (PLIN), all of them key molecules for the regulation of lipid metabolism (Ghaben & Scherer, 2019). In fish, using primary cultures of preadipocytes, two stages along the adipogenic process have been also identified at a transcriptional level: proliferation of preadipocytes and differentiation into mature adipocytes after the addition of a differentiation medium (Bou et al, 2017), demonstrating that the cells present a conserved expression of the genes described for mammals (Figure 6).

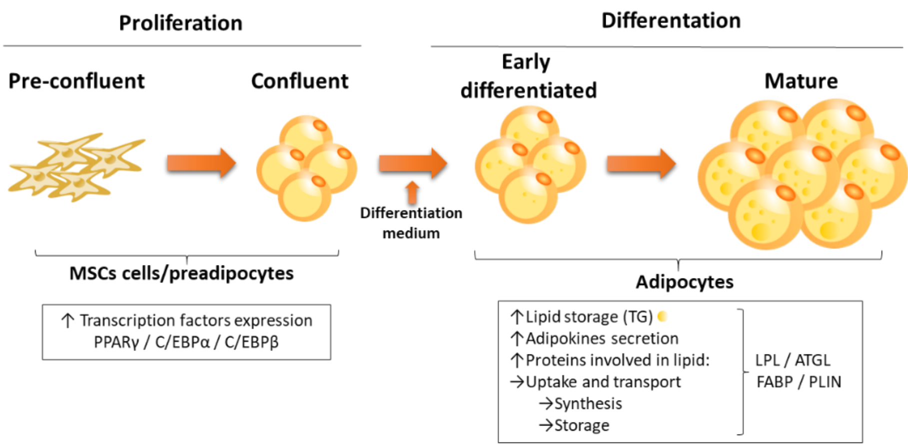



**Figure 6.** Adipogenesis *in vitro* of multipotent mesenchymal stem cells (MSCs) / preadipocytes that differentiate into adipocytes. Adipocyte differentiation is sustained using differentiation medium. During proliferation, cells express transcription factors as peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT-enhancer-binding proteins alpha and beta (C/EBPα and C/EBPβ), and in the differentiation phase cells are expressing lipogenic enzymes including lipoprotein lipase (LPL) and adipose triglyceride lipase (ATGL), fatty acid binding proteins (FABP), and high levels of the lipid-droplet-associated protein perilipin 1 (PLIN). Adapted from Salmerón (2018).

During the process of adipogenesis, PPARγ is known as the master regulator, being necessary during all the differentiation period (Rosen & Spiegelman, 2000). Its action is coordinated forming a heterodimer with the cis-retinoic acid receptor alpha (RXRα) that afterwards, binds to promoters of genes related to fatty acid and glucose metabolism (Lefterova et al, 2014). In fish, preadipocytes have been described to express the *pparg* gene before differentiation indicating the possible pre-commitment into the adipogenic linage of resident precursor cells in the vascular fraction of adipose tissue (Bouraoui, Gutiérrez & Navarro, 2008; Todorčević et al, 2010). Moreover, it has been reported their capacity to accumulate lipid droplets inside without the addition of an adipogenic differentiation medium (Salmerón et al, 2013; Vegusdal et al, 2003). When such medium, containing insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (Scott et al, 2011), is added to human preadipocytes in culture, there is an induction

of transcription factor genes as *pparg* and *cebpa*, that initiate the programming and coordinate the expression of adipocyte-specific genes (Gustafson et al, 2015). In fish preadipocytes *in vitro*, is needed to add an adipogenic medium as in mammals although particularly also a lipid source to induce fully differentiation of adipocytes, thus activating PPARγ through natural ligands and enabling the lipid droplet formation (Bouraoui et al, 2008; Salmerón et al, 2013; Vegusdal et al, 2003; Wang, Huang & Wang, 2012).

*In vivo* as well as *in vitro*, adipogenesis requires a tight regulation that involves such a complex network of transcription factors that have demonstrated to be very susceptible to changes (Todorčević & Hodson, 2015); therefore, consciousness about the function of adipose tissue in lipid metabolism, possible changes in nutrition will be key, for the proper development and health of the tissue and therefore the whole animal welfare.

### 1.3.1. Fatty acids and their importance in fish adipose tissue

Due to the low capability of some species to utilize carbohydrates from diet, fatty acids are the major source of energy (β-oxidation) to promote growth, development, swimming and reproduction (Bureau, Kaushik & Cho, 2002; Sargent, Tocher & Bell, 2002). Nowadays, carnivorous fish are fed with increasing lipid content from plant sources that are rich in n-6 and n-9 PUFA in detriment of lipids from marine origin that have higher levels of n-3 HUFA as eicosapentaenoic (EPA; 20:5 n-3) and docosahexaenoic (DHA; 22:6 n-3), therefore implying a change in the fatty acid profiles of reared fish (Sprague, Dick & Tocher, 2016). Fish can present a low, or even absent capacity, especially in marine species, in the synthesis pathway for the conversion activity in LC-PUFA (Sargent et al, 1995; Tocher, 2003) and this low activity is suggested to be because in the marine ecosystem EPA and DHA are in abundance. Therefore, given the low ability of fish to synthesize LC-PUFA, essential fatty acid requirements depend on the species, and the developmental stage among other critical periods (Glenncross, Booth & Allan, 2007; Rønnestad et al, 2013; Tocher, 2010). Besides, the

deficiency of EPA and DHA produces delay in fish growth, can induce mortality and reduces stress resistance, therefore provoking alterations in anatomic field linked to nutritional disorders (Hamre et al, 2013).

Otherwise, the effect of fatty acids at gene expression levels have been elucidated during the last years. It is known that fatty acids and their metabolites can affect expression of genes related to energy metabolism (Jump, 2004; Leaver et al, 2008). Several transcription factors involved in this connection have been described in mammals and with less detail in fish. In teleost, it is well known the participative role of PPARs and the property of being possibly activated by PUFA and LC-PUFA as in mammals (Leaver et al, 2005, 2008). The dietary effects of fatty acids as EPA and DHA on adipose tissue function have been reported in Todorčević et al (2009), where diets supplemented with these fatty acids decreased adiposity in Atlantic salmon by the regulation of TG accumulation in visceral adipose tissue as well it was similarly found in grass carp (*Ctenopharyngodon idella*) (Ji, Li & Liu, 2011). In the grass carp study, an upregulation of the expression of lipolytic genes (*ppara*, among others) and a decrease in lipid accumulation in intraperitoneal adipose tissue was observed.

Regarding *in vitro* fatty acid effects in preadipocytes from mammals and fishes, the results showed either to be anti-adipogenic whilst others found a pro-adipogenic response, with the inhibition, promotion or without presenting any effects in different adipogenic parameters (lipid droplets, TG accumulation, regulation of the expression of adipogenic-related genes, etc.) on preadipocytes differentiation. For example, in isolated mature adipocytes from grass carp incubated 6 hours with EPA and DHA, TG accumulation decreased while increased the expression of lipolytic genes whereas at any stage of maturation of 3T3-L1 adipocytes, others have reported no effect on TG accumulation (Reviewed in Todorčević & Hodson, 2016). These discrepancies in *in vivo* and *in vitro* studies of the effects of fatty acid in adipogenesis and lipid accumulation are likely to be consequence of the different species, doses and time of exposure, or developmental

stages among other conditions tested that make difficult to obtain a universal conclusion. Overall, there is evidence of the direct effect of fatty acids in the key genes involved in the differentiation process as in lipid accumulation, thus the profile of fatty acids present in a diet formulation should be considered as it impacts on lipid metabolism and preadipocytes differentiation.

## 1.4. Lipid metabolism in fish

Lipids are well digested in fish except for fatty acids which melting point is superior to the temperature of fish habitat, and in the same way the melting point of TG will vary depending on its fatty acids' conformation. In Atlantic salmon, trout and carp, PUFA ingested in the form of TG or free fatty acids are well digested, whereas saturated fatty acids (SFA) are less digested, and the proportion decreases with the increase of chain length. Afterwards, the fatty acids absorbed are re-esterified in TG and phospholipids, the firsts can be stored in enterocytes (as temporal reserve) but majorly are included in chylomicrons or in very low-density lipoproteins (VLDL) to be transported to the liver and other tissues. As an exception, in critical situations (e.g. refeeding after starvation) the absorbed fatty acids can be transported directly to the liver bounded to albumin (Biesalski & Grimm, 2007). Once these lipids arrive to the liver, together with the fatty acids *de novo* synthesized in this tissue, join apoproteins and cholesterol and form new lipoproteins. Plasma lipoproteins are responsible for the transport of these lipids from the liver to other tissues and there are three types: the VLDL, the low-density lipoproteins (LDL) and the high-density lipoprotein (HDL) and the major lipid presence in these lipoproteins are n-3 HUFA. During transport, LPL that is found in hepatic and extrahepatic tissues, together with lecithin-cholesterol acyltransferase, are responsible for the intra-vascular conversion of these lipoproteins. The TG from chylomicrons and VLDL are hydrolysed so the fatty acids are released and stored in tissues or used with energetic purpose (Figure 7) (Guillaume, 2004).

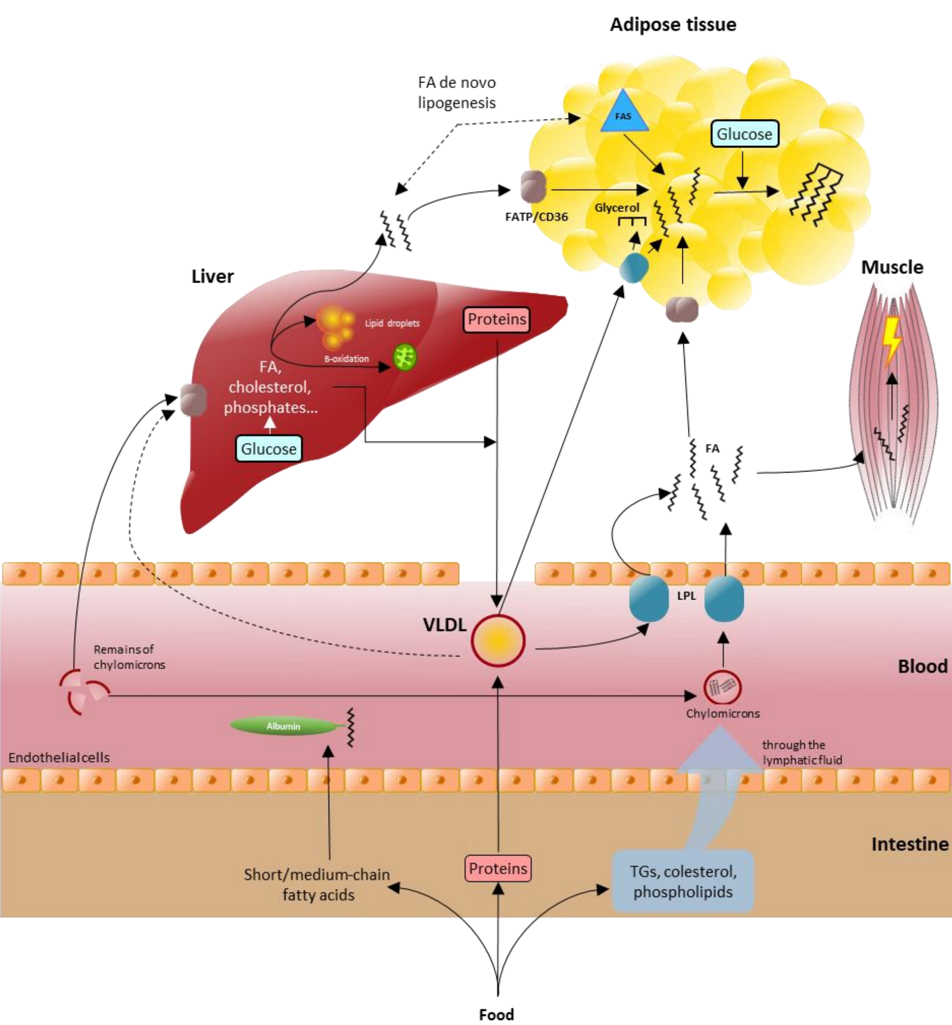



**Figure 7.** Schematic representation of lipid metabolism processes after food intake. Fatty acid (FA); triglycerides (TG); very low-density lipoproteins (VLDL); lipoprotein lipase (LPL); fatty acid transporter (FATP); cluster of differentiation 36 (CD36); fatty acid synthase (FAS). Adapted from Biesalski & Grimm (2007).

TG are formed by fatty acids and glycerol. Fatty acids are obtained from the circulating lipoproteins by lipases or are synthesized by the cell through lipogenesis *de novo* that consists in the synthesis of fatty acid from non-lipid precursors activated by high carbohydrate availability (in Muriel, 2017). In lipogenesis *de novo* there is an upregulation of genes involved such as ATP-citrate lyase, FAS, long chain fatty acid elongase and PPARγ (Strable & Ntambi, 2010).

TG are considered to be the storage form of lipids, that in mammals are accumulated mainly in adipose tissue. In fish is known that lipids can be stored in several tissues as liver, muscle and perivisceral adipose tissue and also, the subcutaneous tissue. Little formations of adipose tissue are created to depot lipids in muscle between the connective tissue of myosepta that separate myotomes. The amount of lipid stored in fish can vary depending on the habitat of the fish, the temperature of water, the stationery time, together with multiple factors as genetics, nutrition, environment among others. In this regard, farmed fish tend to accumulate more lipids in the body than their homologs in the wild, and this can be controlled mainly by the diet administered (Guillaume, 2004).

These lipid depots in mammals and fish, are known to be mobilized mainly by hormone-sensitive lipase (HSL), ATGL and monoacylglycerol lipase (MGL) (Lafontan & Langin, 2009), that hydrolyse the TG, and then, the fatty acids are released to the circulation or retained into the cell where they are oxidised in the mitochondria and peroxisomes to obtain energy. During fish life, lipid depots mobilization is enhanced in some biological conditions as gonad formation and along winter starvation. In these situations, the first lipids mobilized to obtain energy, are those from visceral tissues and if necessary, those in the muscle (Guillaume, 2004).

### 1.4.1. Impact of feed oil sources and temperature

VO have been used as sustainable alternatives to FO, however the different fatty acid composition present in each type of oil, as mentioned above, can change the flesh fatty acid composition, which is one of the most precious values of fish for human diet (De Deckere et al, 1998; Horrocks & Yeo 1999; Hunter & Roberts 2000; ISSFAL 2000; Simopoulos, 1999, 2003). Imbalances in n-3/n-6 PUFA ratio due to the increasing use of VO and therefore a reduction in EPA and DHA, rebound into fish quality and in consequence, fish loses its beneficial effect of contributing n-3 HUFA to human health (Karalazos et al, 2007). Oils as SO, PO, RO and linseed (LO) are the most common VO used in FO substitution. PO

contains high amounts of SFA; RO has a lower level of SFA and is rich in oleic (OA; 18:1 n-9), but also contains linoleic (LA; 18:2 n-6) and alpha-linolenic acids (ALA; 18:3 n-3). Regarding SO and LO, SO presents an 8 percent of ALA and LO is the VO with the highest amount of this fatty acid (50%).

Owning the importance of liver key role in lipid metabolism for being the major site of fatty acid synthesis and of circulating lipids directed to other tissues in the form of lipoproteins (Sissener, 2016), together with the role in lipid storage of adipose tissue, the effects of the substitution of FO on lipid metabolism must be checked in both tissues. In fact, some studies have put in evidence the effects of this substitution by VO sources as in Atlantic salmon, which lipid accumulation in both tissues was increased when fed a diet containing a blend of RO, PO and LO combined with high levels of plant protein (Torstensen et al, 2011). Also, other authors demonstrated that diets with FO substitution by different VO sources impact at distinct level on lipid accumulation (Bell et al, 2001, 2002) making difficult the comparison between different species due to the distinct rearing conditions (temperature, salinity, etc.), duration of challenges, or the fish age among other factors. The studies about effects of lipids on metabolism focused mainly in liver, whereas fewer studies did it on adipose tissue. In Atlantic Salmon, the accumulation of fat around internal organs and in the liver when FM and FO were replaced by high levels of vegetable ingredients was found to indicate a possible deficiency in micronutrients (e.g. methionine) levels, thus suggesting a metabolic imbalance that may affect fish health and especially cardiovascular health (Espe et al, 2015; Torstensen et al, 2011).

In this sense, several studies have researched on oil sources and their differential effects on fat deposition in liver and intestine of distinct fish species (Bell et al, 1995; Caballero et al, 2002, 2004; Olsen, Løvaas & Lie, 1999; Olsen et al, 2000; Tucker et al, 1997). For example, rainbow trout fed with different dietary VO presented more lipid droplets in liver and intestine when compared to fish fed a FO diet (Caballero et al, 2002). In salmon, increased hepatic lipid stores have been observed when fish is fed diets with decreased marine n-3 fatty acids and also a

decreased ratio n-3/n-6 and with increased OA (Jordal, Lie & Torstensen, 2007). Therefore, a clear evidence that dietary FO substituted by VO is inducing changes in lipid metabolism in farmed fish and consequently is compromising the quality and the properties of the product, as well as the welfare of the animal is demonstrated.

These changes in lipid metabolism, rebounding on adipose tissue, can be aggravated with the increase in temperature expected due to global climate change. Temperature is an important factor affecting fatty acid metabolism, and in a general way fish nutrition, physiology and growth (Bendiksen et al, 2003; Bendiksen & Jobling, 2003; Guderley, 2004; Ng, Sigholt & Bell, 2004; Ruyter et al, 2006; Tocher et al, 2004; Torstensen, Lie & Frøyland, 2000). As an example of the combination of the two factors, Ruyter et al, (2006) demonstrated that high levels of FO substitution by SO in the diet of Atlantic salmon reared at low temperature induced fat accumulation in the liver, although diet was observed not to affect lipid accumulation in the intestine, but low temperature increased the intracellular lipid droplets.

Among other aspects to be considered, low temperatures induce in fish an increase in the unsaturation of the fatty acids in the cell membrane and the storage of TG, reducing consistently the amount of SFA and therefore increasing unsaturated fatty acids (UFA) but also monounsaturated (MUFA) ones (Fodor et al, 1995; Hazel and Williams, 1990; Hsieh, Chen & Kuo, 2003; Jobling & Bendiksen, 2003). In this context, a reduction in production of SFA is observed due to the effect of low temperature in the enzymes implied in lipid biosystems thus, as concluded in Atlantic salmon the combination of temperature and feed formulation affect the capacity of holding and deposit n-3 and n-6 essential fatty acids (EFA) (Bendiksen et al, 2003). Concretely, whereas at low temperatures n-3 EFA where properly retained in fish body possibly as thermal acclimation, n-6 were hold in lower quantities implying an increased catabolic degradation.

Therefore, taking into account the key role of liver and the complex regulation of lipid metabolism, to obtain a complete overview of the consequences of FO substitution, it is important to analyse deeper the effects in both, liver and adipose tissues, and to evaluate the changes in tissue functionality in response to temperature changes to assure fish welfare, feed efficiency and quality of the aquaculture product.

## 1.5. Bone tissue and osteogenesis

Bone is a dynamic connective tissue that contributes to body mechanics and muscle attachment, allows movement, and provides protection to vital organs as well as it is a calcium and phosphorus repository (Watkins et al, 2001). Bone is constituted by osteoblasts, osteoclasts (resorption bone cells) and osteocytes, a mineral phase and an organic, mineralized, extracellular matrix (ECM) (Reviewed in Boglione, 2013a) but in some fishes species, including gilthead sea bream, bone is known to be acellular because the non-presence of osteocytes (Kölliker, 1859). In cellular bone, differentiated osteoblasts are embedded within the ECM during bone formation becoming osteocytes trapped by the mineralized matrix (Bonewald, 2011; Franz-Odendaal, Hall & Witten, 2005). These osteocytes function as regulators of two major processes in bone: remodelling or bone repair and modelling or bone adaptation (Bonewald, 2017; Chen et al, 2010; Tatsumi et al, 2007). Once entrapped, osteoblasts conform a compact cellular network that in fully mineralized bone become osteocytes being in small spaces (lacunae) and in continuous communication with surrounding cytoplasmatic processes residing on canaliculi (narrow channels) (Bonewald, 2011; Cao et al, 2011; Franz-Odendaal et al, 2005). On the other hand, acellular bone (anosteocytic) implies absence of osteocytes and a possible explanation suggested that osteoblasts are able to avoid entrapment or alternatively, became entrapped but die and finally are turned fully mineralized (Moss, 1961). Posterior studies suggested that in acellular bones, the osteoblasts seemed to produce ECM only towards the bone surface, avoiding being entrapped by the mineralization front in advanced teleost (Ekanayake & Hall,

1987, 1988; Weiss and Watabe, 1979). In a recent study, it has been observed that osteogenesis in anosteocytic bone, consists on osteoblasts that secrete bone matrix and then become trapped by the secretions, thus dying and being mineralized, although polarised matrix secretion has not been confirmed (Ofer et al, 2019).

Osteoblasts differentiate from mesenchymal stem cells (MSCs) in a process known as osteogenesis and are mononucleated cells recruited for bone formation whose functions are to synthesize, secrete, organize, and mineralize the bone matrix, or osteoid (Aubin, Turksen & Heersche, 1993; Rodan & Noda, 1991). Bone ECM is a blend of collagen type I (COL1A1) as well as fibronectin (Fib1a) among other structural fibers and contains osteopontin (OP), osteonectin (ON) and osteocalcin (OCN) as non-collagenous proteins (Aubin & Turksen. 1996; Young et al, 1993). In fish bone, matrix mineralization fluctuates depending if the bone is acellular or cellular, if the fish swims actively or not, and besides the aquatic environment (marine or freshwater) (Danos & Staab, 2010; Dean & Shahar, 2012; Fiaz et al, 2012; Meunier & Huysseune, 1991; Sfakianakis et al, 2011).

Osteogenesis is the process by which MSCs, as precursor cells, proliferates and differentiate into mature osteoblasts after induction of key transcription factors expression, although they also possess the ability to differentiate into other cell types (Pittenger et al, 1999; Yteborg et al, 2010). One of the main transcription factors involved in the initial stages of osteogenesis is runt related transcription factor 2 (Runx2) that controls osteoblast linage determination and the expression of osteogenic genes (Matsubara et al, 2008). Afterwards, osteoblasts proliferation rate decreases while it starts the expression of osteogenic markers such as alkaline phosphatase (ALP) (also known as tissue non-specific alkaline phosphatase, TNAP), secreted by early osteoblasts (during the matrix maturation phase) and osteocalcin (OCN), which is secreted by late osteoblasts (during the mineralization phase) (Figure 8) (Reviewed in Infante & Rodríguez, 2018). Osteoblasts produce the bone ECM or osteoid, in which key components, are subsequently responsible for mineral deposition regulation including ON, OP and OCN among others as

seen also in fish (Chen et al, 2015; Fonseca et al, 2007; Vieira et al, 2013, Ytteborg et al, 2012).

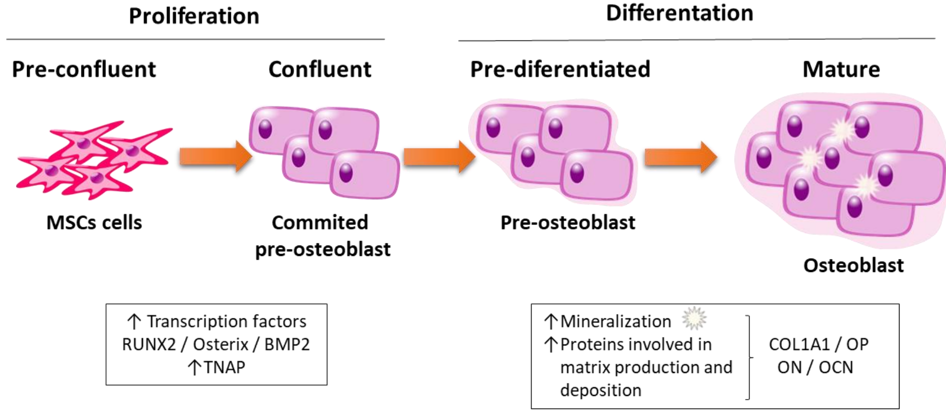



**Figure 8.** Osteoblast differentiation, maturation and key factors involved. Osteogenesis *in vitro* of multipotent mesenchymal stem cells (MSCs) that differentiate into mature osteoblasts. During proliferation, cells express transcription factors genes as runt-related transcription factor 2 (Runx2), osterix and bone morphogenetic protein 2 (BMP2) and tissue non-specific alkaline phosphatase (TNAP). In the differentiation phase, cells are expressing proteins involved in matrix production and mineralization such as collagen type 1A (COL1A1), osteopontin (OP), osteonectin (ON) and osteocalcin (OCN). Adapted from Vieira et al (2013).

This process is susceptible to be compromised by different factors either *in vivo* or *in vitro*, as mutations in the gene coding for COL1A1 have been related to osteogenesis imperfecta in mammals (Duy et al, 2016; Gajko-Galicka, 2002). Moreover, some drugs have demonstrated to affect COL1A1 and ON *in vitro* (Humphrey, Morris & Fuller, 2013) consequently inducing a possible bone loss according to the bone diseases observed in patients that used this drug (Nakken & Taubøll, 2010). Furthermore, pharmacological doses of vitamin A (retinoic acid) in cultured primary rat calvarial osteoblasts decreased osteoblast proliferation enhancing osteogenesis with increased levels of TNAP activity and expression of OP; thus, suggesting an effect in craniofacial development by pathological induction of osteogenesis (Song et al, 2004). Therefore, considering the findings in mammals, further research is necessary to elucidate the effects of genetics,

ambient or nutritional issues that can impact on fish bone, the responsible for providing protection to vital organs.

### 1.5.1. Skeletal malformations in fish

Skeletal anomalies in farmed fish are a major problem in aquaculture concerning economical, biological and animal welfare issues. The deformed specimens have to be manually discarded during production, used for fish meal or as fillets, supposing high loses for the enterprises (Boglione et al, 2001, 2003, 2009; Cahu, Zambonino-Infante & Barbosa, 2003a; Cahu, Zambonino-Infante & Takeuchi, 2003b; Castro, Pino-Querido & Hermida, 2008; Georgakopoulou et al, 2007; Hilomen-Garcia 1997; Koumoundouros et al, 1997a, 1997b; Lall & LewisMcCrea 2007; Le Vay et al, 2007; Lijalad & Powell 2009; Matsuoka 2003). In marine aquaculture production, high mortalities in fish at early stages (20-85%) have been found to be linked to large economic losses. In this sense, skeletal deformities can affect on average, 7-20% of the juveniles produced, and in some conditions, this incidence has been shown to elevate up to 45-100% (Georgakopoulou et al, 2010). Therefore, the decrease of skeletal anomalies is necessary to reduce production costs (Theodorou & Venou, 2012) thus, there is the need of improving knowledge about factors affecting bone development as genetics and epigenetics among others.

Deformities suppose negative effects on fish welfare and as commented above economical losses, because visually these species commercialized as a whole could compromise the consumer perception of aquaculture. Added to these aspects, there is in addition an ethical issue, since the deformities have been described as a symptom of disadvantageous conditions for fish culture in terms of impaired feeding and swimming performances, lowering feeding and growth rates but also compromising resistance *versus* stress and pathogens (Reviewed in Boglione, 2013b). This prevalence highlights the need of improving knowledge in biotic (genetics and epigenetics) and abiotic factors (temperature, type of culture, etc.) that can be responsible for the appearance of anomalies along rearing.

Therefore, several studies have focused on elucidating the possible factors that can arouse the development of deformities in farmed fish in several species including rainbow trout (Aulstad & Kittelsen, 1971); gilthead sea bream (Paperna et al, 1977); European seabass (Barahona-Fernandes, 1978); ayu (*Plecoglossus altivelis*) (Komada, 1980). Improvements of abiotic (Divanach et al, 1997; Georgakopoulou et al, 2010; Sfakianakis et al, 2006) and nutritional (Izquierdo, Socorro & Roo, 2010; Lewis-McCrea & Lall, 2010) conditions have been widely evaluated to reduce the incidence of deformities in these species.

Anomalies have been documented in different skeletal tissues as in vertebra, operculum, head, fins, etc. Curvatures, dislocations, shortening and twisting are vertebral anomalies that have been reported in several farmed species, that in serious cases can develop in macroscopic deviations of the column as it can be V- or Λ-shaped dorsal–ventral curvature named as lordosis and kyphosis respectively and lateral curvature or scoliosis and the lordosis-scoliosis-kyphosis syndrome (LSK). The presence of column deformities in farmed gilthead sea bream are known to be responsible of the devaluation of this species in the market reaching loses up to 1 euro per kilogram (Lee-Montero et al, 2015). On the other hand, anomalies in the operculum (reduction or folding) can provoke the exposure of the branchial arches and consequently compromising fish health. In this regard, in farmed gilthead sea bream almost 80% of the individuals presented operculum deformities in several studies (Andrades, Becerra & Fernández-Llebrez, 1996; Verhaegen et al, 2007). Contrarily to the bounded terminology in vertebral anomalies, cranium anomalies present different affectations in several parts of the head as for example gill cover, jaws, etc. without specific nomenclature. Typical jaw anomalies that have been suggested to develop mainly during early larval stages (Koumoundouros, 2010), comprise pugheadness, cross-bite and reduction or elongation of the lower jaw (Reviewed in Boglione, 2013b).

Some of the factors that have been recently studied as causative of malformations are genetics and epigenetics. These studies have focused on the heritability of some skeletal anomalies, gene mutations or genetic drift in deformed fish, the

phenotypic effects of gene modifications, inbreeding, selective breeding, etc. In this context heritability for vertebral axis anomalies, more concretely lordosis in red porgy (*Pagrus pagrus*) and lack of operculum in gilthead sea bream have been described (Izquierdo et al, 2010; Navarro et al, 2009; Thorland et al, 2007). Moreover, LSK syndrome has been associated with family structure suggesting a possible genetic origin (Afonso et al, 2000).

On the other hand, another key parameter affecting in terms of skeletogenesis during fish larval development is nutrition. Nutrients as lipids, amino acids, vitamins and minerals, which concentration or form in the diet is not balanced or optimal, have been demonstrated to be responsible of the appearance of skeletal deformities (Cahu et al, 2003a, 2003b; Lall & Lewis-McCrea, 2007). The same effect has been described with an unsettled temperature. In fact, an important bottleneck in terms of climate change and proper bone development is temperature, although several environmental factors have been mainly cited as possible candidates to cause skeletal anomalies in farmed fish: fast growth conditions, oil films on water surface, water conditions, $O_2$/$CO_2$ levels, pH, salinity, among others.

Water temperature is a critical factor due to it has significant effects on poikilotherm fish growth, development and survival rates (Georgakopoulou et al, 2010). Specifically, in gilthead sea bream, the first study about effects of temperature on bone development dates from 1991 (Polo, Yúfera & Pascual, 1991), where it was demonstrated that in early stages, water temperature affects this process causing abnormalities in jaws betwixt others. Despite the aetiology of skeletal deformities remain uncertain, increased temperature during fish rearing has been demonstrated an important causative factor (Boglione & Costa, 2011; Boglione, 2013b; Ytteborg et al, 2012). In this sense, abnormal growth of muscle as well as deformation of vertebral bodies in fish larvae consequently leading to spinal deformities, are the effects of fluctuations in water temperature (Davidson et al, 2011; Wang & Tsai, 2000). Several species have demonstrated the compromising effects of increased water temperature not only related to global

warming but also, by the hyperthermia regimes used to obtain faster growth as in Atlantic salmon. In this species, gene transcription in osteoblasts and chondrocytes demonstrated to be altered and directly correlated with the increased number of deformities and structural/compositional modifications in the bone tissue (Ytteborg et al, 2010a). In this respect, Atlantic salmon at elevated water temperatures suffered an upregulation of a matrix metalloproteinase 13 (MMP13), an enzyme involved in the degradation of the ECM (Grini et al, 2011). On the contrary but in line with the same conclusion, a downregulation in the expression of ECM genes (*col1a1, on, ocn,* etc.) was observed in the same species in Ytteborg et al (2010b).

Furthermore, temperature not only affects bone development and osteogenesis, but also fish growth and metabolism, including digestibility of some components of the diet. And, as mentioned above, nutrition has also been demonstrated to impact on bone development. In this regard, in sea bass larvae, fatty acids have been described to affect genes regulating bone development leading to skeletal malformations (Villeneuve et al, 2005a).

### 1.5.2. Fatty acids and their importance in fish bone tissue

Regarding nutrients, especially fatty acids can serve as an energy source, and as demonstrated in mammals (Poulsen, Moughan & Kruger, 2007) some specific LC-PUFA have several essential roles in metabolism and in bone metabolism. In mammals, fatty acids have been described to affect bone by different pathways (Figure 9): in a direct mode interacting with bone nuclear or membrane receptors; indirectly by the effects produced in other tissues and their connection with bone; or by activating an immune response that also affect skeletal tissue (Wauquier et al, 2015).

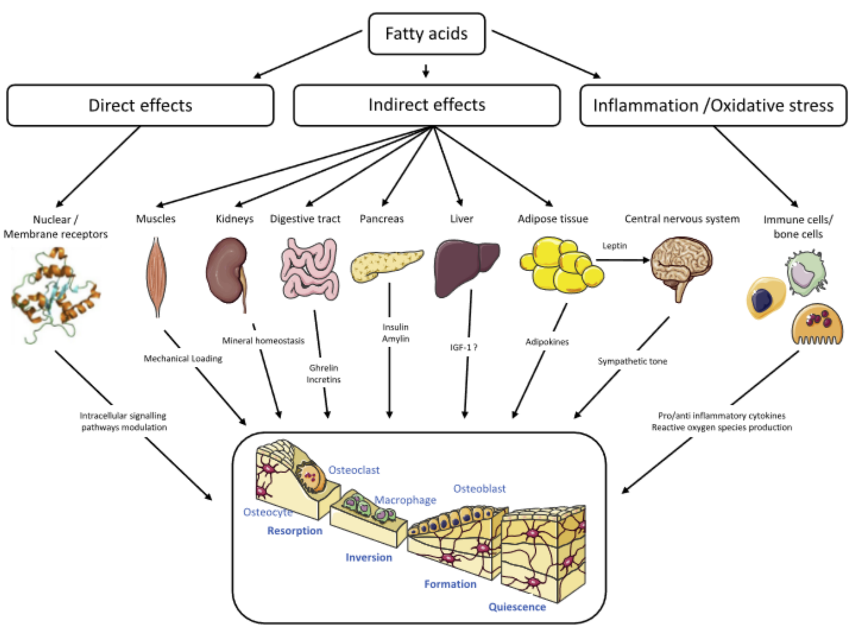



**Figure 9.** Fatty acids and their connections with bone remodelling by direct or indirect effects in mammals (Wauquier et al, 2015).

Among the different affectations by EFA deficiencies, lordosis has been described together with fatty liver, intestinal steatosis and bleeding from gills (NRC, 2011; Tacon 1996); however, the studies focused on the relationship between EFA deficiencies and skeletal development are quite scarce. Fingerlings of grass carp fed a diet without EFA presented higher incidence of a column displacement in a specific region, which prevention was possible with the addition of 1% linoleic acid (LA) to the diet (Takeuchi, Okano & Kobayashi, 1991). Also, in milkfish larvae (*Chanos chanos*), the addition of DHA in adequate doses to live food was demonstrated to reduce the appearance of opercular anomalies in juveniles (Gapasin & Duray 2001). Besides, DHA was demonstrated to have beneficial effects in the reduction of a 50%, the number of fishes presenting vertebral fusion or cranial anomalies in red porgy larvae (Izquierdo et al, 2010).

Concerning the mechanisms of action, dietary PUFA and their metabolites have demonstrated to change metabolism, cell differentiation and growth through the control of transcription factors' gene expression in mammals (Jump, 2004) and in

fish (Oku & Umino 2008; Ytteborg et al, 2010d). High dietary levels of n-3 LC-PUFA have been shown to induce deformities (e.g. supernumerary vertebrae among other vertebral affectations and cephalic anomalies), negatively affecting fish growth and survival by the modulation of some transcription factor genes; thus, accelerating osteoblast differentiation (upregulation of *rxr* and *bmp4*) in European seabass larvae (Villeneuve et al, 2005b, 2006). Alterations in gene expression were found by Viegas et al (2012) in *bmp2, op* and *tnap,* where DHA seemed to stimulate mineralization, while EPA and arachidonic acid (ARA) inhibited the process in *in vitro* osteoblasts derived from gilthead seabream bone, therefore showing different cell phenotypes.

## 1.6. Plasticity of mesenchymal stem cells

Osteoblasts and adipocytes derive from a common precursor in mammals, presenting MSCs plasticity between both cell lineages, which can imply, in some cases, diseases due to an imbalance betwixt the two types of cells in mammals (e.g. osteoporosis). Several transcription factors have been revealed to control osteogenesis and adipogenesis, and when these genes have been silenced or overexpressed have demonstrated to be key in the control of plasticity between the two lineages (Figure 10). In mammals, MSCs have been described as cells that have the ability to change their linage depending on the surrounding microenvironment thus, an induction of adipogenesis will suppose an inhibition of osteoblastogenesis and *viceversa* (e.g. PPARγ can inhibit osteogenesis by suppressing Runx2 expression) (Berendsen & Olsen, 2013a, 2013b).

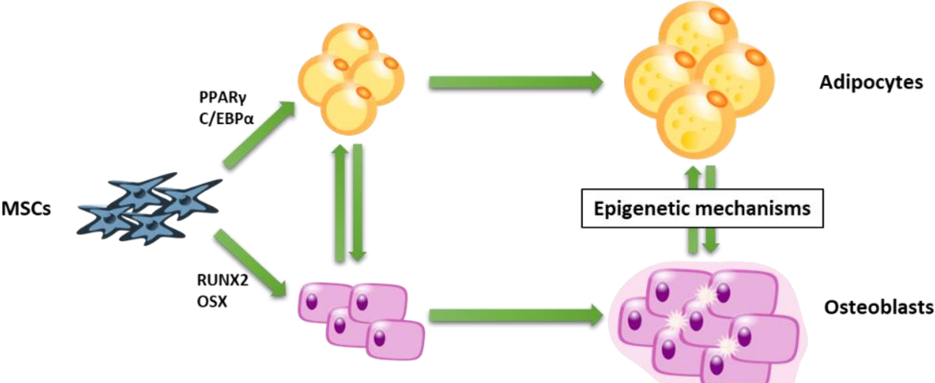



**Figure 10.** Osteoblast-adipocyte lineage plasticity in mammals. During osteogenesis, cells express transcription factors genes as runt-related transcription factor 2 (Runx2) and osterix. During adipogenesis, cells express transcription factors genes as peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα). The determination of lineage commitment of these mesenchymal stem cells (MSCs) is regulated by the balance between osteogenic and adipogenic transcription factors and epigenetic mechanisms. Adapted from Berendsen & Olsen (2013b).

Nowadays, the plasticity of MSCs to differentiate into lineages different from the original tissue has been proved with culture models of cells established from fish adult tissues as fat and bone (Capilla et al, 2011; Salmerón et al, 2013; Ytteborg et al, 2010d, 2015). In fish, cellular determination and transdifferentiation (epigenetic mechanisms) has been suggested to be involved in the appearance of diseases as vertebral malformations in salmon (Helland et al, 2006; Ytteborg et al, 2010a, 2010b, 2010c). In this context, the metabolic surrounding of these cells can be altered by dietary conditions among other factors (Figure 11) and this seems to affect bone formation as it has been demonstrated that fatty acids can affect osteoblasts and also MSCs. In mammals, it has been proved that misadjusted dietary ratios of n3/n-6 can modify bone health and fat content as a consequence of the cellular changeover (Watkins et al, 2001). Furthermore, in mammals PUFA deficiency altered the ECM synthesis and facilitated bone demineralization and resorption (Watkins, Li & Seifert, 2006). In this sense, diets based on SO have induced a reduction in vertebra size in salmon (Gil Martens et al, 2010). Moreover, high levels of ARA have shown to reduce *in vivo* and *in vitro* bone mineralization

in zebrafish (*Danio rerio*) and gilthead sea bream (de Vrieze et al, 2014: Viegas et al, 2012). In this context, it must be important to consider the multiple factors that can control MSCs differentiation such as the different chemical factors used to induce differentiation *in vitro*, but also *in vivo*, where the fate of this cells can be affected by physical and biological factors (Chen et al, 2016) Therefore, diet is also a factor that can affect bone, and this is an important issue to take into account in diet formulations now that aquaculture is improving feeds with new source ingredients.

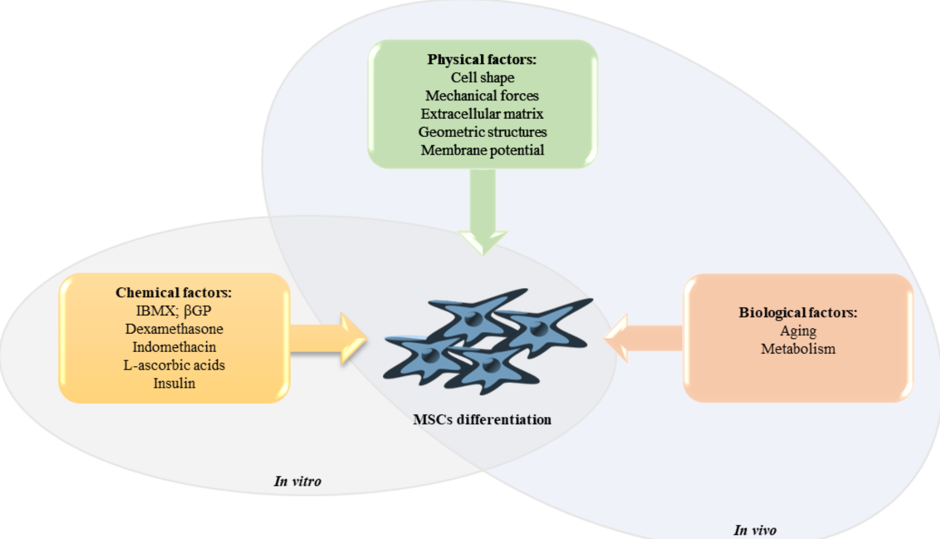
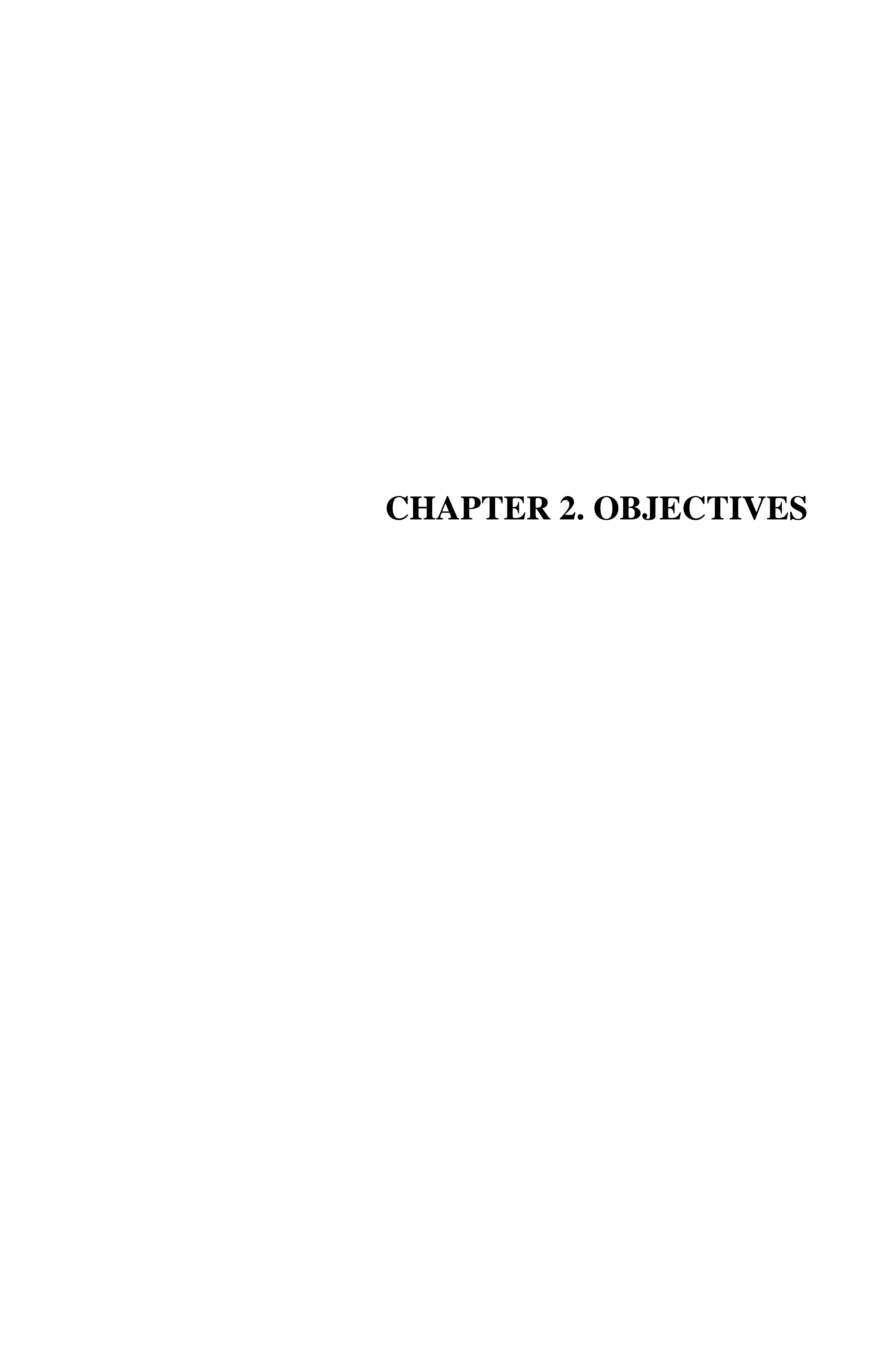



**Figure 11.** Factors controlling MSCs differentiation. IBMX: isobutylmethylxanthine; βGP: β-glycerophosphate. Adapted from Chen et al (2016).



# CHAPTER 2. OBJECTIVES

This thesis aims to characterize the differentiation process of adipocytes and osteoblast cells to then describe the effect of vegetable oils and their common fatty acids *in vivo* and *in vitro* in the adipocyte metabolism and fatty content of the fish through the study of specific lipolytic and lipogenic markers; as well on skeletal functionality, both in habitual or increased temperature. Thus, getting tools to progress sustainably in cultivation of gilthead sea bream and rainbow trout, facing the challenges as improvement of diets and adaptation to climate change to which aquaculture must respond but at the same time assuring the obtention of product with optimum quality.

Specific aims:

1. Characterization of gene expression pattern during the preadipocyte differentiation to mature adipocyte in rainbow trout and gilthead sea bream. **Articles I and II.**

2. Characterization of gene expression pattern during the mesenchymal stem cell *in vitro* differentiation to osteoblast in gilthead sea bream, during development *in vivo* and *in vitro* plasticity of the cells to differentiate into another linage**.** **Articles II, III and IV.**

3. Effects of fatty acids characteristic from fish and vegetable oil on *in vitro* osteoblast from gilthead sea bream, on deposition and lipid mobilization in cultured adipocytes and on lipid metabolism in force-fed rainbow trout. **Articles IV and V.**

4. Effects of temperature changes on the mineralization and skeletal development, deposition and mobilization of lipid stores in cultured osteoblasts and on *in vivo* gilthead sea bream respectively. **Articles III and VI.**

5. Characterization of gene expression pattern in malformed skeletal structures of gilthead sea bream. **Article VII.**

6. Effects of diets of maximum replacement of fish oil with vegetable oils in gilthead sea bream at high temperature. **Article VI.**

Articles:

I – Gene expression pattern during adipocyte differentiation in rainbow trout (*Oncorhynchus mykiss*).

II - Adipogenic gene expression in gilthead sea bream mesenchymal stem cells from different origin **(2016)**. ***Frontiers in Endocrinology, 7,* 113.**

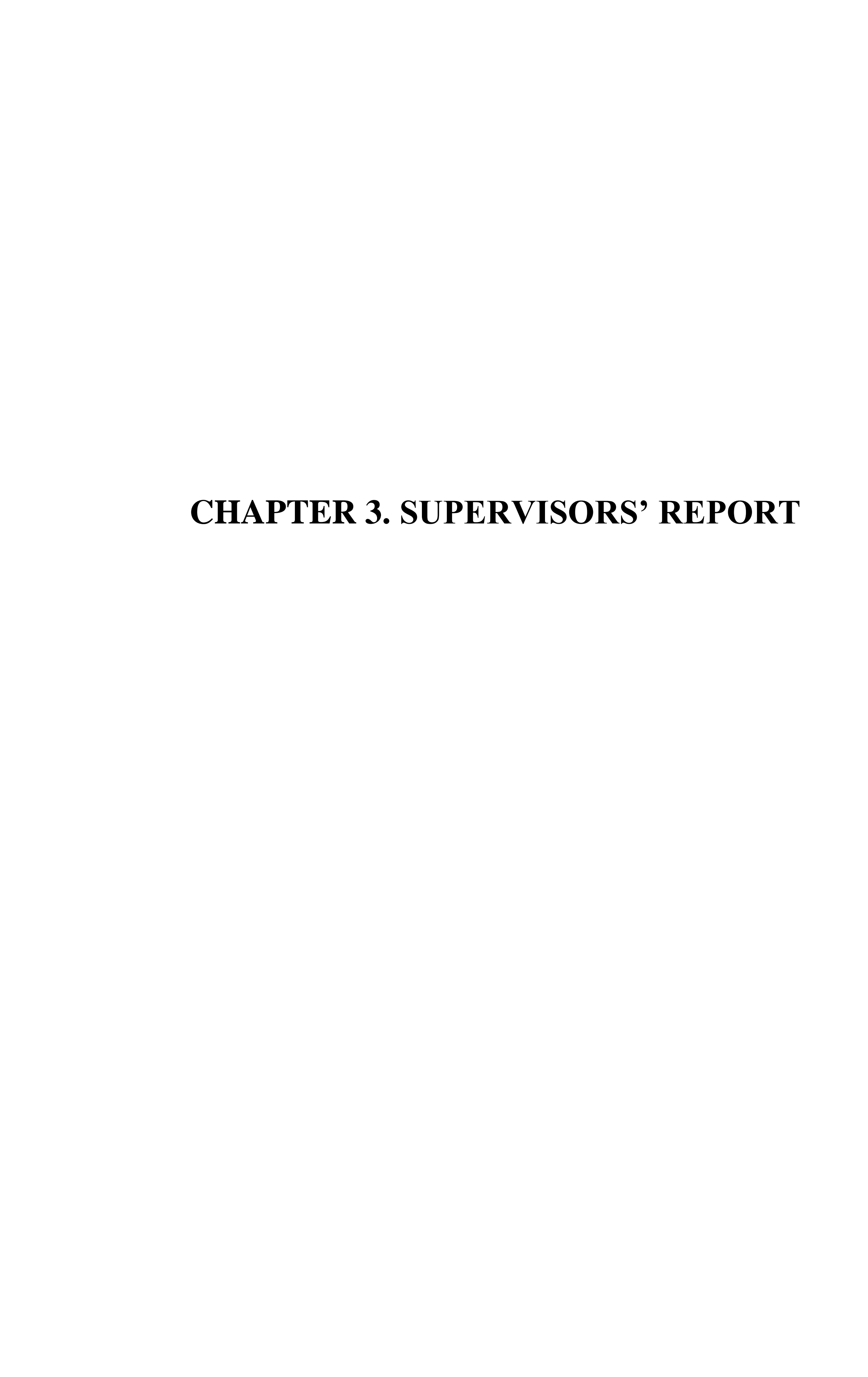
III - Temperature responsiveness of gilthead sea bream bone; an *in vitro* and *in vivo* approach **(2018)**. ***Scientific Reports 8,* 11211.**

IV – Fatty acids from fish or vegetable oils promote the adipogenic fate of mesenchymal stem cells derived from gilthead sea bream bone potentially through different pathways **(2019)**. ***Plos One, 14*, 4, e0215926.**

V - Short-term responses to fatty acids on adipogenesis *in vitro* and lipid metabolism *in vivo* in rainbow trout (*O. mykiss*).

VI - Vegetable oils as a good alternative for optimal growth of gilthead sea bream under ocean warming.

VII - Gene expression analyses in malformed skeletal structures of gilthead sea bream (*Sparus aurata*) **(2019). *Journal of Fish Diseases, 42*, 8, 1169-1180.** (Front Cover. *Journal of Fish Diseases, 42*(8), i-i).



# CHAPTER 3. SUPERVISORS' REPORT

**Department of Cell Biology,**
**Physiology and Immunology**
Faculty of Biology

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Tel: 934 021 531

La Dra. Encarnación Capilla Campos i la Dra. M. Isabel Navarro Álvarez, com a directores de la tesi doctoral presentada per la Natàlia Riera Heredia titulada "**Fatty acids and temperature effects in fish adipogenesis and osteogenesis: *in vitro* and *in vivo* approaches**" manifesten la veracitat del factor d'impacte i la implicació de la doctoranda en els articles científics publicats o pendents de publicació presentats en aquesta tesi.

La Natàlia Riera ha participat de forma molt activa en l'elaboració dels articles en tots els aspectes, tal i com queda reflectit en la relació d'autors, ja que consta com a primera autora en tots excepte un d'ells (article II). Així mateix, la Natàlia ha contribuït de manera destacada en el plantejament i la realització dels experiments, obtenció de dades, anàlisis de resultats, així com en la redacció i elaboració dels esmentats articles. L'article 1 deriva en part d'una estada de 3 mesos realitzada per la Natàlia al laboratori del Dr. Iban Seiliez de l'INRA a Saint-Pée sur-Nivelle (França), i que forma part d'un projecte més ampli que també engloba estudis sobre l'autofàgia. En l'article 2, tot i que no consta com a primera autora, la doctoranda va realitzar molts dels processos d'anàlisis i obtenció dels resultats, així com la seva interpretació. Finalment, es van realitzar també en col·laboració, l'article III amb el grup de la Dra. Deborah Power del CCMAR (Faro, Portugal) que va aportar part del disseny experimental, i l'article VI amb el Dr. Ramon Fontanillas del grup d'I+D+I de l'empresa de pinsos Skretting (Stavanger, Noruega).

**Article I: Gene expression pattern during adipocyte differentiation in rainbow trout (*Oncorhynchus mykiss*).**
Autors: Riera-Heredia, N., Lutfi, E., Balbuena-Pecino, S., Gutiérrez, J., Capilla, E. and Navarro, I.
Revista: per determinar
Factor d'impacte: -
Estat: En preparació

**Article II: Adipogenic gene expression in gilthead sea bream mesenchymal stem cells from different origin.**
Autors: Salmerón, C., Riera-Heredia, N., Gutiérrez, J., Navarro, I. and Capilla, E. (2016)
Revista: ***Frontiers in Endocrinology*** 7: 113. doi: 10.3389/fendo.2016.00113
Factor d'impacte i quartil: **3.675 Q2**
Estat: Publicat

**Article III: Temperature responsiveness of gilthead sea bream bone: an *in vitro* and *in vivo* approach**.
Autors: Riera-Heredia, N., Martins, R., Mateus, A.P., Costa, R.A., Gisbert, E., Navarro, I., Gutiérrez, J., Power, D.M. and Capilla, E. (2018)
Revista: ***Scientific Reports*** 8: 11211. doi: 10.1038/s41598-018-29570-9
Factor d'impacte i quartil: **4.011 Q1**
Estat: Publicat

**Article IV: Fatty acids from fish or vegetable oils promote the adipogenic fate of mesenchymal stem cells derived from gilthead sea bream bone potentially through different pathways.**
Autors: Riera-Heredia, N., Lutfi. E., Gutiérrez, J. Navarro, I. and Capilla, E. (2019)
Revista: ***PLoS ONE*** 14: e0215926. doi: 10.1371/journal.pone.0215926
Factor d'impacte i quartil: **2.776 Q2**
Estat: Publicat

**Article V: Short-term responses to fatty acids on adipogenesis *in vitro* and lipid metabolism *in vivo* in rainbow trout (*Oncorhynchus mykiss*).**
Autors: Riera-Heredia, N., Lutfi, E., Sánchez-Moya, A., Gutiérrez, J., Capilla, E. and Navarro, I.
Revista: ***International Journal of Molecular Sciences***
Factor d'impacte i quartil: **4.183 Q2**
Estat: Pendent d'enviar

**Article VI: Vegetable oils as a good alternative for optimal growth of gilthead sea bream under ocean warming**.
Autors: Riera-Heredia, N., Sánchez-Moya, A., Fontanillas, R., Gutiérrez, J., Capilla, E. and Navarro, I.
Revista: ***Aquaculture***
Factor d'impacte i quartil: **3.022 Q1**
Estat: Pendent d'enviar

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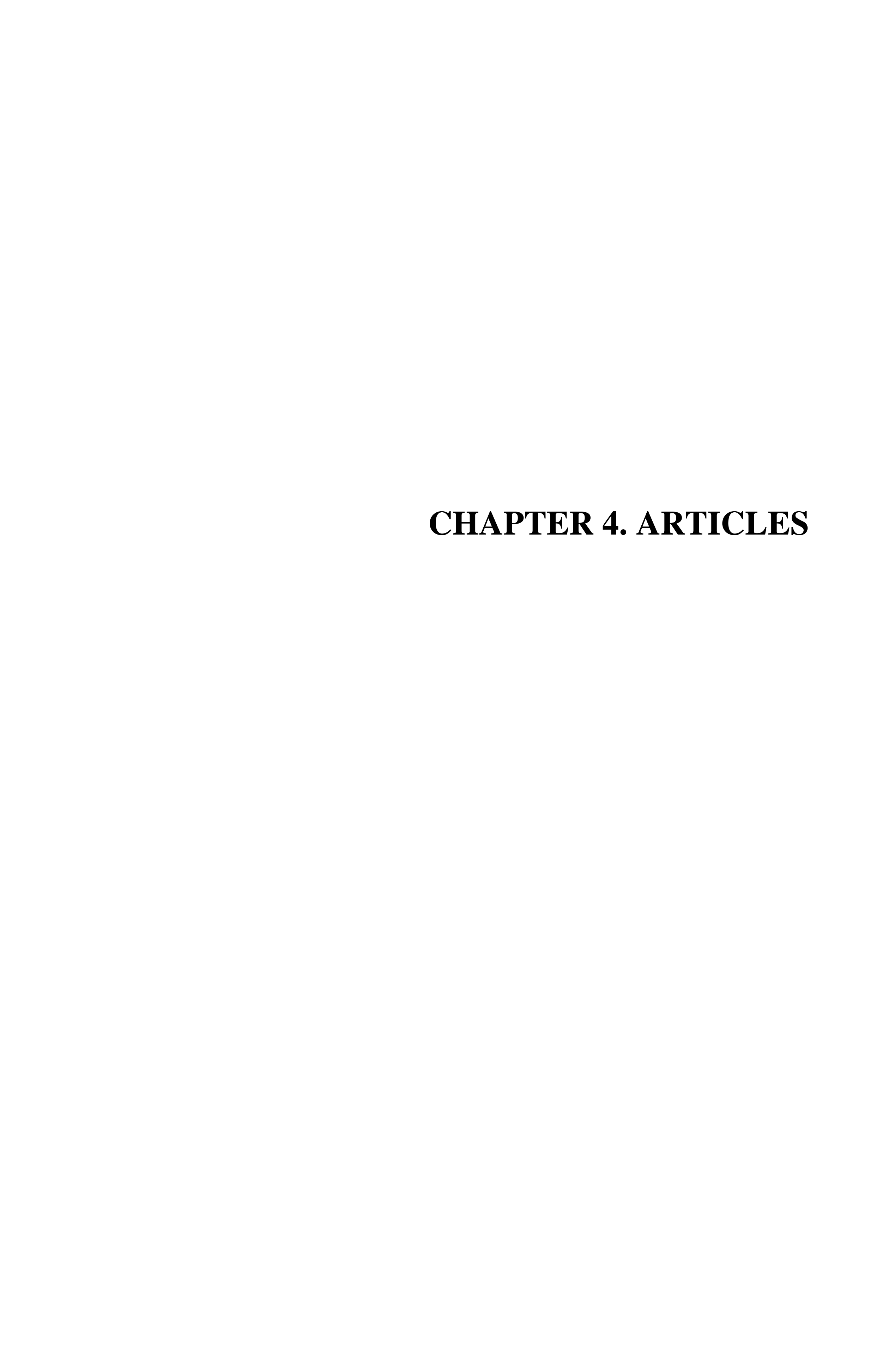

# CHAPTER 4. ARTICLES

# ARTICLE I

# **Gene expression pattern during adipocyte differentiation in rainbow trout (*Oncorhynchus mykiss*)**

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## Abstract

Adipogenesis, in fish as in mammals, is a tight regulated process that involves a complex network of transcription factors, enzymes and lipid transporters. In rainbow trout primary cultured preadipocytes, two well-differentiated phases have been described at a transcriptional level along this process: preadipocytes proliferation and differentiation into mature adipocytes. In fish, a lipid component is required to be added to the differentiation medium to induce the maturation of adipocytes in culture allowing lipid droplet formation and the activation of the key transcription factors responsible of adipogenesis. Afterwards, the organized expression of specific genes as lipogenic enzymes, and genes coding for lipid binding proteins follow thus, along the the programming of adipocyte differentiation. Nevertheless, information on the transcriptional profile during the very early stages of fish adipocyte differentiation is still missing. In this context, a change in the morphology from a fibroblastic to a more rounded shape was observed in the cells during differentiation, together with increased intracellular lipid accumulation. With the induction of adipogenesis, the cells demonstrated transient activation of various transcription factors (i.e. *cebpa* and *cebpb*) that in turn, modulated the expression of genes involved in lipid metabolism, being this cascade responsible for the regulation of the synthesis (*fas*), uptake (*fatp1* and *cd36*), accumulation (*plin2*) or mobilization (*hsl*) of lipids in the mature adipocyte. Thus, this cell system is a good model to study adipogenesis in fish and to establish potential links between whole body energy regulation and adipocytes metabolism in this important aquaculture species.

**Keywords:** adipose tissue, adipogenesis.

## 1. Introduction

In mammals, the existence of white, beige and brown adipose tissue depots has been demonstrated (Lee et al, 2014), whereas only white adipose tissue (AT) has been described in teleost fish (Salmerón, 2018). AT is formed mainly by adipocytes and is involved in the control of energy homeostasis regulating lipid turnover and metabolism through secretion of different adipokines that affect AT itself and other tissues (Esteve Ràfols, 2014; Konige et al, 2014; Rutkowski et al, 2015; Salmerón et al, 2015a,b; Saltiel, 2001; Sánchez-Gurmaches et al, 2012). AT lipid content is balanced between lipogenesis and lipolysis, with production of triglycerides (TGs) for storage and lipolysis, and by breaking these TGs either for distribution to other tissues or, to be metabolized and used as source of energy by mitochondrial oxidation in the same AT (Salmerón, 2018). In situations of positive energy balance, AT grows by hypertrophy, with adipocytes increasing its lipid content and size (Muir et al, 2016). In this sense, AT hypertrophy in rainbow trout (*Oncorhynchus mykiss*) has been described to be consequence of the ingredients contained in vegetable diets, with the obtention of less functional/responsive adipocytes (Cruz-García et al, 2011).

Moreover, a second type of AT growth exists: hyperplasia, consisting in the formation of new adipocytes from precursor cells (Hausman et al, 2001) also known as adipogenesis. This process requires a complex transcriptional cascade to finally express genes associated with lipid uptake, transport and synthesis, characteristic of the mature adipocyte (Den Broeder et al, 2015; Rosen & MacDougald, 2006). In mammals, adipogenesis comprises a two-step developmental process consisting on the commitment of multipotent cells to the adipocyte linage and the further differentiation phase comprising the conversion of these cells into fully functional mature adipocytes (Rosen & Spiegelman, 2000). During the first phase, cells are morphologically like fibroblasts and known to express transcription factors as peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT-enhancer-binding proteins alpha and beta (C/EBPα and C/EBPβ). On the other hand, in the differentiation phase, cells present a more

rounded shape and express lipogenic enzymes like lipoprotein lipase (LPL), fatty acid binding proteins (FABP), and high levels of the lipid-droplet-associated protein perilipin 1 (PLIN) (Ghaben & Scherer, 2019) among other genes. The two well-differentiated phases of preadipocytes proliferation and differentiation into mature adipocytes have been also described in rainbow trout primary cultures of preadipocytes at a transcriptional level (Bou et al, 2017). In the same study, proliferation phase was characterized by an upregulation of genes involved in basic cellular and metabolic processes and cellular remodelling and in the terminal differentiation phase were upregulated genes involved in energy production, lipid and carbohydrate metabolism as well as genes implied in the formation of the lipid droplets.

During adipogenesis, PPARγ is known as the master regulator (Rosen & Spiegelman, 2000), which forms an heterodimer with the cis-retinoic acid receptor alpha (RXRα) that binds to promoters of genes related to fatty acid and glucose metabolism (Lefterova et al, 2014). In human cultured preadipocytes, an increased induction of transcription factor genes from *cebp* family increase *pparg* expression and altogether initiate the programming of adipocyte differentiation, starting the coordinated expression of specific genes when the differentiation medium is added (Gustafson et al, 2015), which usually contains insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) (Scott et al, 2011). Particularly in fish, the addition of a lipid mixture into the differentiation medium is necessary to induce the fully differentiation of adipocytes in culture, enabling lipid droplet formation and the activation of PPARγ through natural ligands (Bouraoui et al, 2008; Salmerón et al, 2013; Vegusdal et al, 2003; Wang et al, 2012). In fish, before adipocyte differentiation is evident, precursor cells express *ppar*, indicating the possible pre-commited adipogenic linage of stromal vascular cells (Bouraoui et al, 2008; Salmerón et al, 2016; Todorčević et al, 2010). Differentiated adipocytes from fish store lipid and express adipocyte marker genes as *transcription factors*, lipogenic enzymes, as well as those coding for proteins related to lipid binding (Salmerón, 2018). Thus, adipogenesis is a complex process that requires a tight

regulation and involves a complex network of transcription factors, enzymes and lipid transporters, in fish as in mammals.

The increase in knowledge of the adipogenic process and its regulation in fish can be key in aquaculture. In fact, some cultured species have experienced excessive fat accumulation, being perceived as an undesirable trait by the consumers and presenting negative effects in production, and fish health and welfare. However, despite primary cultures of preadipocytes have been established from several species (Vegusdal et al, 2003; Oku et al, 2006; Bouraoui et al, 2008; Salmerón et al, 2013), knowledge on the gene expression pattern during fish adipogenesis is still scarce. Thus, this study aims to characterize the cascades of gene expression that are involved in this process to obtain deeper insights into the dynamics of adipocyte differentiation in rainbow trout.

## 2. Material and methods

### 2.1. *Animals and ethics statement*

Adult rainbow trout (*Oncorhynchus mykiss*) approximately 250 g in weight were obtained from the fishery "Troutfactory" (Lleida, Spain) and were acclimated to the facilities in the Faculty of Biology at the University of Barcelona before use. Fish were kept in 400 L fiberglass tanks under a 12 h light/12 h dark photoperiod and were fed *ad libitum* twice daily with a commercial diet (Optiline, Skretting, Burgos, Spain). Fish were fasted for 24 h in order to avoid contamination from the gastrointestinal tract during adipose tissue extraction. Before sacrifice by cranial concussion, fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS222) (Sigma–Aldrich, Tres Cantos, Spain). All animal handling procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by Ethics and Animal Care Committee of the University of Barcelona, following the regulations and procedures established by the Spanish and Catalan governments (permit numbers CEEA OB35/17).

2.2. *Primary culture of adipocyte cells*

All cell culture reagents were purchased from Sigma–Aldrich (Tres Cantos, Spain) and Life Technologies (Alcobendas, Spain) and all plastic items were obtained from Nunc (LabClinics, Barcelona, Spain). Cells were isolated from rainbow trout adipose tissue and cultured according to the previously established procedure by Bouraoui et al. (2008). The cells were seeded at a density of $2 \times 10^4$ cells/cm$^2$ in 6 well plates (9.6 cm$^2$) in growth medium containing L15 with 0.1 % fetal bovine serum and 0.01 % antibiotic antimycotic and were maintained at 18 ºC. After confluence (day 7), cells were induced to differentiate by means of a growth medium supplemented with 10 μg/mL insulin, 0.5 mM IBMX, 0.25 μM dexamethasone and 10 μL/mL lipid mixture. Media were changed every 2 days during the whole procedure. Samples were collected at day 3 (preadipocytes) and every day from day 7-11 of culture (maturing adipocytes). The cells were daily assessed with a Zeiss Axiovert 40C inverted research grade microscope (Carl Zeiss Inc., Germany) equipped with a Canon EOS 1000D digital camera to check the morphology and ensure that they followed the already described developmental process (Bouraoui et al, 2008).

2.3. *RNA extraction, cDNA synthesis and real-time quantitative PCR (qPCR) analyses*

At different culture days (3, 7, 8, 9, 10 and 11), the cells were lysed with a cell scraper and TRI Reagent (Applied Biosystems, Alcobendas, Spain) in a total volume of 500 μL per each two wells. Total RNA was extracted according to the manufacturer's recommendations, was dissolved in DEPC-treated water (RNase-free), quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and stored at −80°C. To eliminate any residual genomic DNA, total RNA (1 μg) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain), following the manufacturer's instructions.

The selected key genes implicated in adipogenesis and energy metabolism regulation as transcription factors or nuclear receptors, enzymes and fatty acid transporters listed in Table 1 were analysed by qPCR including the reference genes elongation factor 1 alfa (*ef1a*), *b-actin* and ubiquitin (*ub*). qPCR analyses and preliminary validation assays were performed as described in Riera-Heredia et al, (2019). The stability of the reference genes as well as the mRNA levels of expression of the genes of interest calculated relative to the most stable ones (geometric mean of *b-actin* and *ub*) according to the Pfaffl method (Pfaffl, 2001) were determined using the CFX Manager Software implemented in the CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain).

**Table 1.** Primers used for real-time quantitative PCR. F, forward primer; R, reverse primer; Ta, annealing temperature; Acc. Num., accession number. Liver X receptor (*lxr*); Fatty acid synthase (*fas*); Hormone sensitive lipase (*hsl*); Fatty acid transport protein 1 (*fatp1*); Cluster of differentiation 36 (*cd36*).

| Gene | Primer sequence (5'→3') | Ta (ºC) | Acc. Num. |
|---|---|---|---|
| *cebpa* | F: TGTGGCGATAAAGCAAGAGC<br>R: CTGGTGGGAATGGTGGTAGG | 57 | DQ423469.1 |
| *cebpb* | F: CACAAAGTGCTGGAACTGGC<br>R: TGGCACAGCGATAAATGGGT | 60 | FR904306.1 |
| *pparg* | F: GCCAGTACTGTCGCTTTCAG<br>R: TCCATAAACTCAGCCAGCAG | 60 | HM536192.1 |
| *rxr* | F: AAAGAGCGCAGTGAGAACGA<br>R: TGTAGGTCTCGGTCTTGGGT | 55 | AJ969439.1 |
| *lxr* | F: TGCAGCAGCCGTATGTGGA<br>R: GCGGCGGGAGCTTCTTGTC | 62 | NM_001159338 |
| *lpl* | F: TAATTGGCTGCAGAAAACAC<br>R: CGTCAGCAAACTCAAAGGT | 59 | AJ224693 |
| *fas* | F: GAGACCTAGTGGAGGCTGTC<br>R: TCTTGTTGATGGTGAGCTGT | 54 | tcaa0001c.m.06_5.1.om.4 |
| *hsl* | F: AGGGTCATGGTCATCGTCTC<br>R: CTTGACGGAGGGACAGCTAC | 58 | TC172767 |
| *fabp11a* | F: CATTTGAGGAGACCACCGCT<br>R: ACTTGAGTTTGGTGGTACGCT | 60 | NM_001124713.1 |
| *fatp1* | F: AGGAGAGAACGTCTCCACCA<br>R: CGCATCACAGTCAAATGTCC | 60 | CA373015 |
| *cd36* | F: CAAGTCAGCGACAAACCAGA<br>R: ACTTCTGAGCCTCCACAGGA | 62 | AY606034 |
| *plin2* | F: GATGGCAATGAGGCAGAGAACA<br>R: AGGCAGAGTGGCTAAGGGACAG | 60 | CB494091.p.om.8 |
| *ef1a* | F: TCCTCTTGGTCGTTTCGCTG<br>R: ACCCGAGGGACATCCTGTG | 58 | AF498320 |
| *b-actin* | F: ATCCTGACAGAGCGCGGTTACAGT<br>R: TGCCCATCTCCTGCTCAAAGTCAA | 61 | AJ438158 |
| *ub* | F: ACAACATCCAGAAAGAGTCCAC<br>R: AGGCGAGCGTAGCACTTG | 58 | AB036060 |

2.4. *Statistical analyses*

Data normality and homoscedasticity were assessed using Shapiro–Wilk and Levene's test, respectively. For multiple mean comparisons among days in culture of normal distributed data, one-way ANOVA was used followed by Tukey's or Dunnett's T3 *post hoc* tests in case of homogeneous or heterogeneous variance data, respectively. When data did not fit normal distribution, the non-parametric Kruskal–Wallis test, followed by Mann–Whitney test, were used. Statistical analyses were performed using SPSS Statistics version 22 (IBM, Armonk, NY, USA). Results were presented as mean ± SEM, and $P < 0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

## 3. Results

Adipose tissue-derived precursor cells from rainbow trout where cultured up to day 11, with induction of differentiation with the specific medium at day 7 when confluence was reached. During adipogenesis, cell morphology changed from the typical fibroblastic shape of precursor cells to a more rounded and enlarged shape filled with intracellular lipid droplets (Fig. 1).

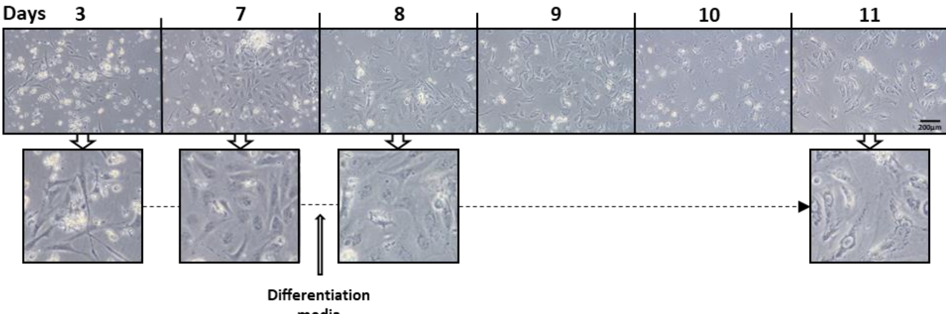


**Fig. 1.** Representative phase-contrast images (20x magnification) and enlarged views of rainbow trout adipocytes at different days of culture from day 3 to day 11.

Moreover, relative gene expression of the key transcription factors driving the differentiation process (*cebpa, cebpb, pparg, rxr* and *lxr*) were analysed, observing

low transcript levels during the commitment phase (days 3-7) and a significant peak in expression at day 8 just 24h after the addition of the differentiation media (Fig. 2A-E). Then, a marked decrease in expression was observed at day 9, maintaining these genes low levels like at day 3, during the rest of the process. In the case of *pparg* expression, despite the overall profile was similar, significant differences were not observed, and the peak at day 8 was softer due to the increasing higher levels of expression already observed during the preadipocyte phase (Fig. 2C).

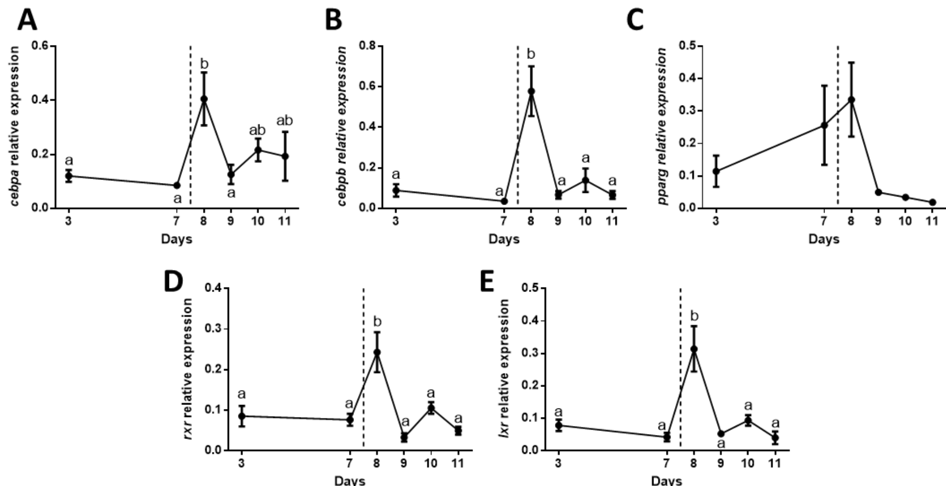


**Fig. 2.** Relative expression of genes coding for transcription factors **(A)** *cebpa*, **(B)** *cebpb,* **(C)** *pparg,* **(D)** *rxr,* and **(E)** *lxr*, in rainbow trout adipocytes at different days of culture from day 3 to day 11. Data are shown as mean ± SEM (n=4-8). Significant differences (p<0,05) among treatments are indicated by different letters. The vertical dotted line indicates the moment of addition of the differentiation medium

Regarding relative expression of genes coding for enzymes involved in lipid metabolism, contrarily to what was found in transcription factors, the patterns observed were different depending on the enzyme. The *lpl* (Fig. 3A) expression pattern was similar to that of the transcription factors showing low levels at days 3 and 7 with a posterior significant peak at day 8 after induction of differentiation, and then decrease rapidly at day 9. During the preadipocyte phase, *fas* (Fig. 3B) presented elevated expression and after the addition of the differentiation medium the expression was quickly decreased from day 9 onwards, whereas *hsl* (Fig. 3C)

showed an upregulated expression at days 7 and 8 with a marked decrease at day 9.

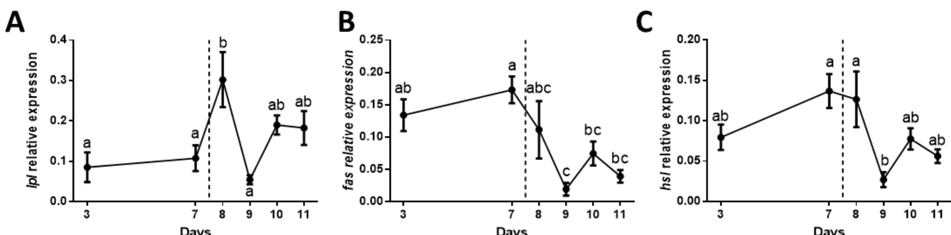


**Fig. 3.** Relative expression of genes coding for enzymes related to energy metabolism **(A)** *lpl,* **(B)** *fas*, and **(C)** *hsl* in rainbow trout adipocytes at different days of culture from day 3 to day 11. Data are shown as mean ± SEM (n=4-8). Significant differences (p<0,05) among treatments are indicated by different letters. The vertical dotted line indicates the moment of addition of the differentiation medium.

Finally, different patterns were also observed concerning the genes involved in lipid transport. Regarding *fabp11* (Fig. 4A), significant differences were not observed but a soft peak could be seen at day 8 to then decrease up to day 11. Low expression levels between days 3 and 9 were observed for *fatp1* (Fig. 4B) with a subsequent significant upregulation at day 10 and a decrease at day 11. For *cd36* (Fig. 4C), expression remained unaltered from day 3 to day 8 to then progressively significantly increase up to 11. A similar pattern of expression was observed for *plin2* (Fig. 4D) which expression remained low at the preadipocyte stage from days 3 to 7 to then significantly increase at day 9 and continue to rise up to day 11.

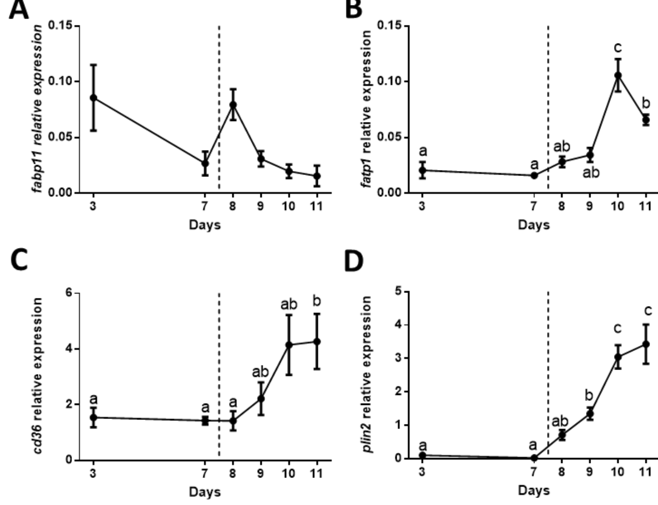

**Fig. 4.** Relative expression of genes coding for fatty acid transporters and proteins related to lipid binding **(A)** *fabp11a*, **(B)** *fatp1*, **(C)** *cd36* and **(D)** *plin2* in rainbow trout adipocytes at different days of culture from day 3 to day 11. Data are shown as mean ± SEM (n=4-8). Significant differences (p<0,05) among treatments are indicated by different letters. The vertical dotted line indicates the moment of addition of the differentiation medium.

## 4. Discussion

Cells isolated from rainbow trout AT can be differentiated into mature adipocytes when the media is supplemented with dexamethasone, IBMX, insulin and lipid mixture (Bouraoui et al, 2008). In mammals, insulin is a powerful inductor of this process through activating *pparg* (Klemm et al, 2001; Nadeau & Draznin, 2004) and, the activation by IBMX and dexamethasone of *cebpb* and *cebpd* induces in turn the expression of *cebpa* and *pparg* as well (Chen et al, 2016). In rainbow trout (Bouraoui et al, 2008; 2012) and in red sea bream (*Pagrus major*) (Oku et al, 2006), insulin has been shown to favour differentiation of cultured preadipocytes, while the addition of lipids into the media as well as the addition of thiazolidinediones are known to induce adipocyte differentiation in fish (Bouraoui et al, 2012; Oku & Umino, 2008). Thus, differentiation media has demonstrated in our study to enhance the transition of the cell morphology from fibroblastic shape to more rounded shape, followed in detail along the different days of culture. These changes indicate the start of adipogenesis with the subsequent lipid accumulation inside the cell as it has been observed in murine cells, Atlantic salmon and gilthead sea bream (Mor-Youssef Moldovan et al, 2019; Vegusdal et al, 2003; Salmerón et al, 2013).

Adipogenesis is known to be a complex process that concerns the integration of many different signalling pathways and transcription factors (Rosen and Macdougald, 2006). A previous study analysing the transcriptome profile in rainbow trout preadipocytes by microarray demonstrated the coordinated expression of functionally related genes during proliferation and differentiation, confirming the relevance of the PPAR signalling pathway and abundance of PLIN2 during the differentiation phase (Bou et al, 2017), although in the last study

a low number of sampling days were analysed. In this sense, the information generated need further investigations about specific genes involved in particular stages of fish adipogenesis. In our study, cultured AT cells from rainbow trout showed similar patterns for the transcription factors evaluated where the expression of *cebpa, cebpb, pparg* and also *rxr* was increased after the induction with the differentiation medium consequently activating adipogenesis to obtain mature adipocytes. Once the process was started, gene expression of this transcription factors was downregulated immediately to remain low during the following days.

Concretely, C/EBPβ together with C/EBPδ, have been reported to transactivate C/EBPα in mammalian models (Cao et al, 1991), thus C/EBPα and C/EBPβ are involved in the initial steps of differentiation, confirmed in our study by its increased expression in rainbow trout cells with the addition of the differentiation medium. Ongoing with these early differentiated cells, an upregulation of *cebpa*, has been described also in Atlantic salmon (Huang et al, 2010; Todorčević et al, 2010). Furthermore, similar results have been reported in protein levels of C/EBPα in differentiated adipocytes from Atlantic salmon and in rainbow trout (Vegusdal et al, 2003; Bouraoui et al, 2008; Bou et al, 2017). Moreover, in salmon preadipocytes it has been showed that cells already express *pparg* before the induction of differentiation possibly indicating that these cells are fated to be adipocytes (Todorčević et al, 2010). In our rainbow trout adipocytes, the increasing higher levels of *pparg* expression already observed during the preadipocyte phase, before the peak after the induction of differentiation, to decrease then to basal levels, is in concordance with the expression pattern found in Atlantic salmon (Todorcevic et al, 2008). Contrarily, in a posterior study in the same species (Todorčević et al,2010), *pparg* presented higher expression in subconfluent cells and after the induction with the differentiation media the expression decreased, whereas in red sea bream the expression remained unaffected during this process (Oku & Umino, 2008). Thus, transcriptional regulation of *pparg* seems to be very sensible to the concrete adipogenic cell conditions and the characteristic dynamics

of the species. At the protein level, Bouraoui et al. (2008) observed that PPARγ levels were higher in maturing cells than in proliferating cells in rainbow trout preadipocytes, therefore confirming that this transcription factor could be essential during the adipogenic process. In the present study, the same pattern of expression as *pparg* was observed in *rxr* with the increased expression after the addition of the differentiation medium to then decrease to basal levels. This similar behaviour must relay in the relationship between both transcription factors, which heterodimerize, to then bind to several promoters of genes involved in fatty acid and lipid metabolism to control their expression (Lefterova et al, 2014). Regarding other transcriptional factors analysed, LXR is a cholesterol sensor (Steffensen & Gustafsson, 2004) that can be regulated by fatty acids (Pawar et al, 2002). In mammals (Mitro et al, 2006; Repa, 2000; Schultz et al, 2000) as well as in salmonids and gilthead sea bream (Cruz-Garcia et al, 2009a; Cruz-Garcia et al, 2009b) has been described to be involved in cholesterol, fatty acid and glucose metabolism. In rainbow trout adipocytes, the expression of *lxr* was similar to the transcription factors described above, thus the presence of lipids in the differentiation media could be activating the expression of this gene to control cholesterol and fatty acid metabolism in the maturing adipocyte.

Afterwards differentiation starts, adipogenic gene expression is then modulated by these transcription factors. In cultured adipocytes from rainbow trout, *lpl* showed an increased expression after differentiation with a subsequent decrease during the rest of the process. In this sense, previous studies in large yellow croaker (Wang et al, 2012), rainbow trout (Bouraoui et al, 2012), and red sea bream (Oku et al, 2006; Oku & Umino, 2008) showed also increased *lpl* expression after the induction of adipocyte differentiation, but maintained after some days of culture. On the other hand, in our adipocytes *fas* and *hsl* presented similar tendencies as *lpl* with a decrease in expression after the induction of differentiation. The same decrease in expression for *fas* was observed in gilthead sea bream adipocyte cells (Salmerón et al, 2016), while contrarily, in salmon (Todorcevic et al, 2010) and red sea bream (Oku & Umino, 2008) the expression increased various days during

the process of adipogenesis. Regarding *hsl,* the decreased expression found was opposite to the observations made in gilthead sea bream (Salmerón et al, 2016). Therefore, one possibility is that the increased *lpl* and decreased *fas* and *hsl* expression observed in our study could be modulated by the addition of the differentiation medium that contains not only hormonal components, but also lipids, putting fatty acids directly available to the cells. Thus, *lpl* could be enhancing lipogenesis, enabling lipid deposition as seen in *in vivo* studies in gilthead sea bream and rainbow trout adipose tissue (Albalat et al, 2007; Saera-Vila et al, 2005); *fas* and *hsl* seems to be inhibited through a negative feedback (Salmerón et al, 2016; Riera-Heredia et al, 2019). These genes associated to lipogenesis (*fas* and *lpl*) and lipolysis (*hsl*) have demonstrated, as discussed above, different patterns during adipocyte differentiation in different species thus, it cannot be discarded that their regulation could be species-specific or modulated at activity level.

Furthermore, genes implied in lipid transport are key in the maturation of preadipocytes and are also modulated by the transcription factors mentioned above. In Atlantic salmon, *fatp1* expression has been described to increase during the differentiation process of adipocytes (Todorcevic et al, 2008; Huang et al, 2010). In rainbow trout adipocytes an increase in the expression of this transporter was observed at 15 and 22 days of culture (Sánchez-Gurmaches et al, 2012). Accordingly, in the present study in rainbow trout adipocytes, *fatp1* expression was found to be increased during adipogenesis, two days after the peak observed for the transcription factors involved in this process. Moreover, *cd36* expression was increased during adipogenesis and higher levels were found at day 11, one day after the *fatp1* peak. In Atlantic salmon and trout AT, *cd36* has been described but also showing a different pattern of expression when compared to *fatp1* in different *in vivo* condition possibly indicating their different role in fatty acid uptake (Torstensen et al, 2009; Sánchez-Gurmaches et al, 2011, 2012). In mammals, FABPs are well-known marker genes in the late stages of differentiation (Vogel Hertzel & Bernlohr, 2000). In Atlantic salmon, expression of *fabp11* has been

described in several tissues and specially found in the visceral fat tissue (Torstensen et al, 2009) but has not been described during adipogenesis. In the present study the expression pattern of *fabp11* did not present significant differences possibly because at day 11 the adipocytes were still not fully mature. In fact, Huang et al (2010) found that *fabp11* increased expression was characteristic from mature salmon adipocytes at day 21, although the dynamics of the whole process of adipogenesis is slower than in trout. Finally, *plin2* was expressed at highest levels at days 10 and 11 in adipocytes from rainbow trout, in agreement with that reported previously in the same cells where increased expression at transcriptional and protein levels in the late stages of differentiation was observed (Bou et al, 2017). In this sense, PLIN2 has been described to be essential for lipid transport due its presence in the lipid droplet in 3T3-L1 adipocytes (Love et al, 2015). Thus, these lipid transporters are fundamental for the increased lipid accumulation occurring during adipogenesis, which has been also corroborated through lipid staining in different species including rainbow trout (Bouraroui et al, 2008 and the present study), gilthead sea bream (Salmerón et al, 2013), Atlantic salmon (Todorcevic et al, 2010) and grass carp (Li, 2011).

With all that, adipogenesis has been demonstrated to be a complex process in preadipocyte cultures from different fish species as well as in mammals (Rosen & MacDougald, 2006; Rutkowski et al, 2015). Cells change the morphology from fibroblastic to more rounded shape and lipid internalization and accumulation through different transporters takes place. In trout pre adipocytes, the transient activation of various transcription factors after induction by differentiation media seems to be crucial to trigger adipogenesis. In turns, these transcriptional changes also modulate the expression of genes involved in lipid metabolism that regulate in the last term the synthesis, uptake or mobilization of lipids inside the mature adipocyte cell. Thus, our model has described adipogenic markers and intermediators in this aquaculture fish species and therefore, the base to found possible potential links between whole body energy regulation and adipocytes.

## 5. Authors’ contributions

EC and IN conceived the idea and designed the experiments. NRH, EL and SBP performed the cell cultures. NRH performed the analytical procedures and drafted the manuscript. All authors interpreted the data, revised the manuscript and approved the final version.

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# ARTICLE II

**Adipogenic gene expression in gilthead sea bream mesenchymal stem cells from different origin (2016).**

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# Adipogenic gene expression in gilthead sea bream mesenchymal stem cells from different origin

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**Running title:** Adipogenesis of *Sparus aurata* MSCs

**Abstract**

During the last decades, adipogenesis has become an emerging field of study in aquaculture due to the relevance of the adipose tissue in many physiological processes and its connection with the endocrine system. In this sense, recent studies have translated into the establishment of preadipocyte culture models from several fish species, lacking sometimes information on the mRNA levels of adipogenic genes. Thus, the aim of this study was to determine the gene expression profile of gilthead sea bream (*Sparus aurata*) primary cultured mesenchymal stem cells (MSCs) from different origin (adipose tissue and vertebra bone) during adipogenesis. Both cell types differentiated into adipocyte-like cells accumulating lipids inside their cytoplasm. Adipocyte differentiation of MSCs from adipose tissue resulted in down-regulation of several adipocyte-related genes (such as *lpl*, *hsl*, *pparα*, *pparγ* and *gapdh2*) at day 4, *gapdh1* at day 8, and *fas* and *pparβ* at day 12. In contrast, differences in *lxrα* mRNA expression were not observed, while *g6pdh* levels increased during adipocyte maturation. Gapdh and Pparγ protein levels were also detected in preadipocyte cultures; however, only the former increased its expression during adipogenesis. Moreover, differentiation of bone-derived cells into adipocytes also resulted in the down-regulation of several adipocyte gene markers such as *fas* and *g6pdh* at day 10 and *hsl*, *pparβ* and *lxrα* at day 15. On the other hand, the osteogenic genes *fib1a*, *mgp* and *op* remained stable, but an increase in *runx2* expression at day 20 was observed. In summary, the present study demonstrates that gilthead sea bream MSCs from both adipose tissue and bone differentiate into adipocyte-like cells, although revealed some kind of species- and cell lineage-specific regulation with regards to gene expression. Present data also provide novel insights into some of the potential key genes controlling adipogenesis in gilthead sea bream that can help to better understand the regulation of lipid storage in fish.

**Keywords:** MSCs, adipogenesis, adipocyte, bone, *Sparus aurata*.

## 1. Introduction

Traditionally adipose tissue was considered a mere energy store, synthesizing and accumulating triglycerides during caloric excess periods, and releasing fatty acids and glycerol when needed, as under nutritional restriction. However, this changed with the discovery of the adipose tissue-produced hormone leptin in 1994 (Zhang et al., 1994). Adipocytes and cells in the stromal vascular fraction of adipose tissue produce many hormones, cytokines and other molecules, with more than 50 described to date also in fish (Kling et al., 2009; Lago et al., 2007; Saera-Vila et al., 2007; Salmerón et al., 2015a; Sánchez-Gurmaches et al., 2012; Todorcević et al., 2010; Vegusdal et al., 2003), which act on the central nervous system and peripheral organs regulating several processes, such as glucose and lipid metabolism (Saltiel, 2001; Tocher, 2003). Thus, it is now recognized that adipose tissue is an active contributor to the regulation of whole-body energy homeostasis.

Adipose tissue grows by increasing the size of existing adipocytes (hypertrophy), its number based on the formation of new adipocytes from precursor cells (hyperplasia), or both (Hausman et al., 2001). These two adipocyte developmental types occur not only during the early life stages but also throughout life (Rosen and Spiegelman, 2014; Weil et al., 2012). In addition to adipocytes, the adipose tissue also comprises a stromal vascular fraction formed by a heterogeneous population of cells, containing mesenchymal stem cells (MSCs) that include multipotent cells with the ability to differentiate into adipocytes, chondrocytes and osteoblasts, among other cell lineages (Bourin et al., 2013; Schäffler and Büchler, 2007). The process of adipocyte differentiation is divided in two steps and is influenced by hormones, growth factors, cytokines and nutrients. First, the multipotent MSCs undergo a process known as determination (Boone et al. 2000; Gregoire et al., 1998). This process results in cells that are morphologically similar to fibroblasts, which appear identical to MSCs, but are only able to differentiate into adipocytes. As a result, the cells in this post-determination state are called preadipocytes or adipoblasts. The second stage, the proper differentiation, consists in the formation of structurally mature adipocytes from preadipocytes and is

commonly known as adipogenesis, where changes in cellular morphology, hormone sensitivity and secretory capacity of the cells occur (Medina-Gómez, 2012; Savopoulos et al., 2011). These changes are regulated through the coordinated expression of mainly transcription factors, which in turn act to activate transcription of genes that produce the adipocyte phenotype (Chen et al., 2016; Reusch et al., 2000). The peroxisome proliferator-activated receptor γ (Pparγ) is the central regulator of adipogenesis and is responsible for activating a number of genes involved in fatty acid binding, uptake and storage, including lipoprotein lipase (*lpl*) or phosphoenolpyruvate carboxykinase among others.

The interest in the adipogenic process and its regulation in fish has increased in the last years because in aquaculture, the excessive fat accumulation experienced by some cultured species is generally perceived as an undesirable trait by the consumers; and also has negative effects in terms of production, product lifetime and fish health. Therefore, several primary cultures of preadipocytes have been established to better understand adipogenesis and its endocrine regulation in fish; including Atlantic salmon (*Salmo salar*) (Vegusdal et al., 2003), red sea bream (*Pagrus major*) (Oku et al., 2006), rainbow trout (*Oncorhynchus mykiss*) (Bouraoui et al., 2008), large yellow croaker (*Pseudosciaena crocea*) (Wang et al., 2012), grass carp (*Ctenopharyngodon idella*) (Li, 2012), gilthead sea bream (*Sparus aurata*) (Salmerón et al., 2013) and common carp (*Cyprinus carpio*) (Ljubojevic et al., 2014). However, knowledge on the gene expression pattern during fish adipogenesis is usually scarce, especially in sparids, with only two microarray studies reported to date in salmonids, one in Atlantic salmon (Todorcević et al., 2010), and one in rainbow trout (Bou et al., 2012).

Adipocytes and osteoblasts arise from a common precursor cell, which after the induction of certain transcription factors differentiates into each one of these two cell types. As mentioned before, Pparγ is the master transcription factor for adipocyte differentiation, while runt-related transcription factor 2 (Runx2) is considered the one regulating osteogenic differentiation (Chen et al., 2016). The prevalence of skeletal malformations in hatchery-reared fish stimulated the

establishment of cell culture models to study the mechanisms of bone formation and development in fish. The osteoblast models described to date have been: two cell lines derived from vertebra and branchial arch of gilthead sea bream (Pombinho et al., 2004), one cell line derived from zebrafish (*Danio rerio*) calcified tissues (Vijayakumar et al., 2013), and three primary cultures; one derived from gilthead sea bream vertebra bone (Capilla et al., 2011) and two of Atlantic salmon, one from white muscle precursor cells (Ytteborg et al., 2010) and one from visceral fat precursor cells (Ytteborg et al., 2015). Using the bone-derived cells from vertebra of gilthead sea bream, we have demonstrated that they are multipotent stem cells as they can be differentiated into either osteoblasts or adipocyte-like cells using an osteogenic or an adipogenic differentiation medium, respectively (Capilla et al., 2011). Nevertheless, the changes that occur in the transcriptional profile of these cells during such processes have not yet been investigated either.

Furthermore, another interest of the study of these cell culture models is that during the last decade in mammals, it has been speculated that the infiltration of bone marrow adipocytes during the development of osteoporosis in the elderly can be due to osteo-adipogenic transdifferentiation (Gao et al., 2014). In fish, the hematopoietic organ is the head kidney and the bone marrow is from the beginning of its development filled with adipocytes, besides nerves, blood vessels and connective tissue cells (Boglione et al., 2013); therefore, we can hypothesize that such a process of adipocyte transdifferentiation from cells of the osteoblastic lineage may be also occurring in the adult, contributing to the whole fat content of the fish. As a first step towards exploring that possibility and using an *in vitro* approach, the aims of the current study were: (1) to determine the transcriptional profile of gilthead sea bream preadipocytes during adipogenesis, and (2) to compare it with the gene expression pattern observed throughout the differentiation of bone-derived MSCs into adipocyte-like cells. To this end, we used primary cell cultures of gilthead sea bream precursor cells obtained from visceral adipose tissue and vertebra bone and analyzed well-characterized

adipocyte differentiation markers and some osteogenic markers at different adipogenic stages.

## 2. Materials and methods

### *2.1. Animal care*

Animal care and experimental procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the Catalan government-established norms and procedures (permit numbers DAAM 7951; CEEA 169/14 and DAAM 6759; CEEA 243/12 for the preparation of primary cultures derived from adipose tissue or bone, respectively).

### *2.2. Fish*

Gilthead sea bream were obtained from a fish farm in Northern Spain and maintained in the animal facilities of the Faculty of Biology at the University of Barcelona. Fish were kept in 200 L fiberglass tanks under 12 h light/12 h dark photoperiod at 21±1ºC, pH 7.5-8, 31-38‰ salinity and >80% oxygen saturation and fed *ad libitum* twice daily with a commercial diet (Skretting España S.A., Burgos, Spain).

### *2.3. Preadipocytes and bone cells cultures*

All plasticware for tissue culture was obtained from Nunc (Barcelona, Spain); and all reagents were purchased from Sigma–Aldrich (Tres Cantos, Spain) unless stated otherwise. Cells were incubated at 23ºC with 2.5% $CO_2$ during the whole duration of the experiments.

#### *2.3.1 Preadipocytes cultures*

Three or four juvenile gilthead sea bream of an average weight of 104 g were used for each culture. Preadipocytes were collected by mechanical disruption and enzymatic digestion of the visceral adipose tissue as described previously (Salmerón et al., 2013), plated at a density of 4.3 x $10^4$ cells/$cm^2$ in gelatin

pretreated 6-well plates and maintained in growth media (GM) composed of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic solution (A/A) and supplemented with 60 mM NaCl. Cells were continuously cultured in GM until day 8, once confluence was reached, and then changed to a differentiation medium (DM) containing GM plus 10 µg/mL porcine insulin, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 0.25 µM dexamethasone, and 5 µL/mL lipid mixture (including cholesterol and fatty acids from cod liver oil) to induce adipocyte differentiation. After 3 days, the culture conditions were changed to an adipocyte medium (AM), consisting of GM plus lipid mixture (5 µL/mL), to keep the cells already differentiating until the end of the culture. Preadipocytes samples were collected at days 4 and 8 of culture (days -4 and 0, respectively), and samples of adipocytes at days 4, 8 and 12 days after induction of differentiation. Prior to harvesting, cells were washed once with phosphate-buffered saline (PBS), recovered with TRI Reagent (Ambion, Alcobendas, Spain) using a cell scraper, then transferred to an RNase-free polypropylene tube and kept at -80ºC until RNA and protein extraction.

*2.3.2 Bone cultures*

A total of 6 juvenile gilthead sea bream of an average weight of 30 g were used for each culture. Bone-derived cells were isolated by mechanical disruption and enzymatic digestion of vertebra bone as described previously (Capilla et al., 2011). Cells and small vertebra fragments were plated with GM supplemented with 19 mM NaCl and 1% fungizone (Invitrogen Life Technologies, Alcobendas, Spain), in a 10 cm culture dish. After 1 week, the fragments were removed and the attached cells collected with 0.25% trypsin–EDTA (Invitrogen Life Technologies, Alcobendas, Spain) and plated into new 10 cm plates with fresh GM. From here, the cells were routinely sub-cultured every time the cells reached about 70–80% confluence and used for a maximum of 10 passages. Differentiation into adipocyte-like cells was achieved as previously described (Capilla et al., 2011). Briefly, 70-80% confluent cells were trypsinized from 10 cm culture dishes, seeded (1 x $10^4$ cells/$cm^2$) and cultured in 6-well plates with GM. The following day (i.e., day 0)

media was changed first to DM and three days later changed to AM using the same experimental settings as explained in 2.3.1. Cell samples for RNA extraction were obtained at days 5, 10, 15 and 20 as described in 2.3.1.

*2.4. RNA and protein extraction*

Simultaneous extraction of RNA and proteins from a single cell sample was performed using TRI Reagent (Ambion, Alcobendas, Spain) and following the manufacturer's recommendations.

*2.4.1 RNA extraction and cDNA synthesis*

Total RNA was dissolved in DEPC-treated water (RNase-free) and stored at −80°C. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and its integrity was analyzed by 1% (m/v) agarose gel electrophoresis. To eliminate any residual genomic DNA, total RNA (250 ng from preadipocytes and 1 µg from bone cells) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain) following the manufacturer's recommendations.

*2.4.2 Protein extraction*

Protein was extracted from the preadipocytes samples only and dissolved in RIPA buffer (Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 1mM, NP-40 1%, Na-deoxycholate 0.25%, PMSF 1mM, $Na_3VO_4$ 1mM, NaF 1mM and protease inhibitor cocktail). Then, samples were homogenized using a "Pellet pestle" for microtubes on ice, mixed in an orbital during 1 h at 4ºC and the supernatant was recovered after centrifugation during 30 min at maximum speed at 4ºC. Total protein concentration was determined (A595 nm) by Bradford assay using bovine serum albumin (BSA) as the standard protein (He, 2011).

*2.5. Quantitative PCR (qPCR) analysis*

In order to characterize the transcriptional profile occurring during adipocyte differentiation in gilthead sea bream, important genes implicated in adipogenesis and energy metabolism regulation were analyzed by qPCR. The genes comprise enzymes: fatty acid syntase (*fas*), *lpl*, glucose-6-phosphate dehydrogenase (*g6pdh*), hormone sensitive lipase (*hsl*), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) 1 and 2; and transcription factors: *pparα*, *pparβ*, *pparγ* and liver X receptor alpha (*lxrα*). Moreover, the expression of four osteogenic genes, fibronectin 1a (*fib1a*), matrix Gla protein (*mgp*), osteopontin (*op*) and *runx2*, was determined in the cells derived from bone. In addition, elongation factor-1α (*ef1α*), ribosomal protein S18 (*rps18*) and 18S ribosomal RNA (*18s*) were tested as reference genes. Quantitative PCR was performed using a CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain) as previously described (Salmerón et al., 2015b). Each qPCR reaction was performed in triplicate in a total volume of 5 µl, containing 2.5 µl of the iTaq Universal SYBR Green supermix (Bio-Rad, El Prat de Llobregat, Spain), 0.125-1.25 ng of cDNA template, 250-500nM of each primer (Table 1), and milliQ water. Samples were amplified as follows: 95ºC for 3 min, and then 40 cycles of 95ºC for 10 s, followed by annealing 57-69ºC for 30 s (primer dependent, Table 1), following by dissociation step from 55ºC up to 95°C with a 0.5°C increase every 5 s. A standard curve dilution series of a cDNA sample pool was constructed to determine the qPCR efficiency of each primer pair (Table 1), which were calculated using the CFX Manager Software (Bio-Rad). No Template Control (NTC), No Reverse Transcription control (RTC) and PCR Control (PCR) were used to determine the overall performance of each qPCR assay. Relative expression levels of the target genes were determined by the Pfaffl method (Pfaffl, 2001) using correction for primer efficiencies and normalizing the quantification cycle (Cq) value of each gene registered during the annealing step to that of *rps18* and *ef1α*, the most stable reference genes among the different culture stages ($p>0.05$) using the CFX Manager Software (Bio-Rad). Data from preadipocytes and bone cells were obtained from 4-5 and 5-6 independent cultures, respectively.

*2.6. Western blotting*

For Western blot analyses 8 µg of protein were loaded in each lane. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, El Prat de Llobregat, Spain) overnight at 4ºC and 100 mA. Reversible Ponceau staining was used as a loading control (Romero-Calvo et al., 2010). The PVDF membranes were blocked with 5% skimmed milk powder in Tris-Buffered Saline and Tween 20 (TBS-T) for 1 h 15 min and probed with a rabbit polyclonal Pparγ (sc-7196) and a goat polyclonal Gapdh (sc-20357) primary antibodies (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:200 overnight at 4ºC on a tube rotator. Membranes were washed in TBS-T and probed with a horseradish peroxidase-conjugated anti-rabbit (sc-2004) or an anti-goat (sc-2020) secondary antibody (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:10000 for 1 h 15 min. Membranes were washed with TBS-T and chemiluminescent detection performed using an enhanced chemiluminescence kit (Pierce ECL Western blotting Substrate; Thermo Scientific, Alcobendas, Spain). Western blotting results were obtained from two independent cultures and band intensities were quantified by scanning densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to Ponceau staining.

*2.7. Oil red O staining*

To evaluate adipocyte differentiation in the bone-derived cells, intracellular neutral lipid accumulation was analyzed by Oil red O staining as explained in (Capilla et al., 2011). Cells were grown as explained in 2.3.2 and samples obtained at days 5 and 15 of culture development. Quantification of cell lipid content was calculated as the absorbance measured at 490 nm divided by the read at 630 nm corresponding to cell protein content, which was obtained after Comassie blue staining for 1 h and dye extraction by incubation of the cells with 85% propylene glycol during 3h at 60ºC (Capilla et al., 2011). Data are presented as fold change relative to day 5 of culture.

*2.8. Statistical analyses*

Data normality and homoscedasticity was assessed using Shapiro-Wilk and Levene's test, respectively. Independent samples Student t-test was used for comparison between two groups. For multiple mean comparisons of normal distributed data, one-way ANOVA was used followed by Tukey's or Dunnett's T3 *post hoc* tests in case of homogeneous or heterogeneous variance data, respectively. When data did not fit normal distribution, the nonparametric Kruskal–Wallis test followed by Mann–Whitney test were used. Statistical analyses were performed using SPSS Statistics version 20 (IBM, Armonk, NY, USA). Results were presented as mean ± SEM. $P<0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

## 3. Results

*3.1. Gene and protein expression profiles during differentiation of preadipocytes*

Gilthead sea bream preadipocytes require the addition of a DM, a mixture of insulin, IBMX, dexamethasone and lipid mixture, to differentiate into mature adipocytes (Salmerón et al., 2013). After differentiation induction, gilthead sea bream preadipocytes start to change its morphology from a fibroblast-like shape to an adipocyte-like form with an enlarged cytoplasm filled with lipids (Salmerón et al., 2013). The transcriptional profile during adipogenesis in gilthead sea bream preadipocytes was analyzed before (days -4 and 0) and after (days 4, 8 and 12) induction of differentiation. The mRNA levels of the early marker of adipocyte differentiation *lpl* decreased significantly from day -4 to day 4 and then gradually increased again (Fig. 1A). *fas* and *g6pdh*, genes participating in the via of *de novo* lipogenesis from glucose and in the pentose phosphate pathway, respectively, showed opposite expression patterns, with *fas* gradually decreasing (Fig. 1B), while *g6pdh* was significantly up-regulated during most of the process (Fig. 1C). The expression of the lipolysis-associated gene *hsl* was significantly higher in

preadipocytes and late differentiated adipocytes relative to cells at day 4 (Fig. 1D). With regards to the adipocyte maturation marker *gapdh*, the expression of both isoforms (*gapdh1* and *gapdh2*) significantly diminished along with differentiation (Fig. 1E and F).

Furthermore, regarding the expression of the transcription factors analyzed, *pparα* and *pparβ*, nuclear receptors that regulate the beta-oxidation of fatty acids, were significantly down-regulated from early and at late stages of differentiation, respectively (Fig. 2A and B). The transcript levels of *pparγ*, the nuclear receptor key in the process of adipocyte differentiation, decreased significantly after the addition of the DM (day 4) but then its expression was maintained along with adipocyte maturation (Fig. 2C). Finally, the mRNA levels of *lxrα*, a nuclear receptor participating in the regulation of cholesterol homeostasis, was stable during the whole adipogenic process (Fig. 2D).

Next, protein expression of Pparγ and Gapdh, was also determined in lysates from undifferentiated (days -4 and 0) and differentiated (days 4 and 12) adipocytes. Results showed that Pparγ tended to increase up to day 4 and then diminished (Fig. 3A), while Gapdh increased steadily during adipogenesis (Fig. 3B).

*3.2. Lipid accumulation during adipogenesis of bone-derived cells*

Bone-derived MSCs of gilthead sea bream can become adipocyte-like cells using the same DM than in preadipocytes (Capilla et al., 2011). Phenotypic changes from the cells with a fibroblast-like appearance to more rounded and lipid-filled cells with the morphological semblance of adipocytes were observed during adipogenesis (Fig. 4A). Differentiation into adipocyte-like cells was tracked by Oil red O staining, which monitors lipid accumulation. Lipid content in the cells was gradually increasing during the process of adipocyte maturation, showing day 15 cells significant differences when compared to day 5 cells (Fig. 4B).

*3.3. Gene expression profile during adipogenesis of bone-derived cells*

The expression of all the genes implicated in lipid metabolism analyzed, such as *fas*, *g6pdh* and *hsl* (Fig. 5B, C and D), was significantly down-regulated during adipogenesis of bone-derived cells. On the other hand, the expression of *lpl*, *gapdh1* and *gapdh2* was unaffected (Fig. 5A, E and F). Moreover, the gene expression of most of the transcription factors determined in the present study (*pparα*, *pparβ* and *lxrα*) was progressively down-regulated (Fig. 6A, B and D), with the exception of *pparγ* that continued stable (Fig. 6C).

With regards to the representative osteogenic genes analyzed, the expression of the three components of the extracellular matrix (*fib1a*, *mgp* and *op*) remained unaltered during the whole process of adipocyte differentiation (Fig. 7A, B and C). On the other hand, the key transcription factor controlling osteogenesis, *runx2*, showed increasing levels during differentiation (Fig. 7D).

## 4. Discussion

MSCs are multipotent cells that through a two-step process, lineage commitment of specific progenitors and maturation, can be induced to differentiate into cells of several tissue types such as osteoblasts or adipocytes (Chen et al., 2016). Local, hormonal and mechanical factors in MSCs, as well as intermediate precursors and differentiated cells, result in the activation of a series of transcription factors and epigenetic mechanisms, which jointly control the balance of adipogenesis and osteoblastogenesis including their transdifferentiation (Berendsen and Olsen, 2014). Gilthead sea bream MSCs isolated from adipose tissue or vertebra bone can be differentiated into mature adipocytes using a medium supplemented with insulin, IBMX, dexamethasone and lipid mixture (i.e. DM) (Capilla et al., 2011; Salmerón et al., 2013). Insulin, the most potent of these inducers in mammals, activates *pparγ* expression (Klemm et al., 2001; Nadeau et al., 2004). IBMX and dexamethasone activate CCAAT enhancer binding proteins *cebpβ* and *cebpδ* expression, respectively (Chen et al., 2016), which in turn induce the expression of *cebpα* and *pparγ*, the master regulators of adipogenesis. In preadipocyte primary cultures of some fish species the addition of lipids in the differentiation cocktail is

required to induce full maturation of adipocytes, most probably since polyunsaturated fatty acids such as docosahexaenoic and eicosapentaenoic acids are natural activators of PPARγ (Grygiel-Górniak, 2014). A lipid mixture containing cholesterol and cod liver oil fatty acids (methyl esters), polyoxyethylenesorbitan monooleate and D-α-tocopherol acetate has been used in Atlantic salmon (Vegusdal et al., 2003), rainbow trout (Bouraoui et al., 2008), large yellow croaker (Wang et al., 2012) and gilthead sea bream (Salmerón et al., 2013). In common carp, the use of linoleic and oleic acid has been employed in combination with T3 and troglitazone (Ljubojevic et al., 2014), whereas in red sea bream (Oku et al., 2006; Oku and Umino, 2008) and grass carp (Li, 2012) the use of a DME/F12 medium containing only linoleic acid was enough to induce adipocyte differentiation.

In the present study, the addition of the DM to the preadipocytes down-regulated the expression of the early marker *lpl*, as it occurs during the early stages of differentiation in Atlantic salmon preadipocytes (Todorcević et al., 2010). However, *lpl* gene expression remained stable during the adipocyte differentiation of bone-derived cells; and in other fish species, such as large yellow croaker (Wang et al., 2012), rainbow trout (Bouraoui et al., 2012) and red sea bream (Oku and Umino, 2008; Oku et al., 2006), its expression was increased after induction of adipocyte differentiation. Thus, the regulation of *lpl* expression could be species-specific or, post-transcriptional mechanisms such as modulation at the activity level cannot be discarded. Fas is an enzyme that regulates the *de novo* biosynthesis of long-chain fatty acids catalyzing the formation of palmitate (Chakravarty et al., 2004). In this study, *fas* mRNA expression decreased gradually during adipogenesis of both MSCs types contrary to that observed in Atlantic salmon (Todorcević et al., 2010) and red sea bream (Oku and Umino, 2008) preadipocytes, where its expression was higher and increased during adipocyte differentiation. In a recent study about *de novo* lipogenesis in Atlantic salmon preadipocytes, Bou and coworkers (2016) showed that the use of palmitate decreases the expression of acetyl-CoA carboxylase (*acc*), the enzyme that catalyzes the formation of malonyl-

CoA necessary for the fatty acid synthesis by Fas. Such data suggested that the synthesis of palmitate mediated by Fas may be blocking its own, and *acc* gene expression, through a negative feedback mechanism, similarly as reported in primary fetal rat calvarial cultured cells where palmitate reduced the expression of *fas* and *pparγ* (Yeh et al., 2014).

G6pdh, an enzyme of the pentose phosphate pathway that produces the NADPH necessary for the biosynthesis of fatty acids and cholesterol, was up-regulated after the induction of differentiation in preadipocytes as it occurs in Atlantic salmon (Todorcević et al., 2010), although its expression decreased during adipogenesis of bone-derived cells. In 3T3-L1 cells, Parks and collaborators demonstrated that *g6pdh* overexpression up-regulates most adipocyte marker genes (such as *fas* and *pparγ*) and elevates the levels of cellular free fatty acids and triglycerides. Consistently, *g6pdh* knockdown via small interfering RNA attenuated adipocyte differentiation reducing lipid droplet accumulation, indicating that proper expression of *g6pdh* is required for adipogenesis as well as lipogenesis (Park et al., 2005). Therefore, the different mRNA expression of *g6pdh* observed during adipocyte differentiation between the two tissues, suggests that G6pdh may be playing different roles during adipogenesis (i.e. fatty acid synthesis and/or oxidation protection) and thus it can be used as a feature to identify adipocytes derived from each tissue type. Moreover, the lipolytic marker *hsl* decreased transiently its gene expression with differentiation to then increase again, in preadipocytes and to a lesser extent in bone-derived MSCs as well, in agreement with a previous study in human preadipocytes where *hsl* mRNA levels rose during adipocyte differentiation (Langin et al., 2005). This result suggests that the increase of lipid storage in the cells during adipogenesis may also promote lipolysis through Hsl in order to control its own intracellular levels of lipids. Furthermore, Gapdh produces the triglyceride glycerol required for the triglyceride synthesis being considered a late adipogenic marker. The gene expression of both isoforms of *gapdh* decreased along with adipocyte differentiation in preadipocytes but was unchanged in bone-derived MSCs, in contrast to that occurred at a protein

level, where Gapdh appeared to increase from preadipocytes to differentiated adipocytes. In grass carp (Li, 2012), Atlantic salmon (Vegusdal et al., 2003) and rainbow trout (Bouraoui et al., 2008), Gapdh gene expression was not determined, but its activity was also increased with adipocyte differentiation suggesting that perhaps some kind of post-transcriptional, translational and/or protein regulation processes may be occurring (Vogel and Marcotte, 2012).

Regarding the analyses of transcription factors, the expression of all *ppars* decreased early (*pparα*), steadily (*pparγ*) or late (*pparβ*) during preadipocyte differentiation and were similarly down-regulated during the differentiation of bone derived-cells with the exception of *pparγ* that remained stable. In previous works with Atlantic salmon, the gene expression of *pparα* and the short form of *pparγ* increased during differentiation, while *pparγ* long was induced during the early phase and decreased at later stages of differentiation (Todorcević et al., 2008). Other studies in Atlantic salmon showed that the gene expression of *pparα* and *pparβ* were unchanged during adipogenesis (Huang et al., 2010), whereas *pparγ* was up-regulated already in subconfluent cells (Todorcević et al., 2010), as observed in our study. In red sea bream, *pparα* mRNA expression increased up to 7 days after induction and then decreased, while *pparβ* and *pparγ* remained unaffected (Oku and Umino, 2008). In grass carp (Li, 2012) and in large yellow croaker (Wang et al., 2012), *pparγ* transcriptional expression increased gradually during cell differentiation. Here, the presence of the Pparγ protein increased from preadipocytes to early differentiated adipocytes, contrary to that occurred at a transcriptional level, suggesting that control at a post-transcriptional level exists. However, the present data about Pparγ protein expression during differentiation, is in agreement with a previous study in rainbow trout preadipocytes where the protein level of Pparγ was higher in mature cells than in proliferating cells, suggesting that Pparγ participates as a transcription factor mostly during the early stages of the adipogenic process (Bouraoui et al., 2008). Finally, gradual down-regulation of *lxrα* mRNA levels was detected during adipocyte differentiation of bone-derived cells, while those were stable in differentiating preadipocytes in

disagreement with other studies in 3T3-L1 cells (Hummasti et al., 2004) and rainbow trout (Cruz-Garcia et al., 2012), where its expression increased during differentiation. Lxr induction in the late wave of adipogenesis and its activation inhibits adipocyte conversion, increases glucose uptake, glycogen synthesis, cholesterol synthesis, and fatty acids efflux (Ross et al., 2002). To sum up, *lxrα* and *g6pdh* during maturation of cells from both tissue types showed a similar pattern of gene expression, suggesting that Lxrα can be involved in the expression of *g6pdh*, regulating the pentose phosphate pathway in gilthead sea bream.

The process of osteogenesis can be divided in three stages, commitment, extracellular matrix production and mineralization. Fib1a, Op and Mgp are components of the extracellular matrix. Fib1a is related to the initial state of osteoblast differentiation (Ribeiro et al., 2010) and in previous studies with gilthead sea bream bone-derived cells its gene expression increased at day 5 after addition of an osteogenic medium and decreased slowly during differentiation (Riera et al., 2014). On the other hand, Op and Mgp are key molecules during the phase of calcification of the extracellular matrix (Chatakun et al., 2014; Lu et al., 2013) and in the gilthead sea bream osteoblast culture its gene expression increased steadily when mineralization of the tissue started (Riera et al., 2014). These findings contrast with the results obtained here in the presence of an adipogenic media, where the expression of these osteogenic genes remained low and without variations, indicating a change in the transcriptomic profile of these cells when turning into adipocyte-like cells. Moreover, Runx2 and Osterix are considered key transcription factors required for osteogenic differentiation of MSCs (Chen et al., 2016). Runx2 promotes cell differentiation into immature osteoblasts inhibiting their commitment to the adipocyte lineage, while Osterix is required for the maturation phase (Chen et al., 2016). Nevertheless, during the *in vitro* osteogenic development of gilthead sea bream bone MSCs differences in *runx2* expression were not observed (Riera et al., 2014). Rat adipose-derived stem cells infected with a recombinant adenovirus carrying the *runx2* gene, decreased the gene expression of *lpl* and *pparγ* and reduced lipid droplet formation (Zhang et al., 2006). In

precursor cells isolated from visceral fat of Atlantic salmon differentiating into adipocytes or osteoblasts, Ytteborg and collaborators found that *pparγ* mRNA was absent in cultures given osteogenic medium, and *runx2* when adipogenic medium was added, suggesting that a similar co-repressing mechanism also exists in fish (Ytteborg et al., 2015). Nevertheless, in our study the gene expression of *runx2* during adipogenesis was high and increased towards the end, explaining perhaps why the *lpl* and *pparγ* mRNA levels in bone-derived cells were not modified.

In summary, in the present study we used primary cultures of MSCs derived from adipose tissue or vertebra bone of gilthead sea bream to investigate the expression profile of lipid metabolism-related genes and transcription factors during adipogenesis. Gilthead sea bream preadipocytes and bone-derived cells were able to differentiate into adipocyte-like cells after addition of a DM. However, the revealed gene expression profile during the process of adipogenesis contradicts some previous findings using similar experimental models in another vertebrate species. The differences between the present and other studies can be related to some kind of species or tissue specific regulation, differences in the composition of the DM used to induce adipogenesis or due to the existence of post-transcriptional, translational and/or protein regulation processes. Future experiments will explore if this cell-lineage determination provokes the complete activation of adipocyte functions, and that the cells are not only able to accumulate lipids, but also have other key characteristics such as adipokines production or sensitivity to lipolytic or lipogenic stimuli as it occurs in mature adipocytes.

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## 6. Authors' contributions

EC and IN conceived the idea and designed the experiments. CS and NRH performed the cell cultures and analytical procedures and drafted the manuscript. All authors interpreted the data, revised the manuscript and approved the final version.

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## Tables and Figures legends

**Table 1.** Primers used for real-time quantitative PCR. *lpl*: lipoprotein lipase; *fas*: fatty acid synthase; *g6pdh*: glucose-6-phosphate dehydrogenase; *hsl*: hormone sensitive lipase; *gapdh1*: glyceraldehyde 3-phosphate dehydrogenase 1; *gapdh2*: glyceraldehyde 3-phosphate dehydrogenase 2; *pparα*: peroxisome proliferator-activated receptor alpha; *pparβ*: peroxisome proliferator-activated receptor beta; *pparγ*: peroxisome proliferator-activated receptor gamma; *lxrα*: liver X receptor alpha; *fib1a*: fibronectin 1a; *mgp*: matrix Gla protein; *op*: osteopontin; *runx2*: Runt-related transcription factor 2; *ef1α*: elongation factor 1 alpha; *rps18*: ribosomal protein S18; *18s*: 18S ribosomal RNA. FW: forward primer; RV: reverse primer; Tm: annealing temperature; bp: base pair; E: amplification efficiency; Acc. Num.: Accession Number.

| Gene | Primer sequence (5'→3') | Tm (ºC) | Product size (bp) | E (%) | Acc. Num. |
|---|---|---|---|---|---|
| *lpl*_FW | GAGCACGCAGACAACCAGAA | 60 | 135 | 98.1 | AY495672 |
| *lpl*_RV | GGGGTAGATGTCGATGTCGC | | | | |
| *fas*_FW | TGGCAGCATACACACAGACC | 60 | 78 | 102.0 | AM952430 |
| *fas*_RV | CACACAGGGCTTCAGTTTCA | | | | |
| *g6pdh*_FW | CAGAATGAAAGATGGGATGGAGTC | 60 | 176 | 102.9 | AY754640 |
| *g6pdh*_RV | TTCAGGTAAATGGCTTCGTTCG | | | | |
| *hsl*_FW | GCTTTGCTTCAGTTTACCACCATTTC | 60 | 122 | 99.6 | EU254478 |
| *hsl*_RV | GATGTAGCGACCCTTCTGGATGATGTG | | | | |
| *gapdh1*_FW | CCAGCCAGAACATCATCC | 60 | 190 | 103.5 | DQ641630 |
| *gapdh1*_RV | GCAGCCTTGACGACCTTC | | | | |
| *gapdh2*_FW | CATGAAGCCAGCAGAGATCC | 57 | 196 | 105.5 | FM145063 |
| *gapdh2*_RV | GGTGGCCGGGTCATATTTC | | | | |
| *pparα*_FW | TCTCTTCAGCCCACCATCCC | 62 | 116 | 104.2 | AY590299 |
| *pparα*_RV | ATCCCAGCGTGTCGTCTCC | | | | |
| *pparβ*_FW | AGGCGAGGGAGAGTGAGGATGAGGAG | 69 | 188 | 108.3 | AY590301 |
| *pparβ*_RV | CTGTTCTGAAAGCGAGGGTGACGATGTTTG | | | | |
| *pparγ*_FW | CGCCGTGGACCTGTCAGAGC | 66 | 171 | 96.0 | AY590304 |
| *pparγ*_RV | GGAATGGATGGAGGAGGAGGAGATGG | | | | |
| *lxrα*_FW | GCACTTCGCCTCCAGGACAAG | 62 | 107 | 88.0 | FJ502320 |
| *lxrα*_RV | CAGTCTTCACACAGCCACATCAGG | | | | |
| *fib1a*_FW | CGGTAATAACTACAGAATCGGTGAG | 60 | 104 | 101.8 | FG262933 |
| *fib1a*_RV | CGCATTTGAACTCGCCCTTG | | | | |
| *mgp*_FW | TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC | 68 | 244 | 81.8 | AY065652 |
| *mgp*_RV | CGGGCGGATAGTGTGAAAAATGGTTAGTG | | | | |
| *op*_FW | AAAACCCAGGAGATAAACTCAAGACAACCCA | 68 | 153 | 85.0 | AY651247 |
| *op*_RV | AGAACCGTGGCAAAGAGCAGAACGAA | | | | |
| *runx2*_FW | ACCCGTCCTACCTGAGTCC | 60 | 122 | 96.1 | JX232063 |
| *runx2*_RV | AGAAGAACCTGGCAATCGTC | | | | |
| *ef1α*_FW | CTTCAACGCTCAGGTCATCAT | 60 | 263 | 85.0 | AF184170 |
| *ef1α*_RV | GCACAGCGAAACGACCAAGGGGA | | | | |
| *rps18*_FW | AGGGTGTTGGCAGACGTTAC | 60 | 164 | 98.3 | AM490061 |
| *rps18*_RV | CTTCTGCCTGTTGAGGAACC | | | | |
| *18s*_FW | TGACGGAAGGGCACCACCAG | 60 | 158 | 91.0 | AY550956 |
| *18s*_RV | AATCGCTCCACCAACTAAGAACGG | | | | |

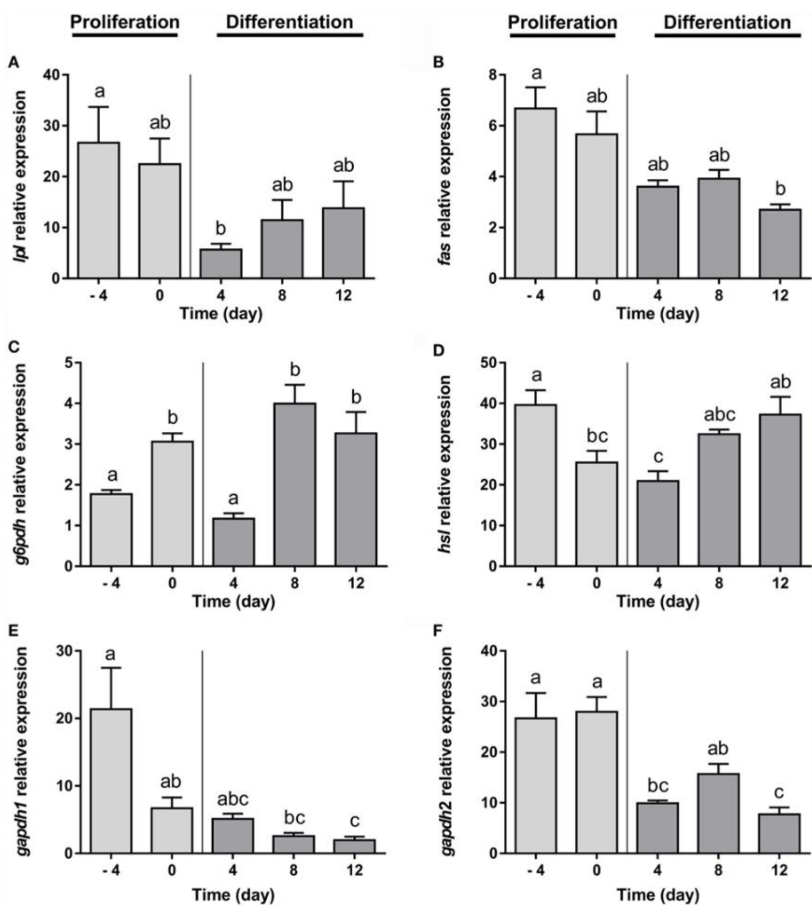


**Figure 1.** Gene expression profile of lipid metabolism-related genes in gilthead sea bream preadipocytes during adipogenesis. The mRNA levels of *lpl* **(A)**, *fas* **(B)**, *g6pdh* **(C)**, *hsl* **(D)**, *gapdh1* **(E)** and *gapdh2* **(F)** were measured by quantitative real-time PCR and normalized to *ef1α* and *rps18*. Samples were taken from preadipocytes (days -4 and 0 of culture) and differentiated adipocytes (days 4, 8 and 12 of culture). Values are means ± SEM, n= 4-5. Bars with different letters are significantly different (P<0.05). *lpl*: lipoprotein lipase; *fas*: fatty acid synthase; *g6pdh*: glucose-6-phosphate dehydrogenase; HSL: hormone sensitive lipase; *gapdh1* and *2*: glyceraldehyde 3-phosphate dehydrogenase 1 and 2, respectively; *ef1α*: elongation factor 1 alpha; *rps18*: ribosomal protein S18.

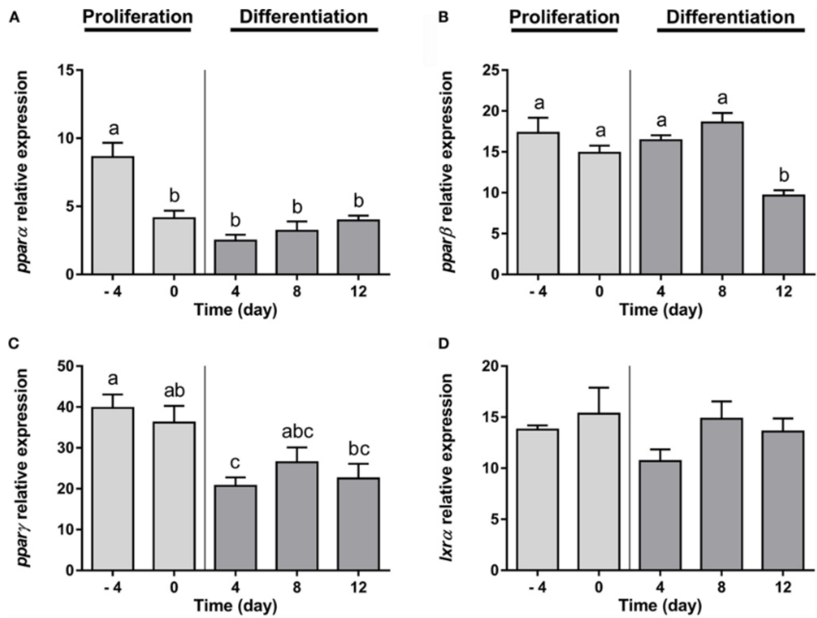


**Figure 2.** Gene expression profile of transcription factors in gilthead sea bream preadipocytes during adipogenesis. The mRNA levels of *pparα* **(A)**, *pparβ* **(B)**, *pparγ* **(C)** and *lxrα* **(D)** were measured by quantitative real-time PCR and normalized to *ef1α* and *rps18*. Samples were taken from preadipocytes (days -4 and 0 of culture) and differentiated adipocytes (days 4, 8 and 12 of culture). Values are means ± SEM, n= 4-5. Bars with different letters are significantly different (P<0.05). *pparα*, *β* and *γ*: peroxisome proliferator-activated receptor alpha, beta and gamma, respectively; *lxrα*: liver X receptor alpha; *ef1α*: elongation factor 1 alpha; *rps18*: ribosomal protein S18.

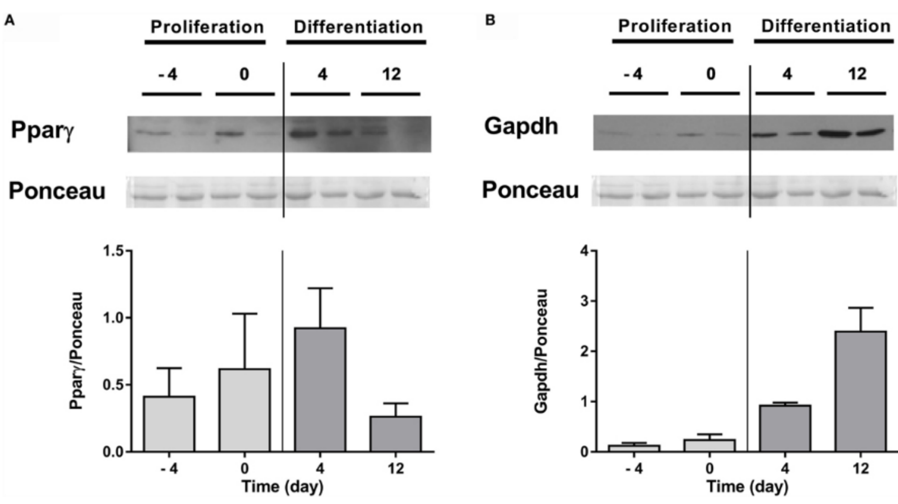

**Figure 3.** Protein expression of Pparγ and Gapdh in gilthead sea bream preadipocytes during adipogenesis. Representative Western blot, corresponding Ponceau and quantification of Pparγ **(A)** and Gapdh **(B)** protein levels in preadipocytes (days -4 and 0 of culture) and differentiated adipocytes (days 4 and 12 of culture). Values are means ± SEM, n=2. Pparγ: peroxisome proliferator-activated receptor gamma; Gapdh: glyceraldehyde 3-phosphate dehydrogenase.

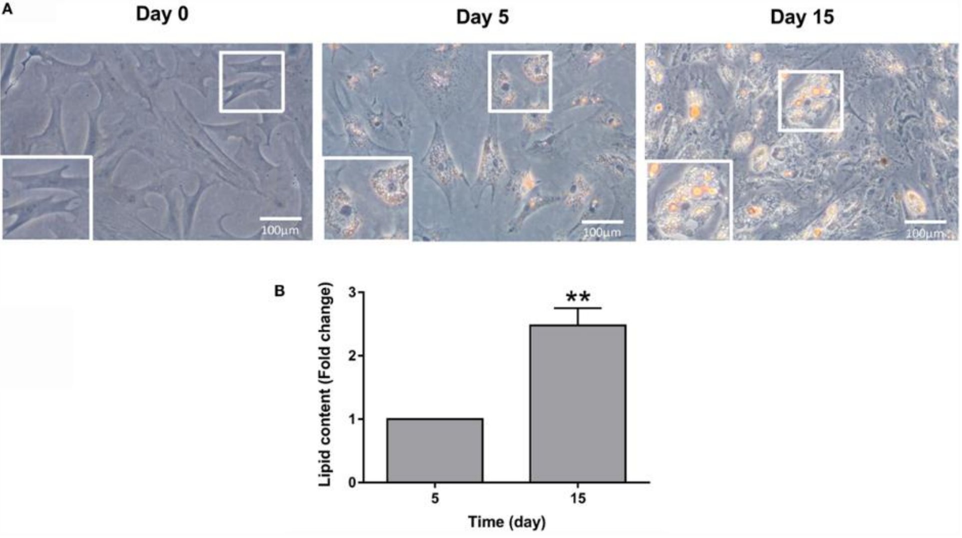


**Figure 4.** Gilthead sea bream bone-derived cells during adipogenesis. **(A)** Representative phase-contrast images of cells stained with Oil red O at days 0, 5 and 15 of culture. Magnification 20x and enlarged views. **(B)** Quantification of Oil red O staining at days 5 and 15 of culture. Values are mean ± SEM of 5 independent experiments with wells run in duplicate and presented as fold change relative to day 5 of culture (** P<0.01).

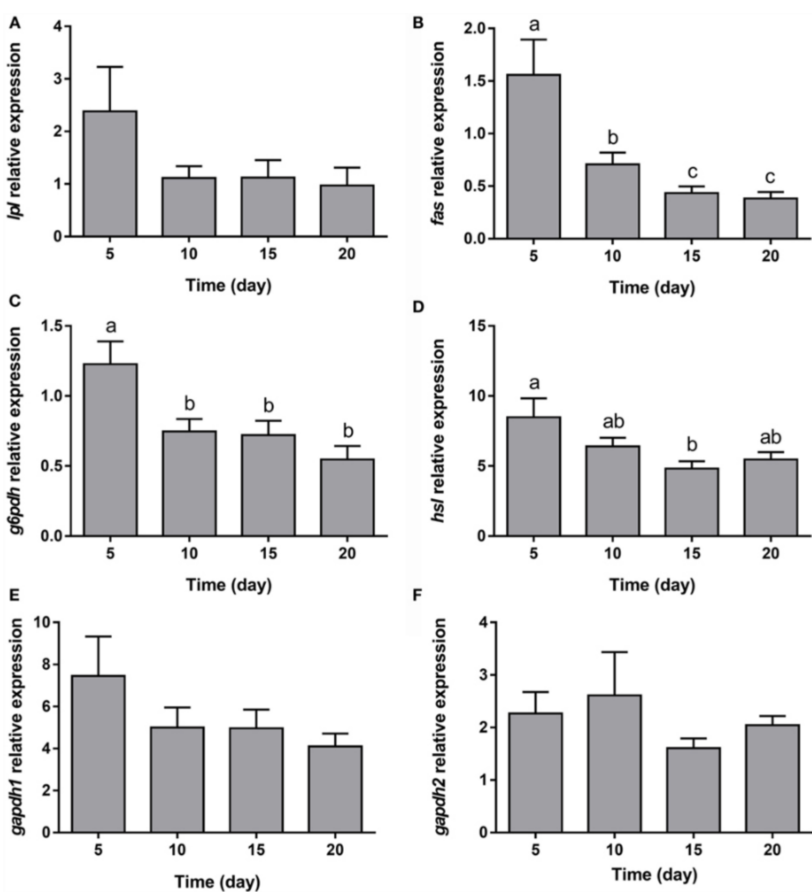


**Figure 5.** Gene expression profile of lipid metabolism-related genes in gilthead sea bream bone-derived cells during adipogenesis. The mRNA levels of *lpl* **(A)**, *fas* **(B)**, *g6pdh* **(C)**, *hsl* **(D)**, *gapdh1* **(E)** and *gapdh2* **(F)** were measured by quantitative real-time PCR and normalized to *ef1α* and *rps18*. Samples were taken from adipocyte-like cells at days 5, 10, 15 and 20 of culture. Values are means ± SEM, n= 5-6. Bars with different letters are significantly different (P<0.05). *lpl*: lipoprotein lipase; *fas*: fatty acid synthase; *g6pdh*: glucose-6-phosphate dehydrogenase; *hsl*: hormone sensitive lipase; *gapdh1* and *2*: glyceraldehyde 3-phosphate dehydrogenase 1 and 2, respectively; *ef1α*: elongation factor 1 alpha; *rps18*: ribosomal protein S18.

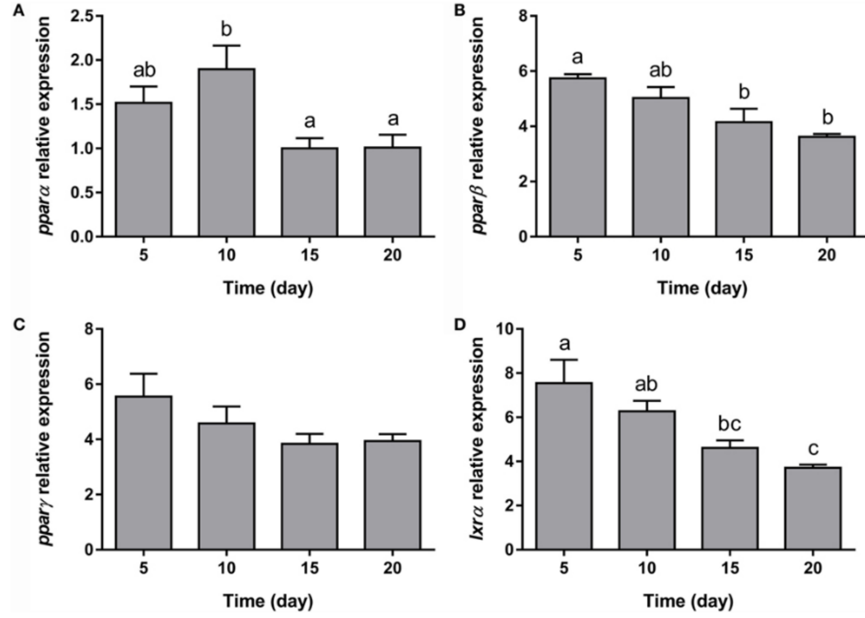


**Figure 6.** Gene expression profile of transcription factors in gilthead sea bream bone-derived cells during adipogenesis. The mRNA levels of *pparα* **(A)**, *pparβ* **(B)**, *pparγ* **(C)** and *lxrα* **(D)** were measured by quantitative real-time PCR and normalized to *ef1α* and *rps18*. Samples were taken from adipocyte-like cells at days 5, 10, 15 and 20 of culture. Values are means ± SEM, n= 5-6. Bars with different letters are significantly different (P<0.05). *pparα*, *β* and *γ*: peroxisome proliferator-activated receptor alpha, beta and gamma, respectively; *lxrα*: liver X receptor alpha; *ef1α*: elongation factor 1 alpha; *rps18*: ribosomal protein S18.

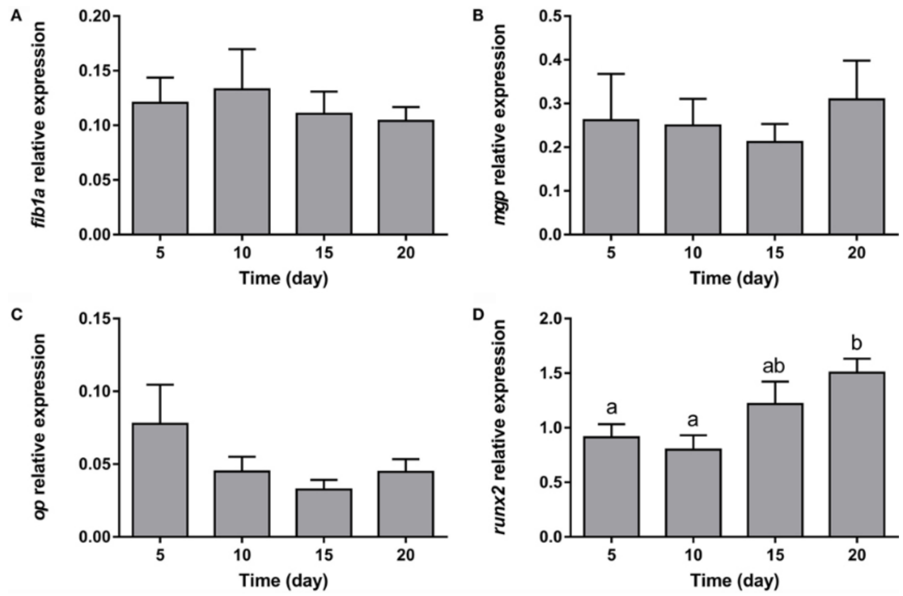

**Figure 7.** Gene expression profile of osteogenic markers in gilthead sea bream bone-derived cells during adipogenesis. The mRNA levels of *fib1a* **(A)**, *mgp* **(B)**, *op* **(C)** and *runx2* **(D)** were measured by quantitative real-time PCR and normalized to *ef1α* and *rps18*. Samples were taken from adipocyte-like cells at days 5, 10, 15 and 20 of culture. Values are means ± SEM, n= 5-6. Bars with different letters are significantly different ($P<0.05$). *fib1a*: fibronectin 1a; *mgp*: matrix Gla protein; *op*: osteopontin; *runx2*: Runt-related transcription factor 2; *ef1α*: elongation factor 1 alpha; *rps18*: ribosomal protein S18.

# ARTICLE III

**Temperature responsiveness of gilthead sea bream bone; an *in vitro* and *in vivo* approach (2018).**

***Scientific Reports 8,* 11211.**

# **Temperature responsiveness of gilthead sea bream bone; an *in vitro* and *in vivo* approach**

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**Abstract**

This study aimed to characterize the molecules involved in osteogenesis in seabream and establish using *in vitro*/*in vivo* approaches the responsiveness of selected key genes to temperature. The impact of a temperature drop from 23 to 13ºC was evaluated in juvenile fish thermally imprinted during embryogenesis. Both, *in vitro*/*in vivo*, *Fib1a*, appeared important in the first stages of bone formation, and *Col1A1*, *ON* and *OP*, in regulating matrix production and mineralization. *OCN* mRNA levels were up-regulated in the final larval stages when mineralization was more intense. Moreover, temperature-dependent differential gene expression was observed, with lower transcript levels in the larvae at 18ºC relative to those at 22ºC, suggesting bone formation was enhanced in the latter group. Results revealed that thermal imprinting affected the long-term regulation of osteogenesis. Specifically, juveniles under the low and low-to-high-temperature regimes had reduced levels of *OCN* when challenged, indicative of impaired bone development. In contrast, gene expression in fish from the high and high-to-low-temperature treatments was unchanged, suggesting imprinting may have a protective effect. Overall, the present study revealed that thermal imprinting modulates bone development in seabream larvae, and demonstrated the utility of the *in vitro* MSC culture as a reliable tool to investigate fish osteogenesis.

## 1. Introduction

Bone formation in fish does not occur during somitogenesis and the first skeletal structures appear after hatching. These structures consist of dermal bone (formed by intramembranous ossification) and cartilage replacement bone. The skeleton of the jaw and mouth only form after mouth opening with the onset of exogenous feeding. Subsequently, the first vertebrae appear in larvae at around 4 mm in body length, and only after early flexion (6 mm) does mineralization of these structures start to take place [1-4]. An almost fully mineralized skeleton is evident in larvae of 16 mm body length [1] or at about 30 days post-hatching [4]. Concerning the molecules involved in the formation and turnover of bone, transcriptomes from gilthead sea bream (*Sparus aurata*) gill arch and vertebra have recently been generated and revealed homologs of many mammalian skeleton-related transcripts [5]. Many of the identified genes corresponded to transcription factors that control osteoblast differentiation (i.e. runt-related transcription factor 2 (Runx2/Cbfa1), osterix/Sp7), or components of the extracellular matrix (ECM), including structural proteins, collagens, proteoglycans, as well as non-collagenous proteins that regulate mineral deposition such as osteonectin/SPARC (ON), osteopontin/Spp1 (OP) and osteocalcin/BGP (OCN). The study by Vieira et al., [5] unveiled the conservation of transcripts present in bone and cartilage of gilthead sea bream compared to mammals, although the tissue and cell specific localization of these transcripts remains to be established. However, with the increasing number of *in vitro* fish cell models developed in recent years [6,7], including the gilthead sea bream primary culture of mesenchymal stem cells (MSCs) derived from vertebrae, established by our group [8], it is now possible to characterize at a cellular level the role of identified candidate markers during osteoblastogenesis.

Gilthead sea bream is one of the most important farmed species in Spain, which is ranked fourth in total production volume in the Mediterranean area and third in the European Union [9]. Although the production of gilthead sea bream in aquaculture systems is well consolidated, the high incidence of skeletal deformities is still an important bottleneck for the sustainability of this industry. Moreover, since this

species is commercialized as whole fish, skeletal anomalies lead to a downgrade in the quality of the product and a reduction in its value. The most common deformities found in gilthead sea bream are those that affect the opercular complex, vertebral column and haemal or caudal body regions [10-12], which may result in ca. to 50% of lost production at the end of the hatchery phase [13]. These deformities may also reduce growth rate, increase mortality and negatively impact animal welfare [14].

The aetiology of skeletal deformities is uncertain, but temperature is one of the most important abiotic factors linked to this problem and fish grown at elevated temperatures exhibit increased levels of vertebral anomalies [13-15]. In fact, fluctuations in water temperature are associated with abnormal muscle growth and heterochrony of muscle formation, whereas temperature effects on bone growth have been associated with deformation of the vertebral bodies in fish larvae, which leads to spinal deformities [16,17]. In addition, body shape and meristic characters (i.e. dorsal spines) are also significantly affected by environmental temperature during the early life stages of the European sea bass, *Dicentrarchus labrax* [18]. In Atlantic salmon (*Salmo salar*), faster growth induced by hyperthermia significantly modified gene transcription in osteoblasts and chondrocytes, which was associated with an increased number of deformities and modified bone tissue structure and composition [19].

In the Mediterranean Sea, gilthead sea bream is frequently exposed to severe temperature changes during winter, which is one of the causal factors of winter syndrome in reared populations [20]. This pathology causes chronic mortality during the coldest months of the year and acute death episodes when the water temperature rises again [21,22]. Mortality rates are usually around 7-10% of the fish stock, although in some very acute cases, they may be as high as 80% [23]. Winter syndrome is a multifactorial condition that triggers a stress response [24,25], depresses the immune system [21,26], affects several tissues including the skeletal muscle, exocrine pancreas, liver and digestive tract [20,25,27,28], and may cause lesions in the brain and kidney [23]. The effect of thermal imprinting in embryos and larvae and on

the response of the skeleton to a temperature drop (to simulate winter conditions) was recently studied in adult gilthead sea bream [29]. Overall, the study reported that although thermal imprinting failed to modify bone homeostasis in optimal ambient water temperatures, it did change the bones responsiveness during a cold challenge.

Following on from our previous work, in the present study, we addressed the hypothesis that, modifications in the skeleton due to water temperature are linked to its effect on the gene expression of key osteogenic molecules during development. To this end, we analysed *in vivo* how thermal regimes during development influenced the expression of genes involved in osteogenesis and used an *in vitro* primary culture of MSCs from juvenile gilthead sea bream vertebrae to investigate how temperature modified bone tissue specific gene expression. We then assessed *in vivo* how thermal regimes experienced during early development influenced the expression of bone tissue specific genes in adults exposed to a cold challenge.

## 2. Results

### *2.1. Bone cells development and temperature effects in vitro*

Orthologs of mammalian osteogenic genes have recently been identified in gilthead sea bream by transcriptome analysis, however their specific expression in bone cells during the process of osteoblastogenesis remains elusive. The transcriptional profile of gilthead sea bream bone-derived MSCs differentiating into osteoblasts under mineralizing conditions induced by incubation with an osteogenic medium (OM) was compared to that of cells growing in growth medium (GM) for 20 days and is presented in Figure 1. A significant interaction between days in culture and media was observed for fibronectin 1a (*Fib1a*), Bone Morphogenetic Protein 2 (*BMP2*) and *OP* mRNA levels. Nevertheless, significant differences were not observed with regard to transcript abundance through time in cells growing in GM for any of the genes studied (Fig. 1). Moreover, in cells incubated in OM, the gene expression of *Runx2*, a key transcription factor driving

osteogenesis of MSCs, and of collagen type 1 alpha-1 (*Col1A1*), and tissue non-specific alkaline phosphatase (*TNAP*), did not vary significantly as differentiation progressed (Fig. 1A, 1D and 1H). Matrix Gla protein (*MGP*) showed similar results, but probably as an effect of the culture media (P= 0.029), an increase in its expression was observed at day 20 in cells growing in OM (Fig. 1G). *Fib1a* expression decreased gradually during MSCs differentiation, and significantly higher mRNA levels were found in the OM group compared to the GM group at days 5 and 10 (Fig. 1B). In contrast, *BMP2*, *ON* and *OP* expression increased during culture under mineralizing conditions and significantly higher mRNA levels were detected at day 20 relative to the previous days (Fig. 1C, 1E and 1F). A significant increase in transcript abundance in the OM group compared to the GM group was noted for *OP* at days 15 and 20 (Fig. 1F), and for *BMP2* and *ON* at day 20 (Fig. 1C and 1E).

The expression of osteogenic gene transcripts from cells incubated in OM at 18 and 28ºC in comparison with the control temperature of 23ºC is represented as a heat map in Figure 2. The relative expression data obtained at each of the three temperatures can be found in Table S1. For most genes, *MGP*, *TNAP*, *BMP2*, *Fib1a* and *OP*, expression was down-regulated in response to an increase or reduction in temperature. However, this is probably not a generalized non-specific response to change in temperature, since the other osteogenic gene analysed (*ON*) showed a contrary pattern. Similarly, heat shock protein 90b (*HSP90b*) gene expression increased in the first 6 h after temperature challenge and then subsequently decreased. In contrast, mRNA levels of *HSP30* and *ON* responded differently to temperatures. At 18ºC the *HSP30* mRNA levels increased over time, while at 28ºC they fell; the gene expression of *ON* was opposite to *HSP30*, since at 18ºC mRNA levels decreased over time and at 28ºC they increased (Fig. 2).

### *2.2. Bone development and temperature effects in vivo*

Since cell differentiation and proliferation is not always proportional to bone/cartilage formation by osteoblasts/chondrocytes or resorption by osteoclasts,

the most suitable predictors of bone mineralization available are: i) the abundance of ECM proteins, ii) the activity of cell-type specific enzymes, or iii) the expression levels of genes encoding these proteins. The expression of key osteogenic genes during embryonic and larval development of gilthead sea bream exposed to two different rearing temperatures was studied and is presented in Figure 3.

A significant interaction between developmental stage and rearing temperature was observed for all genes studied except *TNAP*. *Fib1a* and *BMP2* had their highest expression during somitogenesis at both temperatures, although expression was significantly higher in the high temperature group (HT, 22ºC) compared to the low temperature group (LT, 18ºC), at 1 somite stage for *BMP2* and at 10 somites stage for *Fib1a* (Fig. 3A and 3B). The transcription of *Fib1a* and *BMP2* at both temperatures decreased progressively until hatching, after which they remained stable. In contrast, *Col1A1* and *ON* transcript abundance was low during somitogenesis and increased from hatching up to the early flexion stage in both HT and LT groups (Fig. 3C and 3D). Furthermore, although in the latter stages the mRNA levels of these genes were always higher in the HT group, statistically significant differences were only detected at hatch. The *OP*, *MGP* and *OCN* mRNA levels in the LT and HT groups were very low up until hatch but at mouth opening (*MGP*) and early flexion (*OP* and *OCN*) a significant increase in transcript abundance existed between the two groups, although it should be noted that only 2 samples (pools of larvae) were available for analysis for the latter developmental stage (Fig. 3E, 3F and 3G). Finally, *TNAP* transcripts were very abundant up until hatch and then were significantly down-regulated after mouth opening in both LT and HT groups, and were not significantly different between the temperature groups (Fig. 3H).

The analysis of gene expression in bone from 7-month old gilthead sea bream exposed to the four thermal regimes (LT, HT, low-to-high temperature LHT or high-to-low temperature HLT) was not significantly different between the treatment groups (Fig. 4). However, when fish were exposed to a shift in water

temperature (23 to 13ºC) a significant reduction in expression was observed for *OCN* in the LT and LHT groups (Fig. 4F).

## 3. Discussion

In the present study we aimed to characterize the molecules involved in osteogenesis in gilthead sea bream and to determine how temperature modified their expression using both *in vitro* and *in vivo* approaches. Moreover, we also investigated the temperature-induced gene responses in bone tissue by challenging juvenile fish that had undergone thermal imprinting during their embryonic and larval stages.

Differentiation of gilthead sea bream bone-derived MSCs into osteoblasts *in vitro* requires the addition of an OM. Shortly after induction, the cells started to change their morphology from a fibroblast-like form to a geometrical shape, and mineralizing nodules became progressively more evident [8], indicating the characteristic stages of lineage commitment, ECM production and ECM mineralization. These results are similar to those reported by Fernández et al., [30] using another fish bone-derived cell line. These processes are illustrated in the present study by cell morphology and at a transcriptional level (Fig. 5A). Lineage determination and differentiation of osteoblasts from MSCs involves multiple regulatory actors, specific transcription factors, environmental conditions and mineral availability. Among the transcription factors, Runx2 is crucial and it is well-known that it is required for osteoblast differentiation in mammals [31] and coordinates the expression of genes such as *OP*, *OCN* and alkaline phosphatase [32,33]. In fish, Runx2 is also an important early indicator of the osteogenic capacity of cells [15] and several authors have shown in fish and mammals that *Runx2* expression increases at very early stages [34-37]. However, in the present study we did not observe significant changes in *Runx2* expression in the cell cultures, which may be because sampling only occurred at day 5. In fact, regulation of *Runx2* levels in gilthead sea bream MSCs undergoing differentiation into osteoblasts was recently detected 6 h after induction (Riera-Heredia, unpublished data). The

secreted factor BMP2 is involved in bone commitment and is a widely used growth factor for *in vitro* bone induction [38,39], although lineage determinant effects of BMPs on MSCs are highly dependent on receptor type and dose [40]. Contrary to expectations, increased *BMP2* expression was not observed in early bone cell cultures. Nonetheless, BMP2 has also been associated later in the process of ECM mineralization and bone nodule formation during MSCs differentiation in mammals [41,42], which is coherent with the *BMP2* mRNA expression profile detected in the gilthead sea bream MSC cultures described herein. Furthermore, a similar expression pattern was reported in a gilthead sea bream chondrocyte-like VSa13 cell line, since *BMP2* gene expression was also strongly induced during mineralization, but did not occur in the osteoblast-like VSa16 cell line [43].

Concerning ECM structural elements, Fib1a is produced by osteoblasts and accumulates in the ECM; its expression is higher during the early stages of osteoblast differentiation and declines during cell maturation [44-47]. In our MSC cultures, *Fib1a* expression was highest at day 5 and progressively decreased thereafter, confirming a likely role in the first stages of osteogenesis and cell adhesion [46-48]. Col1A is a component that comprises 90% of the bone ECM, it is expressed during osteoblast differentiation together with some non-collagenous proteins (i.e. ON and OP) and serves as a useful marker of early mineralization. ON acts as a modulator of cell–matrix interactions and has been recognized as a mediator of the early phase of ECM production [49] and OP is involved in ECM mineralization by regulating calcium phosphate deposition and in bone remodelling by mediating osteoclast attachment to the mineralized ECM during resorption [50-53]. Notwithstanding this timely role, the expression of *ON* and *OP* usually increases during maturation [15,54], as occurred in our cells, which presented a significant up-regulation by day 20, confirming that these molecules seem to be required for bone mineralization in fish. The results for *OP* agreed with those reported in control *versus* mineralized VSa13 and VSa16 fish cell lines [55], although under the same conditions *ON* was also shown to be down-regulated by the same authors. During this initial phase of ECM production, an increase in *MGP* and

*TNAP* concomitant with *Col1A1,* was expected based on the homology between fish and mammalian systems [56,57]; nevertheless, higher *MGP* mRNA levels were only found at day 20 in OM cells relative to cells growing in GM in our study, but no changes in *TNAP* expression were found. This may indicate that cells during this period were more prone to mineralize than to differentiate as suggested by the *MGP* and *TNAP* mRNA levels; only at later stages of differentiation, has mineralization been found to be negatively regulated by increased *MGP* expression [6].

Summarizing the *in vitro* characterization analysis, Fib1a appears to be involved in the regulation of the very early stages of the bone cells in culture, possibly in the establishment of the osteoid, while at later stages BMP2, ON, OP and probably MGP, appear to play a role in the consolidation of osteoblast maturation and mineralization of the ECM. Interestingly, the gene expression profiles reported in the present study during embryonic and larval development (Fig. 5B) are concordant with those obtained in our *in vitro* culture model, with the exception of BMP2, demonstrating the relevance and robustness of this *in vitro* system for studies of osteogenesis in this fish species. Furthermore, the gene expression profiles reported herein for *BMP2*, *ON*, *OP* and *OCN* during embryogenesis also agree with those previously presented for this fish species [43,55,58-60].

*Fib1a* had the highest levels of expression during the somite stages, whereas *Col1A1* and *ON* transcript levels were increased at hatch, confirming their important role in structuring the bone ECM [46-48] and establishing ECM production [49,54], respectively. Such up-regulation at hatch could be related *in vivo* with the early formation of the cranial skeleton (neurocranium and spachnocranium) and the caudal fin elements that are of fundamental importance for the proper development of sensory systems, exogenous feeding and survival [61], since at this stage the vertebral column and fins are not yet differentiated [1,3]. Then, the onset of bone mineralization at mouth opening was marked by increased transcript levels of *MGP* and *OP*, supporting their central role in osteoblast maturation and ECM mineral deposition, mainly in the already formed viscerocranial structures. Finally,

*OCN* was significantly up-regulated at early notochord flexion *in vivo* in agreement with its major osteogenic role, although due to the low sample number (n = 2) available in the present study for this stage, further confirmatory analysis is required. The up-regulation of *OCN* could be associated with the appearance at this developmental stage of skeletal structures formed by intramembranous ossification (i.e. vertebral centra, neural and haemal spines) and the mineralization of cartilage replacement bones (i.e. hypurals, neural and haemal arches) since at this time the notochord is still not segmented [1-3]. In fact, although we used whole larvae for gene expression analysis and OCN is not exclusively present in skeletal tissues [31], it is the most abundant non-collagenous protein in the bone ECM and is considered a late marker of osteoblast differentiation, essential for appropriate maturation of hydroxyapatite crystals and bone mineralization [62-64]. Probably this is why this molecule was undetected in our *in vitro* cultures, because the level of mineralization/maturation of the cells was too low to induce its expression.

Temperature is a primary factor affecting fish larval development, and rearing fish at temperatures outside their optimal range, which in the case of temperate Sparids is below 15ºC and above 22ºC, leads to increased skeletal anomalies [65]. Specifically, for gilthead sea bream, although the usual hatchery temperatures are between 16 and 18ºC [66], rearing at 22ºC for the entire larval period has been proposed since larvae have less malformations and also have enhanced growth [67]. Overall, while the mechanisms controlling osteogenesis in fish are not fully clarified, it is generally accepted that temperature changes transcription in bone-forming and cartilage-forming cells.

When the responsiveness of gilthead sea bream bone-derived MSCs to a change in temperature was evaluated, an increase in *HSP30* and *HSP90b* transcripts was observed, which may be associated with exposure to stressful conditions as previously reported [68-70]; thus confirming the potentially compromised status of these cells. Most of the osteogenic genes studied in the bone-derived MSCs were down-regulated in response to either an increase or decrease in temperature. A reduction in the osteogenic genes *Col1A1*, *ON* and *OCN* has previously been

reported in rat osteoblast primary cultures exposed to hypothermia [71], and also when Atlantic salmon primary muscle satellite cells that were induced to differentiate into osteoblasts *in vitro* were exposed to a sudden rise in temperature [7]. Thus, although a change in temperature (both an increase and decrease) caused an apparent generalized reduction in gene transcription in our cells, the response was gene specific, as has previously been reported [7,71]. In this sense, our results showed that *ON* was primarily up-regulated in response to temperature change. ON is an ECM glycoprotein that has also been described as a heat shock protein having chaperone-like properties [72,73]. In this context, we cannot eliminate the possibility that the rapid response of *ON* to temperature may be an initial cellular response to stress that is linked with its role in the prevention of collagen denaturation, as has previously been suggested [74]. Overall, the *in vitro* results suggest *ON* gene expression in fish bone may be a candidate indicator of stressful conditions, while the decrease in expression of the other genes may be indicative of a temperature induced check in the osteogenic process.

To evaluate the effects of temperature *in vivo*, fish were reared under two different thermal regimes, and lower expression of all osteogenic genes studied in the embryo and larvae incubated at 18ºC (LT) compared to fish kept at 22ºC (HT) was encountered. Interestingly, the gene expression patterns throughout development were not modified due to incubation temperature, probably since both thermal regimes were within the optimal temperature range for gilthead sea bream rearing [65]. In this sense, although bone development might be accelerated in the HT fish, the consequences with regard to the incidence of malformations is not known. Moreover, in all cases the differences in transcript levels were most significant at the developmental time at which the corresponding gene had its peak of expression: at the stages of 1 and 10 somites for *BMP2* and *Fib1a*, respectively, at hatching for *Col1A1* and *ON* and at early flexion for *OP* and *OCN*. In the latter case substantial differences in gene transcript expression were detected, but since only 2 samples (each corresponding to a pool of larvae) were available for this developmental stage, further confirmation is required. Since the potential

temperature effects on fish from different experimental treatments were corrected by sampling using developmental stage as a reference, these data indicate that the most temperature sensitive developmental period is embryogenesis, when imprinting mechanisms can influence the expression of osteogenic genes (i.e. epigenetic modification of chromatin and histones, DNA methylation) [75]. Furthermore, the results of our study indicate that, in common with skeletal muscle and other physiological systems, the skeleton of gilthead sea bream is affected by a short-term change in temperature during embryogenesis. Moreover, in various teleost species the embryonic rearing temperatures produced persistent effects by affecting gene expression in adults even when grown at a common temperature from hatch onwards [76-78].

Mateus et al., [29] recently demonstrated in gilthead sea bream that the temperature regime applied during early development caused thermal imprinting with long-term effects on bone turnover and gene expression. The present study reinforces the previous observations and identifies, using gene markers as a proxy and an *in vitro* culture model, the molecular and cellular effects. In fact, juvenile fish imprinted with the LT and LHT thermal regimes had reduced transcription of some genes in the bone when the water temperature fell from 23 to 13ºC, suggesting bone formation and mineralization was checked and this may be another negative consequence of winter syndrome. The significant differences observed for *OCN* agree with that reported previously [29], where the cold challenge in these fish caused a reduction in the bone calcium content and the relative abundance of *OCN* and osteoglycin, a small leucine-rich proteoglycan found in the ECM of bone. In the same study [29], the LHT and LT groups also had the most notable down-regulation of the endocrine factors controlling bone growth (i.e. insulin-like growth factor 1 and glucocorticoid and thyroid receptors). The reduced expression of some key regulatory and osteogenic factors was proposed to be indicative of impaired responsiveness of bone, suppressing osteoblast differentiation and affecting bone homeostasis and remodelling [79,80]. In support of this notion, hyperthermic Atlantic salmon showed down-regulation of ECM genes

(i.e. *Col1A1*, *ON* and *OCN*) and at the juvenile stage had shorter and less mineralized vertebral bodies and a higher rate of skeletal malformations than fish reared under a normal temperature regime [19]. On the other hand, mRNA levels of all the genes under the HT and HLT treatments remained similar irrespective of the rearing temperature of the juveniles. This may show the possible beneficial effects for the skeletal tissue in juveniles and adults, of thermal imprinting during embryogenesis. We hypothesize that under the present experimental conditions, epigenetic mechanisms such as modifications in chromatin and histones or DNA methylation were in place to maintain the expression of the osteogenic genes that had a higher transcript abundance in the HT and HLT groups during their early development, whereas the changes caused by temperature imprinting in the LT and LHT groups were less beneficial. In view of the variable response of the gene transcript expression to a drop in temperature in the different thermally imprinted groups, we conclude that the response is specific and not generalized to either, i) reduced water temperature or ii) the reduced food intake in the 13ºC group (1% *versus* 3% of the controls at 23ºC). The specificity of the response of the thermally imprinted fish to a temperature drop was further supported by the similar level of abundance of the reference genes (also in the *in vitro* study), in the control and challenged fish, and this counters the idea that the results obtained arose from a global repression in transcription due to temperature/nutrient reduction.

Overall, the present study provides novel and detailed data characterizing the gene networks regulating the process of osteogenesis in developing gilthead sea bream exposed to thermal imprinting. We further demonstrated the potential of the gilthead seabream MSCs *in vitro* system as a reliable model to investigate osteogenesis and revealed that it mirrors accurately the data obtained with *in vivo* models. Thus, this study provides, not only better knowledge with regards to bone development and temperature induced effects, but also presents a promising tool for future studies aimed at unravelling the mechanisms underlying the high incidence of skeletal deformities in aquaculture.

## 4. Methods

### *4.1. In vitro studies*

#### *4.1.1. Primary cultures of bone-derived cells from gilthead sea bream*

Gilthead sea bream were obtained from a fish farm in the North of Spain (Tinamenor S.L., Pesués) and maintained in the animal facilities of the Faculty of Biology at the University of Barcelona. Fish were kept in 200 L fiberglass tanks under a 12 h light/12 h dark photoperiod and fed *ad libitum* twice daily with a commercial diet (OptiBream™, Skretting, Burgos, Spain). All animal handling procedures were carried out in accordance with the guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 243/12 and DAAM 6759). Primary cultures of gilthead sea bream bone-derived MSCs were performed as previously described [8]. Briefly, six fish (average weight 23 g) per culture were used, they were sacrificed by a blow to the head, and the vertebral column was removed and cleaned. The vertebrae were diced-up into smaller fragments using a scalpel and then, two subsequent digestions of 30 and 90 min, respectively, were performed with 0.125% Type II collagenase. Next, the tissue fragments obtained were washed with Dulbecco's Modified Eagle Medium (DMEM) supplemented with a 1% antibiotic/antimycotic solution (A/A) and plated with complete GM composed of DMEM with 10% fetal bovine serum, 1% Fungizone and 1% A/A and incubated at 23ºC and 2.5% $CO_2$. After 1 week, the vertebrae fragments were removed, and the cells collected with 0.25% trypsin-EDTA and routinely sub-cultured for a maximum of 10 passages. All plastic ware was obtained from Nunc (Barcelona, Spain). The trypsin–EDTA and Fungizone solutions were from Invitrogen (El Prat de Llobregat, Spain), and all the other reagents were purchased from Sigma-Aldrich (Tres Cantos, Spain).

#### *4.1.2. Characterization of osteogenesis in vitro*

In order to characterize the stages of development of the cell cultures, trypsinised cells in suspension were counted and plated in 6-well plates with GM at a density

of $10^5$ cells per well (n=8 independent experiments). On the following day (day 0), the media was changed to grow the cells under control (GM) or mineralizing conditions using an OM, which consisted of GM supplemented with 50 µg/ml L-ascorbic acid, 10 mM β-glycerophosphate and 4 mM $CaCl_2$. These cell cultures (GM and OM) were then sampled at different time points (days 5, 10, 15 and 20) with pools of 2 wells collected into 1 ml of Tri Reagent® solution (Life Technologies, Alcobendas, Spain) and stored at -80ºC until gene expression analyses were performed.

#### *4.1.3. Differentiating bone cells at three temperatures*

Cultivated bone-derived cells were grown in OM until day 13 to have differentiated cells in a partly mineralized ECM (n=7 independent experiments). Then, the cell plates were divided into three different temperature groups (time 0). One group was kept at 23ºC (control), a second group was transferred to an incubator at 18ºC and a third group was moved to 28ºC. Cell samples from the three temperature groups were thereafter harvested for gene expression analyses at the following time points: 1, 6, 24 and 48 h after the temperature challenge. Results from cells incubated at the two experimental temperatures (18 and 28ºC) were analysed relative to cells maintained at the control temperature of 23ºC and standardized (i.e. standard score normalization). A heat map with these values was then generated using PermutMatrix version 1.9.3. [81].

### *4.2. In vivo studies*

#### *4.2.1. Animals and ethics statement*

Rearing of gilthead sea bream embryos, larvae and juveniles was performed at the Institute for Aquaculture and Food Technology Research, (IRTA, Sant Carles de la Ràpita, Spain), in a temperature-controlled seawater recirculation system (IRTAmar™). Animal handling procedures were carried out in accordance with the guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of IRTA (4998-T9900002).

### *4.2.2. Embryogenesis characterization at two temperatures*

The effect of temperature on embryonic and larval development was determined by rearing fish under a constant temperature of 18ºC (LT) or 22ºC (HT). Fertilized eggs (110 mL/tank) of gilthead sea bream (fertilization rate of 92%) were maintained in duplicate 30 L tanks from embryogenesis up until the larval-juvenile transition. A detailed description of the experiment is provided in García de la serrana et al., [75]. Samples of each stage, 6 per tank, were removed by quickly netting the larvae, sacrificed with an overdose of anaesthetic (150 mg/L of MS-222; Sigma, Tres Cantos, Spain) and snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde. Embryos and larvae were sampled at: 1 (n = 6), 10 (n = 6) and 25 somites (S., n = 6), hatch (H., n = 6), mouth opening (M.O., n = 6) and early notochord flexion (E.F., n = 2), and to compensate for the potential effects of temperature on the developmental progression, the samples of fish from the two different experimental groups were collected using developmental stage as the reference.

### *4.2.3. Temperature drop challenge*

For this experiment, fish reared under four different thermal regimes during embryonic and larval development were studied. A schematic representation of the experimental trial and sampling details can be found in Mateus et al., [29], where biometric and bone homeostasis parameters are reported. After hatching, the temperature was either maintained (LT, 18ºC or HT, 22ºC groups, respectively) or changed from 22 to 18ºC (HLT) or 18 to 22ºC (LHT) up until the larval-juvenile transition. Juvenile fish were then transferred to duplicate 2000 L tanks per treatment connected to a recirculating sea water system (5-10% water renewal per day, IRTAmar™) at 22-23ºC and were fed 3% body mass (w/w) with a commercial diet (OptiBream™, Skretting, Spain).

For the cold challenge experiment, fish were age matched (7 months' post-hatch), although significant differences ($P<0.001$) in weight and length existed between the fish reared under the different thermal regimes during embryogenesis as

previously reported [29]. Duplicate tanks of fish from the four different thermal regimes, were either, a) maintained at the same temperature of 23 ± 1ºC or b) exposed to a temperature challenge of 13 ± 1ºC for 15 days. The circuit consisted of 200 L fibreglass tanks in a semi-closed sea water system at pH 7.5-8.0, 35-36‰ salinity and > 80% oxygen saturation and maintained under a 12 h light/12 h dark photoperiod. Fish from the control group were fed at the rate of 3% body weight daily using a commercial diet (OptiBream™), while fish from the cold challenge were fed at a rate of only 1%, and uneaten food was siphoned daily from the bottom of the experimental tanks. These feeding ratios were adjusted to the differences observed in intake due to rearing temperatures during acclimation to keep the tanks clean and with good water quality conditions. Ten fish from each condition were sacrificed with an overdose of phenoxyethanol (450 ppm) and samples of vertebrae were collected. No mortality occurred during the experimental trial and the gilthead sea bream exhibited no signs of distress during the experiment.

### *4.3. Gene expression analyses*

#### *4.3.1. RNA extraction and cDNA synthesis*

Total RNA was isolated using Tri Reagent® following the manufacturer's instructions and quantified using a NanoDrop2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain), and the quality analysed by 1% (w/v) agarose gel electrophoresis. One µg of total RNA per sample was DNase treated (Life Technologies, Alcobendas, Spain) and used to synthesise first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Vallès, Spain), following the manufacturers' instructions.

#### *4.3.2. Quantitative real-time PCR (qPCR)*

The qPCR assays were conducted according to the requirements of the MIQE guidelines [82] using a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). Prior to the analyses, the specificity of the reaction, absence of primer-dimers, as well as the most appropriate cDNA working dilution for each assay was determined by running a dilution curve with a pool of samples. Reactions were

performed in triplicate (methodological replicates) and contained cDNA, iQ SYBR Green Supermix (Bio-Rad) and 250 nM (final concentration) of sense and antisense primers (Table 1). The protocol consisted of 1 cycle of 3 min at 95ºC and 40 cycles of 10 s at 95ºC and 30 s at 55-68ºC (primer dependent, see Table 1), followed by an amplicon dissociation analysis from 55 to 95ºC with a 0.5ºC increase every 30 s. A single peak was observed for each of the qPCR reactions and this confirmed reaction specificity. SYBR Green fluorescence was recorded during the annealing-extending phase of cycling. Target transcript abundance was analysed using the delta-delta Ct method [83] with the CFX Manager Software (Bio-Rad). In the *in vitro* studies, gene expression results were normalized using the geometric mean of ribosomal protein S18 (*RPS18*) and elongation factor 1 alpha (*EF1a*) for the culture development experiment and ribosomal protein L27 (*RPL27*) and ubiquitin (*Ub*) for the temperature experiment. *RPS18* and *RPL27* were used to standardise the gene expression during the *in vivo* experiments of embryogenesis and the adult temperature challenge, respectively. In each case, the reference gene(s) selected were those for which stability was confirmed by running the GeNorm algorithm implemented in the CFX Manager Software (Bio Rad).

*4.4. Statistical analyses*

Statistical analyses of all parameters were performed in SPSS Statistics version 20 (IBM, Armonk, NY, USA). Normality was analysed using the Shapiro-Wilk test and homogeneity of variance using a Levene's test. Statistical significance was assessed by two-way analysis of variance (two-way ANOVA) followed by Tukey *post-hoc* test. Significant differences were taken at $p<0.05$ for all statistical tests performed. Data are presented as mean ± standard error of the mean (s.e.m.). Significant differences through time within groups (i.e. days in culture, developmental stages or thermal regimes) are shown with different letters and significant differences between treatments (i.e. culture media or rearing temperature) are shown with an asterisk.

*4.5. Data availability*

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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**Competing interests’ statement**

No competing interests (financial or non-financial) have been declared by any author.

**Author contributions statement:** D.M.P. and E.C. conceptualised the study; N.R-H. performed all the *in vitro* cultures and corresponding laboratory analyses; E.G. run the *in vivo* trials; R.M., A.P.M. and R.A.C. performed the laboratory analyses corresponding to the *in vivo* experiments; N.R-H., D.M.P. and E.C. analysed and interpreted the data; I.N., J.G., D.M.P. and E.C. acquired funding; N.R-H., E.G., J.G., D.M.P. and E.C. drafted and critically reviewed the manuscript. All authors read and approved the final paper.

## Figures and figure legends

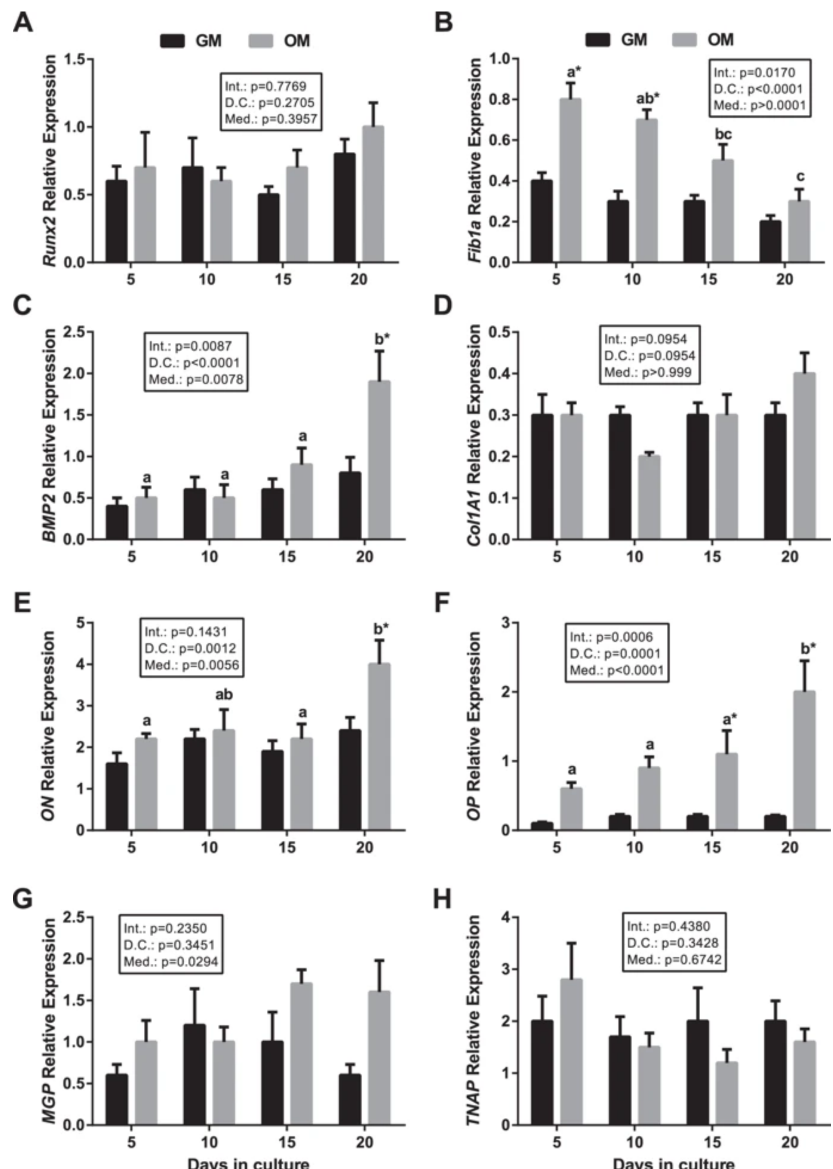



**Figure 1. Expression of osteogenic genes in gilthead sea bream differentiating bone-derived primary cell cultures *in vitro*.** Quantitative gene expression relative to the geometric mean of *RPS18* and *EF1α* for (**A**) *Runx2*, (**B**) *Fib1a*, (**C**) *BMP2*, (**D**) *Col1A1*, (**E**) *ON*, (**F**) *OP*, (**G**) *MGP* and (**H**) *TNAP* in cells cultured in growth (GM) or osteogenic (OM) media at days 5, 10, 15 and 20. The results are shown as the mean ± s.e.m. (n=5-8). Different letters indicate significant differences throughout time within groups (upper case for GM and lower case for OM) and asterisks indicate significant differences between groups at each culture day (p<0.05). Int.: Interaction, D.C.: Days in culture, Med.: Media.

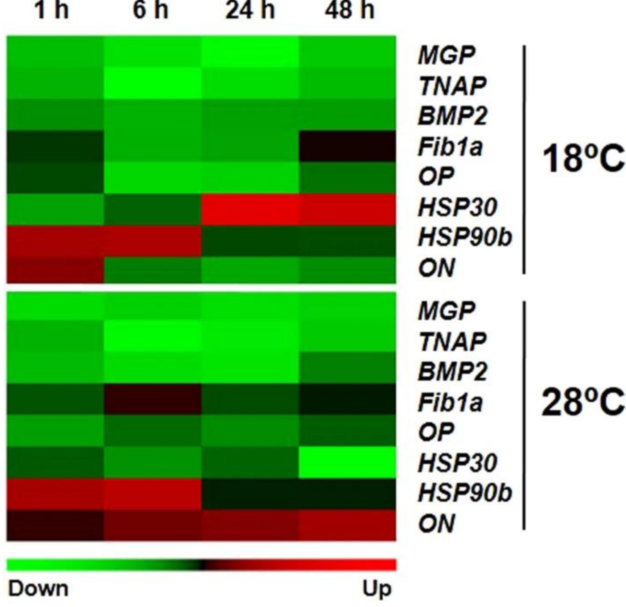



**Figure 2. Heat map representing changes in osteogenic genes expression in gilthead sea bream bone-derived primary cell cultures incubated at different temperatures.** Quantitative gene expression was relative to the geometric mean of *RPL27* and *Ub* for *MGP*, *TNAP*, *BMP2*, *Fib1a*, *OP*, *HSP30*, *HSP90b* and *ON*. Changes in gene expression were determined in cells after 13 days growing in osteogenic medium (OM) and incubated at two different temperatures (18 and 28ºC) and normalized in relation to the control temperature of 23ºC at 1, 6, 24 and 48 h (n=5-8). Rows (mRNA transcripts) in the heat map were standardized following a standard score normalization. Red and green shading, respectively, indicate the highest and lowest expression levels, as specified in the scale bar at the bottom of the figure. Each block represents the average standard-score normalization for the 5-8 cultures sampled at each time-point.

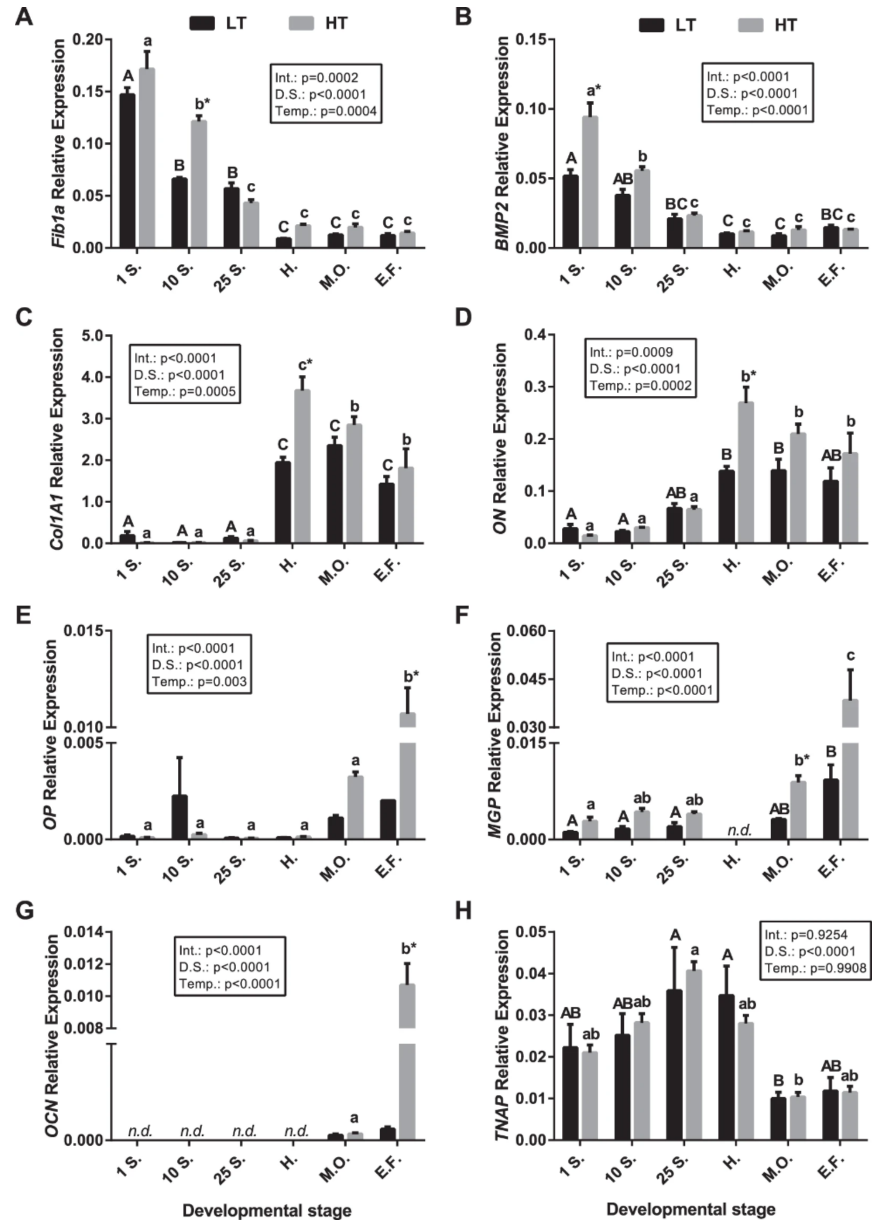



**Figure 3. Expression of osteogenic genes in gilthead sea bream embryos and larvae reared at two different temperatures.** Quantitative expression relative to *RPS18* of (**A**) *Fib1a*, (**B**) *BMP2*, (**C**) *Col1A1*, (**D**) *ON*, (**E**) *OP*, (**F**) *MGP*, (**G**) *OCN* and (**H**) *TNAP* at different developmental stages (1 somite [1 S.], n = 6; 10 somites [10 S.], n = 6; 25 somites [25 S.], n = 6; hatch [H.], n = 6; mouth opening [M.O.], n = 6; early flexion [E.F.], n = 2) in fish reared at two temperatures (18 [LT] and 22ºC [HT]). Results are shown as the mean ± s.e.m. Different letters indicate significant differences through time within groups (upper case for LT and lower case for HT) and asterisks indicate

significant differences between groups at each developmental stage (p<0.05). Although it should be taken into account that only 2 samples (each corresponding to a pool of larvae) were available for the developmental stage of early flexion. n.d.: Non-detected, Int.: Interaction, D.S.: Developmental stage, Temp.: Temperature.

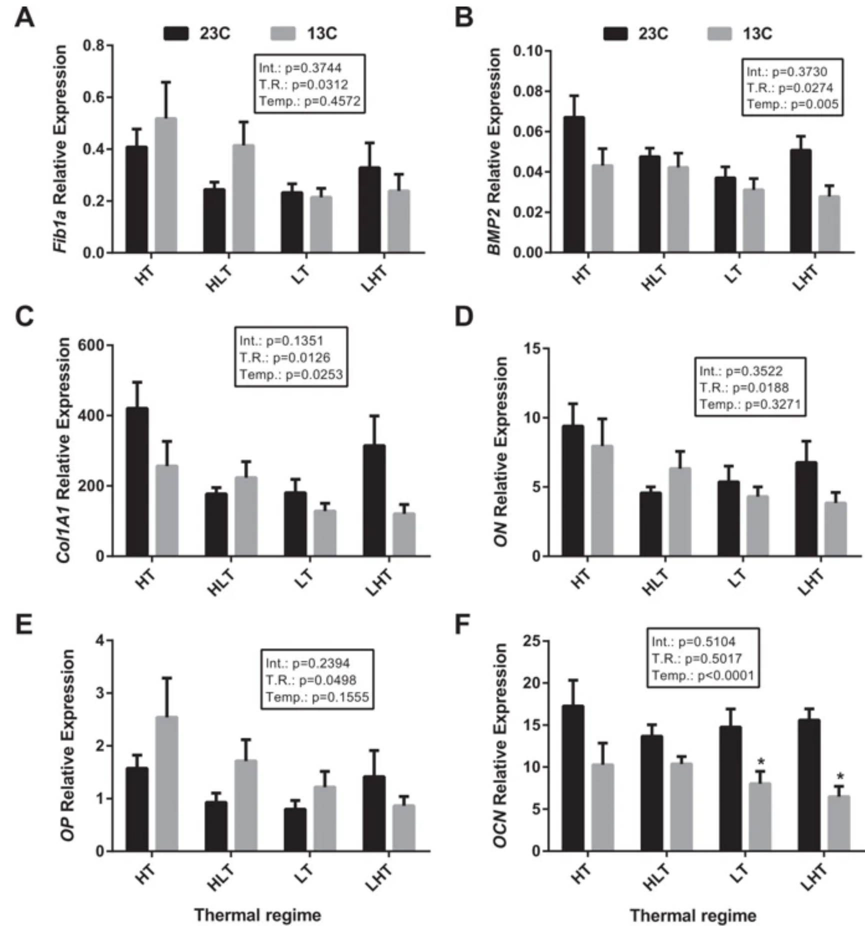
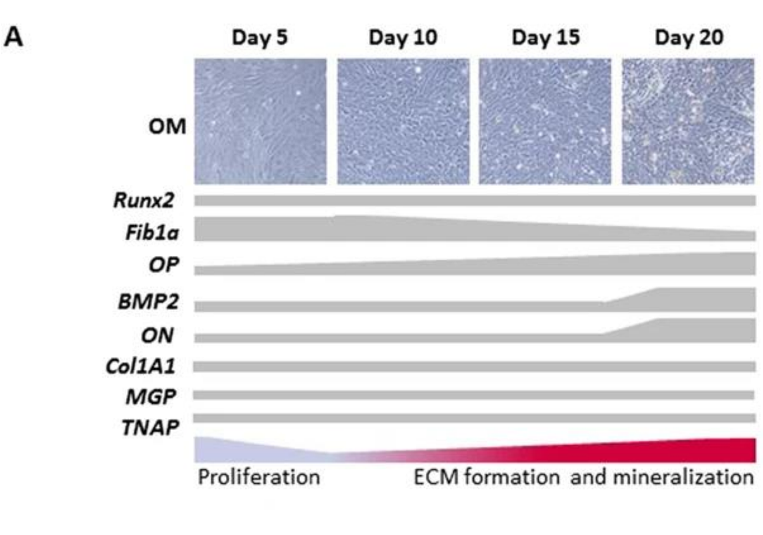



**Figure 4. Expression of osteogenic genes in gilthead sea bream juveniles after a temperature challenge *in vivo*.** Quantitative expression relative to *RPL27* of (**A**) *Fib1a*, (**B**) *BMP2*, (**C**) *Col1A1*, (**D**) *ON*, (**E**) *OP* and (**F**) *OCN*. Quantitative PCR was performed with samples from gilthead sea bream reared at different temperature regimes during embryogenesis (LT [18-18ºC]; LHT [18-22ºC]; HT [22-22ºC]; HLT [22-18ºC]) and then maintained at 23ºC (LT n=9, LHT n=10, HT n=9, HLT n=9) or challenged with a drop in water temperature from 23ºC to 13ºC (LT n=7, LHT n=9, HT n=9, HLT n=9) at 7 months' post-hatch. Results are shown as the mean ± s.e.m. No significant differences were observed among thermal regimes within each temperature treatment (23 or 13ºC), but asterisks indicate significant differences between temperature treatments for each thermal regime (p<0.05). Int.: Interaction, T.R.: Thermal regime, Temp.: Temperature. Variation in sample number for

different experimental groups was due to the low yield and quality of RNA extracted from some samples.



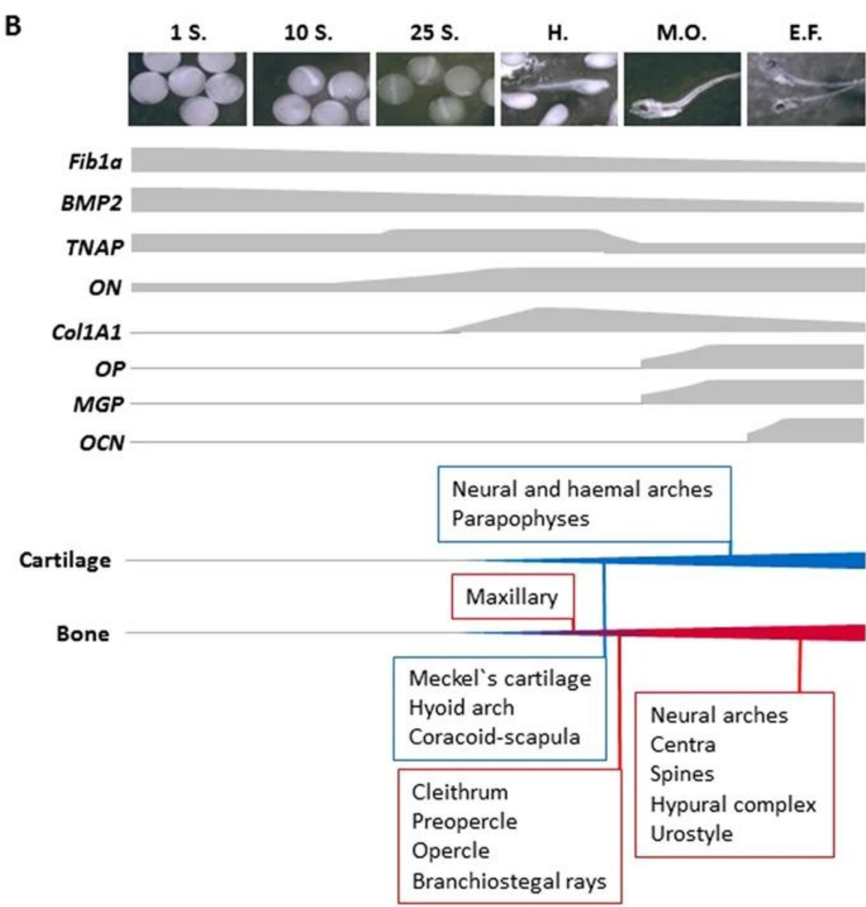



**Figure 5. Schematic representation summarizing the expression of osteogenic genes in gilthead sea bream *in vitro* and *in vivo* assays.** Representative images of (**A**) bone-derived primary cell cultures growing in osteogenic medium (OM) at days 5, 10, 15 and 20, and (**B**) gilthead sea bream at different developmental stages during embryogenesis (1 somite [1 S.]; 10 somites [10 S.]; 25 somites [25 S.]; hatch [H.]; mouth opening [M.O.]; early flexion [E.F.]). In **A**, the processes of proliferation and extracellular matrix (ECM) mineralization according to Capilla et al., [8] are schematically indicated with the thick to narrow transition or vice versa representing the approximate rate during culture development. In **B**, the developmental osteology of gilthead sea bream indicating the main skeletal structures that are formed is shown [1-3]. Cartilage is represented by the blue line and endochondral and dermal bone formation is represented in red; the blue - red transition represents the onset of endochondral (cartilage replacement bone) formation. The narrow to thick transition of the line for cartilage or bone formation represent the increasing number of structures that develop during development. In sea bream, skeletogenesis begins soon after hatching with the development of cartilaginous and bony structures necessary for exogenous feeding, vision, opening and closing the mouth, expanding and narrowing the oral and branchial areas and propulsion. The comparison between the *in vitro* and *in vivo* results shows that of the genes analysed *Fib1a* is most important during the early development of bone, which is in agreement with its more structural role, while *ON*, OP, *MGP* and *OCN* are more abundant in later stages when the deposition of mineral needs to be tightly regulated for the proper formation of bone. In contrast, *BMP2*, *Col1A1* and *TNAP* had divergent expression patterns between the *in vitro* and *in vivo* situation probably due to their diverse roles in tissues during development.

**Table 1. Primers used for real-time quantitative PCR.** F, forward primer; R, reverse primer; Tm, annealing temperature; Acc. Num., accession number.

| *Gene* | *Primer sequence (5'-3')* | *Tm (ºC)* | *Acc. Num.* | *Efficiency (%)* |
|---|---|---|---|---|
| ***BMP2*** | F: GGAGAAGCAGCGTGGATTAAACACGAAT<br>R: GGCCTGCGCCTCAGTCCAAACATATT | 68 | AY500244 | 103.9 |
| ***Col1A1*** | F: GAGATGGCGGTGATGTGGCGGAGTC<br>R: GCCTGGTTTGGCTGGATGAAGAGGG | 68 | DQ324363 | 91.7 |
| ***EF1α*** | F: CTTCAACGCTCAGGTCATCAT<br>R: GCACAGCGAAACGACCAAGGGGA | 60 | AF184170 | 94.2 |
| ***Fib1a*** | F: CGGTAATAACTACAGAATCGGTGAG<br>R: CGCATTTGAACTCGCCCTTG | 60 | FG262933 | 101.8 |
| ***HSP30*** | F: GGTGACTGACGGGAAAGAGA<br>R: CTGAGGAGGAGGTGCTGTTC | 60 | GU060312 | 93.1 |
| ***HSP90b*** | F: TTCACGCATGGAAGAAGTTG<br>R: GGTCCACCACACAACATGAA | 56 | DQ012949 | 86.0 |
| ***MGP*** | F:TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC<br>R: CGGGCGGATAGTGTGAAAAATGGTTAGTG | 68 | AY065652 | 89.2 |
| ***OCN*** | F: TCCGCAGTGGTGAGACAGAAG<br>R: CGGTCCGTAGTAGGCCGTGTAG | 60 | AF048703 | 90.7 |
| ***ON*** | F: AGGAGGAGGTCATCGTGGAAGAGCC<br>R: GTGGTGGTTCAGGCAGGGATTCTCA | 68 | AY239014 | 97.1 |
| ***OP*** | F:AAAACCCAGGAGATAAACTCAAGACAACCCA<br>R: AGAACCGTGGCAAAGAGCAGAACGAA | 68 | AY651247 | 95.3 |
| ***RPL27*** | F: AAGAGGAACACAACTCACTGCCCCAC<br>R: GCTTGCCTTTGCCCAGAACTTTGTAG | 68 | AY188520 | 97.4 |
| ***RPS18*** | F: AGGGTGTTGGCAGACGTTAC<br>R: CTTCTGCCTGTTGAGGAACC | 60 | AM490061 | 100.3 |
| ***Runx2*** | F: ACCCGTCCTACCTGAGTCC<br>R: AGAAGAACCTGGCAATCGTC | 60 | JX232063 | 96.1 |
| ***TNAP*** | F: CATCGCAACCCTTTTCACAGTCACCCG<br>R:AACAGTGCCCAAACAGTGGTCCCATTAGC | 68 | AY266359 | 103.2 |
| ***Ub*** | F: CGGAAGTAAGAGGAACCAACAC<br>R: AAGCAGTCAGAATGCAAAGTCA | 56 | AM955423 | 78.4 |

# ARTICLE IV

**Fatty acids from fish or vegetable oils promote the adipogenic fate of mesenchymal stem cells derived from gilthead sea bream bone potentially through different pathways (2019).**

***Plos One, 14*, 4, e0215926.**

# Fatty acids from fish or vegetable oils promote the adipogenic fate of mesenchymal stem cells derived from gilthead sea bream bone potentially through different pathways

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**Short title:** Induction of adipogenesis by fatty acids in sea bream bone MSCs

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**Abstract**

Fish are rich in n-3 long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, thus they have a great nutritional value for human health. In this study, the adipogenic potential of fatty acids commonly found in fish oil (EPA and DHA) and vegetable oils (linoleic (LA) and alpha-linolenic (ALA) acids), was evaluated in bone-derived mesenchymal stem cells (MSCs) from gilthead sea bream. At a morphological level, cells adopted a round shape upon all treatments, losing their fibroblastic form and increasing lipid accumulation, especially in the presence of the n-6 PUFA, LA. The mRNA levels of the key transcription factor of osteogenesis, *runx2* significantly diminished and those of relevant osteogenic genes remained stable after incubation with all fatty acids, suggesting that the osteogenic process might be compromised. On the other hand, transcript levels of the main adipogenesis-inducer factor, *pparg* increased in response to EPA. Nevertheless, the specific PPARγ antagonist T0070907 appeared to suppress the effects being caused by EPA over adipogenesis. Moreover, LA, ALA and their combinations, significantly up-regulated the fatty acid transporter and binding protein, *fatp1* and *fabp11*, supporting the elevated lipid content found in the cells treated with those fatty acids. Overall, this study has demonstrated that fatty acids favor lipid storage in gilthead sea bream bone-derived MSCs inducing their fate into the adipogenic *versus* the osteogenic lineage. This process seems to be promoted via different pathways depending on the fatty acid source, being vegetable oils-derived fatty acids more prone to induce unhealthier metabolic phenotypes.

**Keywords:** eicosapentaenoic acid, linoleic acid, Runx2, PPARγ, adipocyte, hypertrophy

## Introduction

In the last decades, both the world population and the consumption of fish and seafood per capita have increased and will continue to rise. Fish products are rich in n-3 long chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids [1], which are crucial nutrients for overall health [2]. For these reasons, scientific research is indispensable to improve aquaculture production under sustainable conditions, which implies among others, a reduction in the use of fish oil in aquafeeds formulation [3]. The alternatives are vegetable oils, which in contrast to fish oil, are richer in n-6 or n-9 PUFA such as linoleic (LA, 18:2n-6), oleic (18:1n-9) or alpha-linolenic (ALA, 18:3n-3) acids [4]. Moreover, fish (especially marine) may have limited ability to convert $C_{18}$ PUFA to $C_{20/22}$ [4], [5] so, it should be considered that feeding fish with highly substituted diets can result in tissues with lower n-3 LC-PUFA content [6], [7]. Apart from changes in the fatty acid composition of the fish filet [8], [9], [10], dietary vegetable oils in excess can cause adipose tissue and hepatic metabolic alterations [11], [12] or affect the immune system [13], [14]. Besides, low concentrations of dietary EPA and DHA during development, have been related to increased incidence of skeletal malformations [15], [16]. Overall, these can lead to unhealthier or low-quality fish having consequences in aquaculture production.

Fish bone consists, as in other vertebrates, of several cell types including progenitor cells or mesenchymal stem cells (MSCs) that differentiate into osteoblasts after appropriate induction [17], [18]. There are many regulators involved in the process of osteoblastogenesis, but runt-related transcription factor 2 (Runx2), is the main transcription factor controlling lineage determination and osteogenic genes expression [19]. Once differentiated, osteoblasts produce the bone extracellular matrix (ECM) or osteoid, where key components such as osteonectin (ON), osteopontin (OP) and osteocalcin subsequently regulate mineral deposition [20], [21], [22]

Interestingly, mammalian adipocytes can arise from the same MSCs as osteoblasts and a high degree of plasticity has been observed between the two cell lineages, even in very advanced maturation stages [23]. During the onset of the adipogenic process, transcription factors such as CCAAT/enhancer binding protein β and δ (C/EBPβ and C/EBPδ) are activated, which in turn, induce the expression of *cebpa* and peroxisome proliferator-activated receptor γ (*pparg*) [24]. These factors successively promote the transcription of specific genes mainly related with lipid metabolism like fatty acid synthase (*fas*) or the hormone sensitive lipase (*hsl*) [25]. Adipose tissue can grow not only by elevating the cellular number from resident precursors (hyperplasia) but also by increasing the size of existing adipocytes (hypertrophy), by accumulating lipids into their cytoplasm [26]. For that matter, fatty acid transporter proteins like FATP1 or the FAT translocase/CD36, together with lipoprotein lipase (LPL), are relevant actors that facilitate the fat uptake [27]. However, despite being the adipose tissue the largest body energy reserve, considered vital for the maintenance of energy homeostasis [28], its growth by hypertrophy has been associated with less responsive adipocytes to hormones and metabolites (i.e. insulin). This situation in humans derives in hypertrophic obesity and is closely linked to major health issues such as diabetes, hyperlipidemia or cardiovascular diseases [29], [30].

As indicated, the decision of MSCs fate can be affected by cell surrounding microenvironment and might be modulated by endocrine and dietary conditions. Moreover, the signals that induce adipogenesis, at the same time act as inhibitors of osteoblastogenesis, and *vice versa* [23]. Therefore, depending on the stimulus they receive, MSCs differentiate into one or another lineage. In mammals, low dietary n-3/n-6 ratios reduce bone formation and cause greater bone resorption [31], [32], [33], [34]. In the same way, changes in dietary fatty acids could modify the bone health and whole fat content due to this cellular interconversion, but although this is clear in mammals [33] it has not been proved in fish yet. Recently, culture models of MSCs have been established in fish from various adult tissues including fat and bone, and those MSCs have been demonstrated to hold the

plasticity to differentiate into lineages different from the original tissue [18], [35], [36], [37] [38], [39].

In this context, the aim of the present work was to study the effects of the fatty acids EPA and DHA, present mainly in fish oil, and those of LA and ALA, common in vegetable oils (such as soybean, rapeseed and linseed oils), on fat deposition and the expression of both adipogenic- and osteogenic-related genes, in MSCs derived from gilthead sea bream vertebrae. The study hypothesis was that these fatty acids, supplemented in the media, could induce the differentiation of bone-derived MSCs toward the adipogenic *versus* the osteogenic lineage, potentially producing phenotypically different adipocytes depending on the fatty acid source.

## Materials and Methods

### Animals and ethics statement

Gilthead sea bream (*Sparus aurata*) were obtained from the Tinamenor fish farm (Cantabria, Spain) and maintained in the animal facilities of the Faculty of Biology at the University of Barcelona. Sexually immature juveniles of an average weight of 30 g were kept in 200 L fiberglass tanks under a 12 h light/12 h dark photoperiod and fed *ad libitum* twice daily with a commercial diet (Optibream, Skretting, Burgos, Spain). All animal handling procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of the University of Barcelona, (permit numbers CEEA 210/14 and DAAM 6759).

### Primary cultures of bone-derived MSCs and experimental design

Primary cultures of gilthead sea bream bone-derived MSCs were performed as previously described [35]. Briefly, three juvenile gilthead sea bream were used for each culture. The fish were sacrificed by a blow to the head, and bone-derived MSCs were isolated from a piece of vertebra by mechanical disruption and enzymatic (i.e. collagenase) digestion. After several washes, cells and small

vertebra fragments were plated with growth medium (GM) consisting on Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution and supplemented with 19 mM NaCl and 1% fungizone (Invitrogen Life Technologies, Alcobendas, Spain), in 10 cm culture dishes. After 1 week, the fragments were removed, and the attached cells collected with 0.25% trypsin–EDTA (Invitrogen Life Technologies) and plated into new 10 cm plates with fresh GM. From here, the cells were routinely subcultured every time they reached about 70–80% confluence and used for a maximum of 10 passages.

To perform the experiments the cells were seeded at a density of $1 \times 10^4$ cells/cm$^2$ in 24 well plates for the viability test and the lipid quantification assay and in 6 well plates for the gene expression analyses. The fatty acids were applied 3-4 days after plating and the duration of the treatments were of 6 h for gene expression analyses, 24 h to determine viability and 6, 24, 48 and 72 h to quantify lipid accumulation. In all cases, two wells were used for each experimental condition. The fatty acids selected were the following: EPA and DHA since are the most abundant n-3 fatty acids in fish oil sources, and LA and ALA because are essential fatty acids that are present at a high percentage in vegetable oils commonly used in fish feeds (i.e. soya and rapeseed oils). The fatty acids obtained from Cayman Chemical Company (Michigan, USA), were first dissolved in ethanol and used at a final concentration of 200 µM both, individually and in all tested combinations unless stated otherwise. Final concentration of ethanol was very low (below 1%) and did not cause any negative effects in cell viability as confirmed in preliminary assays (ethanol concentrations up to 10% were tested for 24 h, S1 Fig). For the PPARγ antagonists experiment, two commonly used covalent PPARγ ligands T0070907 (2-Chloro-5-nitro-N-4-pyridinyl-benzamide) and GW9662 (2-Chloro-5-nitro-N-phenylbenzamide) were used. These two compounds are referred to as antagonists because they physically block ligand binding by covalently modifying the Cys285 located in an orthosteric pocket embedded in the ligand-binding domain, although they do not have comparable effects with regards to transcription

[40], [41]. Both were obtained from Sigma-Aldrich (Tres Cantos, Spain), diluted with dimethyl sulfoxide (DMSO) and applied together with the fatty acids at a final concentration of 10 µM according to previous literature [42].

MTT cell assay

The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to evaluate cell viability as previously described elsewhere [35]. Briefly, cells from 3-4 independent cultures were incubated the last 3 h of the total 24 h treatment with a final concentration of 0.5 mg/mL of MTT. Then, cells were washed with PBS, resuspended in 250 µL of DMSO per well and absorbance was read immediately using a microplate reader (Infinite 200, Tecan). Cell viability values were obtained from the absorbance measured at 570 nm, with 680 nm as the reference wavelength.

Oil Red O staining

Intracellular neutral lipid accumulation was analyzed by Oil red O (ORO) staining as explained in [35]. Briefly, cells were fixed with 10% formalin for 1 h, subsequently rinsed with PBS, stained with 0.3% ORO prepared in 36% tri-ethyl phosphate for 2 h, and then rinsed with distilled water. Quantification of cell lipid content was calculated as the absorbance measured at 490 nm divided by the read at 630 nm (Infinite 200, Tecan) corresponding to the protein content. The latter was obtained after Comassie blue staining for 1 h and dye extraction by incubation of the cells with 85% propylene glycol during 3 h at 60°C [35]. Data are presented as fold change relative to the control (n=3). The staining effectiveness was evaluated with a Zeiss Axiovert 40C (Carl Zeiss Inc., Germany) inverted research grade microscope equipped with a Canon EOS 1000D digital camera (magnification 20x).

RNA extraction and cDNA synthesis

The cells were lysed with a cell scraper and TRI Reagent (Applied Biosystems, Alcobendas, Spain) in a total volume of 1 mL per each two wells. Total RNA was

extracted according to the manufacturer's recommendations, dissolved in DEPC-treated water (RNase-free), quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and stored at −80°C. To eliminate any residual genomic DNA, total RNA (1 µg) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain), following the manufacturer's instructions.

Quantitative PCR analyses

To characterize the transcriptional profile occurring during the differentiation of bone-derived MSCs into adipocyte-like cells, key genes implicated in osteogenesis, adipogenesis and energy metabolism regulation were analyzed by real-time quantitative PCR (qPCR). The genes evaluated comprise the following: the transcription factor *runx2*, and ECM components: fibronectin 1a (*fib1a*), matrix Gla protein (*mgp*), *op* and *on* for the osteogenic genes. The transcription factors or nuclear receptors: *pparg*, retinoid X receptor (*rxr*) and *cebpb*; the enzymes: fatty acid synthase (*fas*), lipoprotein lipase (*lpl*) and hormone-sensitive lipase (*hsl*); fatty acid transporters: *cd36*, fatty acid transport protein 1 (*fatp1*) and fatty acid binding protein 11 (*fabp11*) for the adipogenic genes. In addition, elongation factor 1 alfa (*ef1a*), ribosomal protein S18 (*rps18*), and beta-actin (*b-actin*) were tested as reference genes.

qPCR was performed using a CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain) as previously described [38]. Each qPCR reaction was performed in triplicate in a total volume of 5 µL, containing 2.5 µL of the iTaq Universal SYBR Green supermix (Bio-Rad, El Prat de Llobregat, Spain), 2 µL of diluted cDNA, 0.125 µL of each primer (250 nM) (Table 1), and milliQ water. Samples were amplified as follows: 95°C for 3 min, and then 40 cycles of 95°C for 10 s, followed by annealing 60-68°C for 30 s (primer-dependent, Table 1), followed by a dissociation step from 55 to 95°C with a 0.5°C increase every 5 s. A standard curve with a dilution series of a cDNA sample pool was constructed to

determine the qPCR efficiency of each primer pair (Table 1), which was calculated using the CFX Manager Software (Bio-Rad). To determine the overall performance of each qPCR assay three control samples were used: no template control (NTC), no reverse transcriptase control (RTC), and PCR control (PCR). Relative expression levels of the target genes were determined by the Pfaffl method [43], using correction for primer efficiencies and normalizing the quantification cycle (Cq) value of each gene, registered during the annealing step to that of *b-actin* and *rps18*, the most stable reference genes among the different conditions ($P > 0.05$) determined using the CFX Manager Software (Bio-Rad). Data were obtained from 4–6 independent cultures.

**Table 1.** Primers used for real-time quantitative PCR. F, forward primer; R, reverse primer; Tm, annealing temperature; Acc. Num., GenBank accession number.

| Gene | Primer sequence (5'→3') | Tm (ºC) | Efficiency (%) | Acc. Num. |
|---|---|---|---|---|
| *runx2* | **F:** ACCCGTCCTACCTGAGTCC | 60 | 104.1 | JX232063 |
| | **R:** AGAAGAACCTGGCAATCGTC | | | |
| *pparg* | **F:** CGCCGTGGACCTGTCAGAGC | 66 | 94.1 | AY590304 |
| | **R:** GGAATGGATGGAGGAGGAGGAGATGG | | | |
| *rxr* | **F:** CCCGGATGCAAAAGGTCTCT | 60 | 99.7 | - |
| | **R:** ATGCTCCAGACACTTGAGGC | | | |
| *cebpb* | **F:** ATGCGCAACTTGGAGACTCA | 60 | 95.5 | - |
| | **R:** GATTAGACAAGCGGCCCAGT | | | |
| *fib1a* | **F:** CGGTAATAACTACAGAATCGGTGAG | 60 | 96.7 | FG262933 |
| | **R:** CGCATTTGAACTCGCCCTTG | | | |
| *mgp* | **F:** TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC | 68 | 101.1 | AY065652 |
| | **R:** CGGGCGGATAGTGTGAAAAATGGTTAGTG | | | |
| *on* | **F:** GTGGTGGTTCAGGCAGGGATTCTCA | 68 | 94.3 | AY239014 |
| | **R:** AGGAGGAGGTCATCGTGGAAGAGCC | | | |
| *op* | **F:** AAAACCCAGGAGATAAACTCAAGACAACCCA | 68 | 91.9 | AY651247 |
| | **R:** AGAACCGTGGCAAAGAGCAGAACGAA | | | |
| *fas* | **F:** TGGCAGCATACACACAGACC | 60 | 95.7 | AM952430 |
| | **R:** CACACAGGGCTTCAGTTTCA | | | |
| *lpl* | **F:** GAGCACGCAGACAACCAGAA | 60 | 108.3 | AY495672 |
| | **R:** GGGGTAGATGTCGATGTCGC | | | |
| *hsl* | **F:** GCTTTGCTTCAGTTTACCACCATTTC | 60 | 92.0 | EU254478 |
| | **R:** GATGTAGCGACCCTTCTGGATGATGTG | | | |
| *cd36* | **F:** GTCGTGGCTCAAGTCTTCCA | 60 | 96.8 | - |
| | **R:** TTTCCCGTGGCCTGTATTCC | | | |
| *fatp1* | **F:** CAACAGAGGTGGAGGGCATT | 60 | 102.7 | - |
| | **R:** GGGGAGATACGCAGGAACAC | | | |
| *fabp11* | **F:** CATTTGAGGAGACCACCGCT | 60 | 107.5 | - |
| | **R:** ACTTGAGTTTGGTGGTACGCT | | | |
| *b-actin* | **F:** TCCTGCGGAATCCATGAGA | 60 | 106.9 | X89920 |
| | **R:** GACGTCGCACTTCATGATGCT | | | |
| *ef1a* | **F:** CTTCAACGCTCAGGTCATCAT | 60 | 97.4 | AF184170 |
| | **R:** GCACAGCGAAACGACCAAGGGGA | | | |
| *rps18* | **F:** AGGGTGTTGGCAGACGTTAC | 60 | 107.3 | AM490061 |
| | **R:** CTTCTGCCTGTTGAGGAACC | | | |

## Statistical analyses

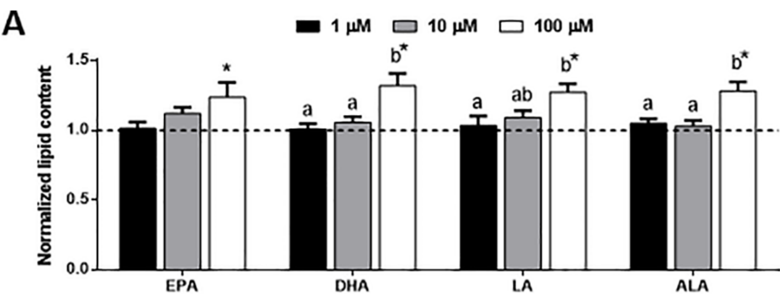
Data normality and homoscedasticity were assessed using Shapiro–Wilk and Levene's test, respectively. Independent samples' Student's t-test was used for comparison between two groups (each experimental treatment *versus* the control). For multiple mean comparisons (among fatty acid treatments) of normal distributed data, one-way ANOVA was used followed by Tukey's or Dunnett's T3

*post hoc* tests in case of homogeneous or heterogeneous variance data, respectively. When data did not fit normal distribution, the non-parametric Kruskal–Wallis test, followed by Mann–Whitney test, were used. Statistical analyses were performed using SPSS Statistics version 20 (IBM, Armonk, NY, USA). Results are presented as mean ± SEM. $P < 0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

**Results**

Fatty acids effects in cell viability and differentiation

Preliminary analyses demonstrated that cell viability was unaffected by the addition of the different fatty acids up to the 200 µM concentration tested (S1 Fig). On the other hand, a dose response was observed with regards to lipid accumulation upon a 48 h treatment with each one of the four fatty acids (EPA, DHA, LA and ALA), showing at the 100 µM concentration significantly higher intracellular lipid content compared to lower doses and the control condition without fatty acids (Fig 1A). Moreover, the images obtained after ORO staining of the cells upon all treatments (EPA and LA shown in Fig 1B as a representation) confirmed this observation, being the 200 µM concentration the one causing higher lipid accumulation and therefore, the one selected for the following experiments. In addition, we could observe in these images the change of cell morphology in response to the treatments, becoming the cells more rounded with an enlarged cytoplasm while losing the fibroblastic shape of MSCs.



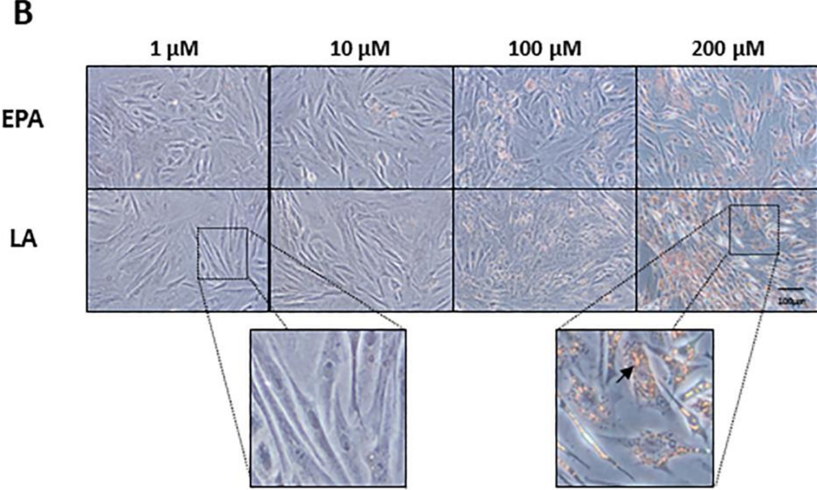
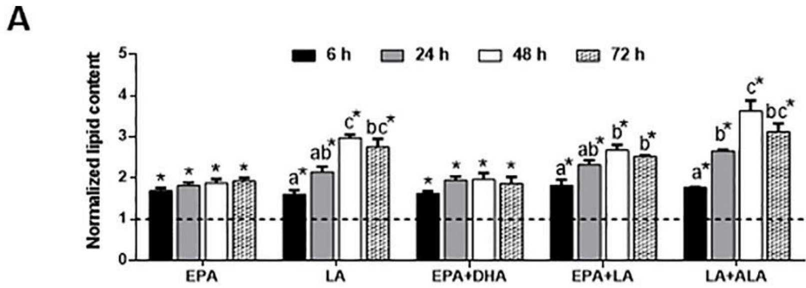



**Fig 1. Dose response of fatty acids on lipid accumulation.** (A) Quantification of lipid content normalized by protein and (B) representative phase-contrast images of gilthead sea bream bone-derived cells after staining with Oil red O. Cells were treated at day 4 with different concentrations of individual fatty acids, or were left untreated as control (dashed line in A) for 48 h. In (A) data are shown as mean + SEM (n=3-4). Significant differences ($p<0.05$) among concentrations are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. In (B) magnification 20x and enlarged views, arrow indicates lipid droplets. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

To further determine the effects through time of selected fatty acids on lipid accumulation, individual treatments with EPA and LA as representatives from the highly present fatty acids in fish and vegetable oils respectively, plus the mixtures EPA+DHA as the fish oil combination, LA+ALA as the vegetable oils one and EPA+LA as the combination containing one fatty acid of each source, were tested at 6, 24, 48 and 72 h. All treatments caused a significant increase in cell lipid content compared to the control condition. Moreover, a time-dependent response up to 48 h (remaining high at 72 h) was also observed by treatments including LA alone or in combination (Fig 2A). The effects of these fatty acids inducing cell lipid accumulation and differentiation (i.e. rounding up) compared to the control condition were confirmed by the microscopic visual evaluation of the culture (Fig 2B). According to these results, the 6 h treatment was selected to evaluate gene

expression in subsequent experiments, in order to observe the immediate effects of these fatty acids at a transcriptional level inducing changes on cell metabolism.



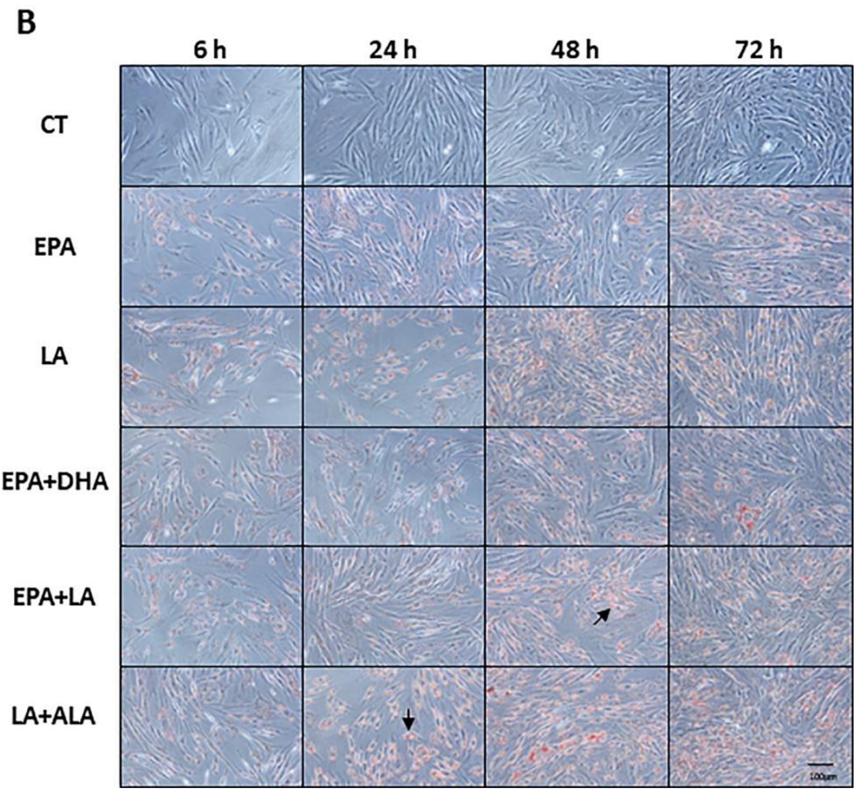
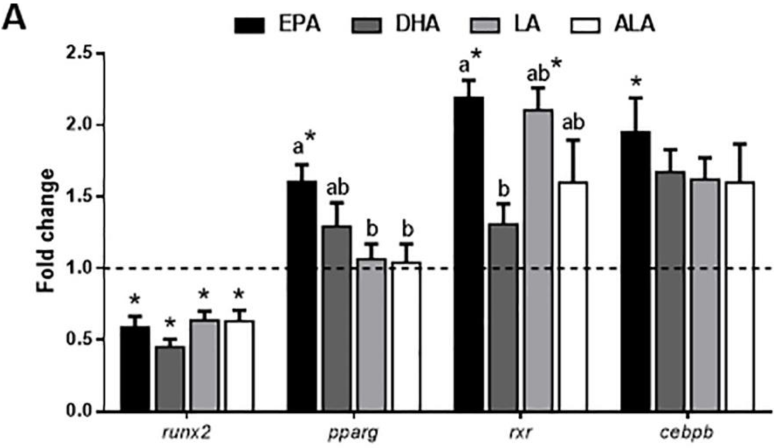




**Fig 2. Time course of lipid accumulation by fatty acids.** (A) Quantification of lipid content normalized by protein and (B) representative phase-contrast images of gilthead sea bream bone-derived cells stained with Oil red O. Cells were treated at day 4 with selected individual or combined fatty acids (200 μM), or were left untreated as control (CT, dashed line in A) for 6, 24, 48 and 72 h. In (A) data are shown as mean + SEM (n=3-4). Significant differences ($p<0.05$) among time-points

are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. In (B) magnification 20x, arrows indicate lipid droplets. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

## Fatty acids effects in gene expression

The gene expression of *runx2*, the key transcription factor of the osteogenic process, was significantly down-regulated by all fatty acids, either applied individually or combined, compared to the control condition, although differences were not observed among treatments (Figs 3A and 3B). Contrarily, the principal genes involved in the first steps of adipogenesis, were up-regulated after EPA treatment, significantly for *pparg*, *cebpb* and *rxr,* and the latter also by LA treatment, when compared to the control. In addition, EPA-treated cells showed significant differences with respect to those treated with LA or ALA for *pparg* gene expression and with DHA as well in the case of *rxr* (Fig 3A). Furthermore, the different combinations caused patterns of expression for these genes according to the fatty acids included in the mixture (Fig 3B). Namely, the combinations containing one (i.e. DHA) or specially the two fatty acids present in fish oils, significantly up-regulated the transcript levels of the adipogenic genes *pparg* and *cebpb,* compared to the control condition and the combination of LA+ALA. In addition, the mRNA levels of *rxr* and *cebpb* were significantly lower in response to the combinations containing LA (especially in the one of LA+ALA), respect to the EPA+DHA mixture (Fig 3B).



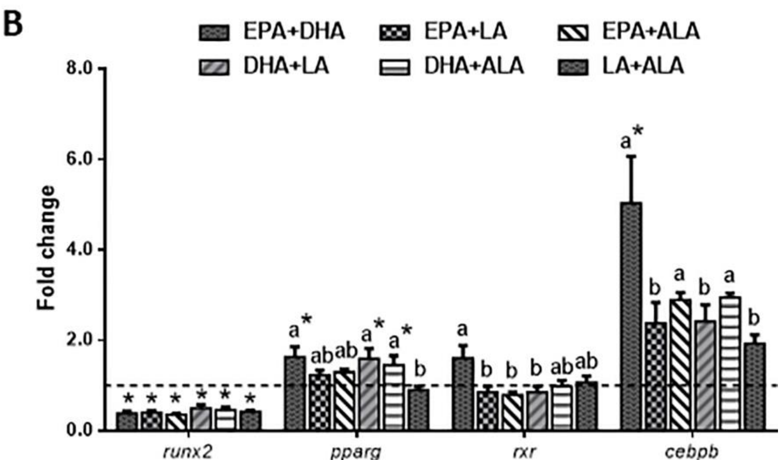
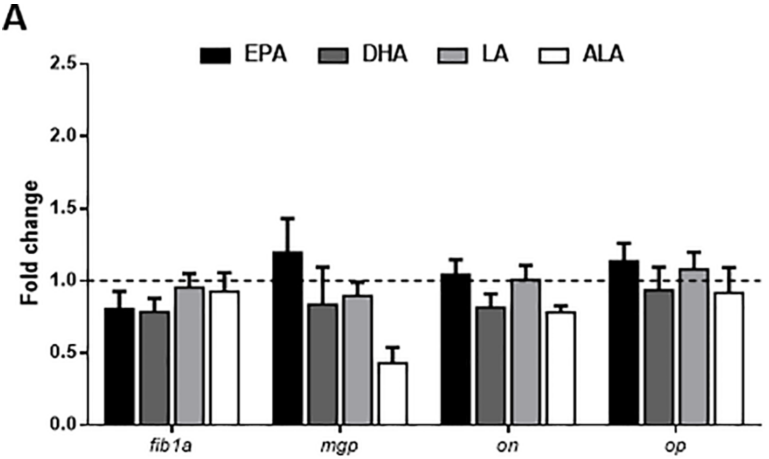




**Fig 3. Fatty acids effects on transcription factors gene expression.** Relative expression of genes related to the processes of osteogenesis (*runx2*) and adipogenesis (*pparg, rxr* and *cebpb*) normalized to *b-actin* and *rps18* in gilthead sea bream bone-derived cells. Cells at day 4 were treated with different (A) individual or (B) combined fatty acids or were left untreated as control (dashed lines) for 6 h. Data are shown as mean + SEM (n=3-4). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

The expression analysis of osteogenic genes involved in ECM formation and/or mineralization showed how these remained unaltered in cells in the presence of fatty acids either applied alone or in combination. Nevertheless, the treatments with two fatty acids combined caused *on* and *op* to have a lower, but not significant expression, compared to the control (Figs 4A and 4B). Moreover, genes encoding lipid metabolism-related enzymes and fatty acid transporters were studied to unravel whether a possible regulation of pro-adipogenic genes was related to the process of differentiation of MSCs into adipocyte-like cells. EPA treatment caused an increase in *hsl* mRNA levels, although only significant when compared to DHA-treated cells, while *fas* and *lpl* remained stable (Fig 5A). Concerning the fatty acid transporters, EPA, LA and ALA significantly up-regulated the mRNA levels of *fabp11* compared to the control (Fig 5B). Even applying combinations of the different fatty acids to the cells, the gene expression of *fas*, *lpl*, *hsl* and *cd36* remained unaffected (Figs 5C and 5D). Nevertheless, the combination of the two fatty acids more common in vegetable oils (LA+ALA), significantly up-regulated the transcript expression of *fabp11* in comparison to the combination with the fatty acids EPA+DHA and the control condition. On the other hand, *fatp1* levels were significantly higher in response to LA+ALA when compared to the combinations containing EPA and either one of these two fatty acids from vegetable oils, but not with the control (Fig 5D).



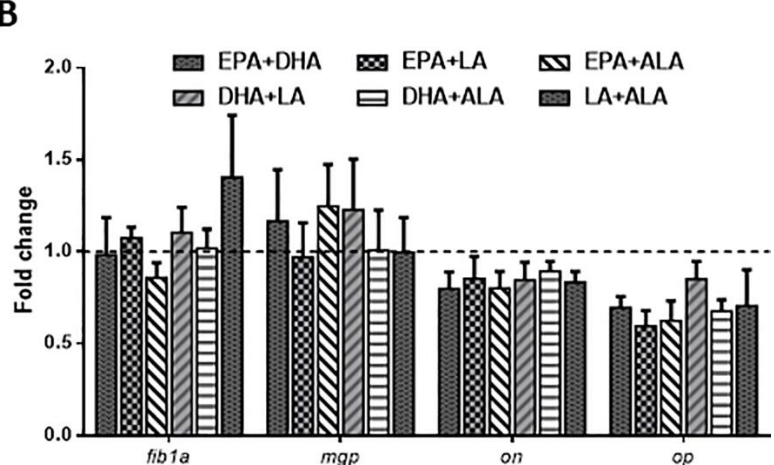



**Fig 4. Fatty acids effects on osteogenic genes expression**. Relative expression of genes related to the process of osteogenesis (*fib1a*, *mgp, on* and *op*) normalized to *b-actin* and *rps18* in gilthead sea bream bone-derived cells. Cells at day 4 were treated with different (A) individual or (B) combined fatty acids or were left untreated as control (dashed lines) for 6 h. Data are shown as mean + SEM (n=3-4). Significant differences (p<0.05) among treatments are indicated by different letters. Asterisks indicate significant differences (p<0.05) with the control. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

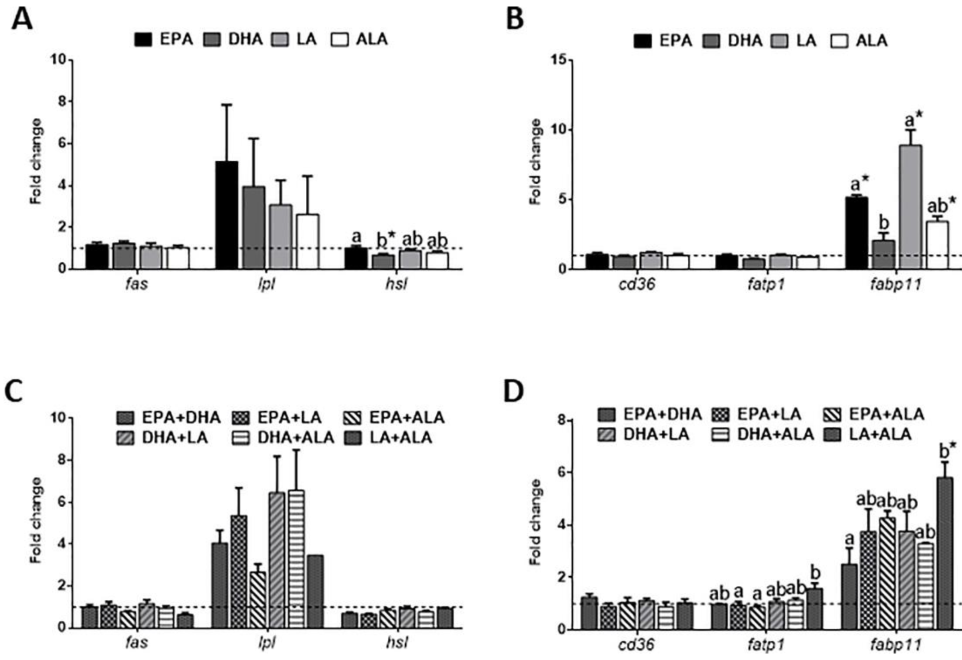
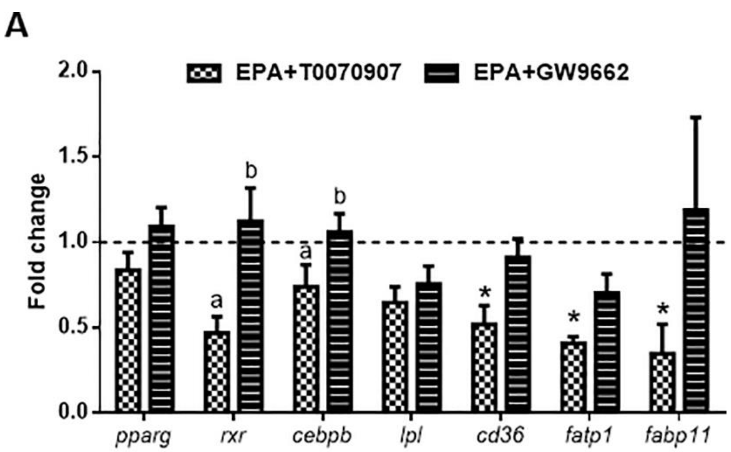



**Fig 5. Fatty acids effects on adipogenic genes expression**. Relative expression of genes related to lipid metabolism, including (A, C) enzymes (fas, lpl and hsl) and (B, D) fatty acid transporters (cd36,

fatp1 and fabp11) normalized to b-actin and rps18 in gilthead sea bream bone-derived cells. Cells at day 4 were incubated with different (A, B) individual or (C, D) combined fatty acids, or were left untreated as control (dashed lines) for 6 h. Data are shown as mean + SEM (n=3-4). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

## Effects of PPARγ antagonists in gene expression

Two different antagonists of PPARγ were applied to cells treated with either EPA or LA, to elucidate the potential different mechanism of action of these fatty acids inducing the differentiation of the bone-derived MSCs into adipocyte-like cells. First, viability assays were performed to assure non-toxicity of the products (S1 Fig). Next, taking into account that the transcriptional effects of each fatty acid alone in comparison to the control were already reported, the condition of each fatty acid in the absence of antagonists was used as the corresponding control in this set of experiments. Cells treated with EPA and T0070907 showed an overall decrease in expression for the genes studied, which was significant in comparison to the treatment with the other antagonist, GW9662 for *rxr* and *cebpb* and, to the control condition for the fatty acids transporters *cd36*, *fatp1* and *fabp11* (Fig 6A). Contrarily, the cells treated with LA and either one of the two antagonists, did not show any significant changes in gene expression (Fig 6B).



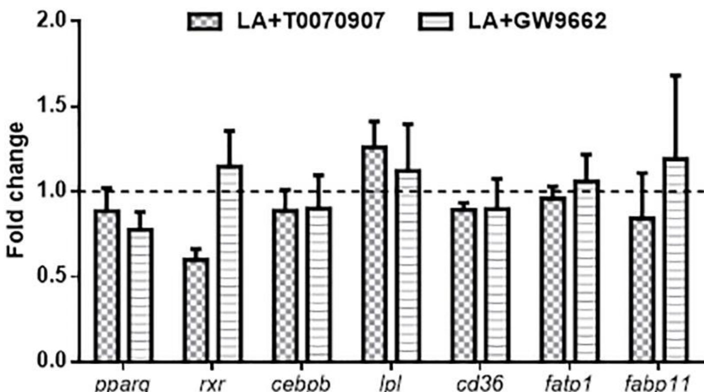



**Fig 6. PPARγ antagonists effects in adipogenic genes expression.** Relative expression of genes related to adipogenesis and lipid metabolism (pparg, rxr, cebpb, lpl, cd36, fatp1 and fabp11) normalized to b-actin and rps18 in gilthead sea bream bone-derived cells. Cells at day 4 were incubated with the fatty acids (A) EPA or (B) LA in the absence (dashed line, used as control) or presence of a PPARγ antagonist (T0070907 or GW9662) for 6 h. Data are shown as mean + SEM (n=3-4). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks show significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; LA: linoleic acid.

## Discussion

This study has focused on the characterization of the likely differential effects of fatty acids typical from fish oil (EPA and DHA) and those most commonly found in vegetable oils (LA and ALA) on cellular plasticity and metabolism. To this end, we used as a model an *in vitro* culture of MSCs derived from vertebra bone of gilthead sea bream (*S. aurata*), one of the most cultivated species in Mediterranean aquaculture. The main objective was to evaluate the lineage-induction potential over the MSCs of these fatty acids, not only for its possible relevance in fish nutrition and welfare, but also to validate the cell system to further study the multipotentiality of piscine MSCs and their regulation.

The first step was to check that the incubation with the fatty acids was not causing any deleterious effect on the cells. Thus, an MTT assay was run showing that none

of the fatty acids significantly affected bone-derived MSCs viability at concentrations up to 200 µM, although a slight rise in viability could be seen with the 100 µM concentrations, maybe related to the increased metabolic activity of the cells along the induced process of differentiation. These results coincide with those in another study, where fatty acid treatments were performed on human MSCs grown in osteogenic media, to see their possible effect on osteoblastogenesis [44]. Also, cell viability was verified after applying different fatty acids (e.g. EPA, DHA and arachidonic) at a 100 µM concentration on the skeletal VSa16 cell line of gilthead sea bream, demonstrating that such treatments can stimulate proliferation without signs of toxic effects [45].

Bone marrow MSCs in mammals retain a high degree of plasticity and their fate is affected by cell culture medium composition [23]. In the present study, accumulation of lipid content induced by fatty acids added to the media was dose-dependent as confirmed through quantification and microscopy observation of the morphological changes, and the intensification of the red aura occupied in the cells by ORO staining. Previously, the ability of MSCs derived from bone to differentiate, in addition to osteoblasts, into adipocyte-like cells was demonstrated, by applying adipogenic media containing two different concentrations of lipid mixture; thus, confirming their multipotentiality [35]. The presence of fatty acids in such specific media seems to be critical to shoot the adipogenic process in undetermined fish cells [37], [46], [47], [48], and this has also been observed in avian adipocyte precursor cells [49]. Accordingly, in the present study, the cells began to accumulate lipids potentially inducing adipocyte-like differentiation in response to the fatty acid treatments. However, LA and the combinations containing this fatty acid were those that produced a greater effect, whereas the combination of the two fatty acids mainly present in fish oil (EPA+DHA) caused lower lipid deposition. Similarly, in mature salmon adipocytes it was observed that fatty acids from vegetable oils (i.e. oleic acid) were able to induce more triacylglycerol accumulation than fatty acids characteristic of fish oils [50]. Other studies have also shown this lower capacity of fatty acids from fish oils to be stored

in adipose cells, such as those performed on 3T3-L1 pre-adipocytes, where DHA reduced dose-dependently fat deposition likely by suppressing lipid filling [51]. Overall, the data suggest that the n-6 PUFA LA may stimulate the uptake and depot of extracellular fats in these adipocyte-like cells, more than the other treatments tested.

Next, to further evaluate the effects of fatty acids on MSCs lineage determination, expression of relevant driving genes was analyzed. *runx2* codifies for a key transcriptional activator that promotes osteoblastogenesis thus, inhibiting the determination and subsequent differentiation of MSCs into other cell lineages [24]. On the other hand, PPARγ, a member of the hormone nuclear receptors family, after interaction with specific ligands such as LC-PUFA, activates the transcription of genes involved in adipogenesis and lipid metabolism determining the adipocyte phenotype of MSCs [25]. Moreover, induction of *pparg* expression can result in the inhibition of differentiation toward osteoblasts, as it has been described in some mammalian studies by acting as a suppressor of *runx2* [23], [52], [53]. Besides, overexpression of *runx2* in rat MSCs derived from adipose tissue produces a decrease in the expression of *pparg* [54]; consequently, these two transcription factors seem to act by negatively regulating each other. In Atlantic salmon, *pparg* is also silenced when the culture medium used is osteogenic, while *runx2* has its expression inhibited in the presence of an adipogenic medium [39]. In agreement with these observations, in our study, all fatty acid treatments, either alone or in combination, down-regulated *runx2*, although only EPA was able to significantly increase *pparg* gene expression. In fact, when treating the gilthead sea bream osteoblast-like VSa16 cell line with EPA, a decrease in the mRNA levels of *runx2* was also reported [45]. Accordingly, an increase in the transcript levels of *pparg* was also caused in human MSCs due to EPA treatment [44]. Despite significant differences were not observed with DHA alone, up-regulation of this key adipogenic gene was found with all combinations containing this fatty acid. Interestingly, both EPA and DHA had been considered for years, natural ligands of *pparg*, and to have greater potency on activating this transcription factor,

compared to the n-6 PUFA (i.e. LA) [55]; so, these findings propose a direct effect of these n-3 LC-PUFA stimulating adipogenesis, as previously described in mammalian models [25]. Furthermore, *cebpb* expression was also up-regulated in response to EPA and by the combination EPA+DHA, supporting that the initiation of the adipogenic process is taking place upon those treatments. In fact, at least in mammals, *cebpb* contributes to stimulate *pparg* expression during early adipogenesis [56], [57]. Moreover, similar results were found when the gene expression of *rxr* was analyzed, since EPA, but also LA, could significantly up-regulate it. The nuclear receptor RXR forms a heterodimer among others with PPARγ, to regulate the transcription of genes related to lipid metabolism, thus also driving adipocyte differentiation [58]. Nevertheless, knowledge on the PPARγ-RXR heterodimers, as well as their response to fatty acids in fish is very limited.

To further evaluate if the bone-derived MSCs are deviated from the osteogenic process when the fatty acid treatments are applied, the expression of various genes related to both early osteogenesis and late mineralization of bone ECM was determined [36]. The mRNA levels of *fib1a*, *mgp*, *on*, and *op* remained constant in response to all the treatments, similarly as in [38], in the same cell model after addition of a standard adipogenic medium. With these results, we could suggest that the osteogenic process, to which these cells were previously predestined in their tissue microenvironment, has stopped. This deregulation of MSCs determination and/or trans-differentiation has been related not only in mammals, but also in fish, with developmental disorders or disease states, such as distraction osteogenesis in rats [59], bone loss in osteoporotic human patients [60] and reduced or malformed vertebrae in Atlantic salmon [61], [62], [63], [64], in which, diet specifically, has been shown as a causative factor [65].

Regarding expression of adipocyte markers, specifically genes that codify for key enzymes such as *fas,* involved in the synthesis of fatty acids, remained stable, maybe due to a direct inhibition of *de novo* synthesis caused by the addition of fatty acids into the culture medium. Similarly, differences could not be found in *lpl* or *hsl* gene expression during differentiation into adipocytes of rainbow trout

cultured pre-adipocytes when the whole transcriptional profile of this process was analyzed [66]. Nevertheless, increased gene expression in the late phases of adipocyte differentiation has been reported for *lpl* in red sea bream [47] and Atlantic salmon [50].

Concerning the genes involved in the uptake and transport of fatty acids, *cd36* is, among others, a target gene of PPARγ [25]. In this context, PPARγ activation was found to induce *cd36* expression and adipocyte differentiation of the arterial rat VSMCs line [67]. In our study, the increased *pparg* mRNA levels at 6 h incubation in response to EPA, but not in *cd36*, suggest a possible delayed up-regulation of the latter. Furthermore, in Atlantic salmon and rainbow trout, the mRNA levels of *fatp1* increased during the differentiation of pre-adipocytes, at earlier stages than *fabp11*, indicating that the former has an important role in the induction of adipogenesis and in the uptake of fatty acids from the environment [27], [68]. In our study, the combination of LA+ALA or LA alone caused an increase in *fatp1* and *fabp11* mRNA levels compared to other combinations. Altogether, these results indicated a more direct stimulation of fat transporters expression by fatty acids of vegetal origin, especially LA, suggesting a possible mechanism of action to induce adipogenesis through enhancing fatty acid uptake. These observations together with the elevated capacity of lipid accumulation when cells were treated with LA are in agreement with a previous study in gilthead sea bream, in which diets with high content in vegetable oils induced adipocyte hypertrophy [12]. On the other hand, DHA and more remarkably EPA, showed a higher potential to stimulate adipogenesis via up-regulation of *pparg* gene expression, thus inducing the adipocyte-like phenotype but with lower lipid content. Hence, since smaller adipocytes have the metabolic advantage of retaining insulin sensitivity and protect other tissues from lipotoxicity according to works in mammals [69], further studies would be of great interest to demonstrate if fish oil-derived fatty acids would lead to healthier cells as well in fish.

To corroborate the differential action of EPA and LA driving adipocyte-like development of bone-derived MSCs through PPARγ activation, their effects in

combination with two PPARγ antagonists were evaluated. According to the results, LA action was unaffected, demonstrating the direct effect of this fatty acid on the transport of lipids due to *fabp11* and *fatp1* up-regulated gene expression. With regards to EPA treatment, the antagonist GW9662 did not cause any change, but the use of the specific antagonist T0070907, triggered a remarkable down-regulation on transcript levels of the three fatty acid transporters and the factors *rxr* and *cebpb*, although not of *pparg*. Accordingly, in mammals GW9662 shows negligible effects on transcription compared to T0070907, which displays properties of an inverse agonist, showing effects on transcription opposite to well-known PPARγ agonists such as [40], [41]. Similarly, the antiobesogenic effect of these antagonists has been shown in zebrafish larvae *in vivo*, although without performing transcriptomic analyses [42], [70]. Overall, the current data would suggest an inhibition of the adipogenic process, including lipid internalization, mediated at least in part by the incapability of EPA to activate PPARγ and the companion transcription factors. In agreement with this hypothesis, other authors using this same antagonist showed a decrease in *cd36* mRNA levels not depending on the increased gene expression of *pparg*, but elevated PPARγ activity [71]. Thus, blockage of EPA action by T00709007 indicates that via the action of this transcription factor, EPA may also up-regulate fat transporters to ultimately stimulate adipocyte differentiation of MSCs. These results agree with the capacity of n-3 LC-PUFA to promote the formation of healthy new adipocytes [72]. An example of a similar scenario may be the antidiabetic treatment with the full PPARγ agonists, the thiazolidinediones (i.e. troglitazone or pioglitazone), which have been shown, at least in rodent models, to favor remodeling of the adipose tissue by promoting pre-adipocyte recruitment for hyperplastic growth [73], [74].

## Conclusions

Gilthead sea bream bone-derived MSCs treated with one or two combined fatty acids undergo morphological and transcriptional changes, increasing lipid accumulation as well as the expression of adipogenic genes while decreasing or maintaining stable those related to the osteogenic process. This confirms the

plasticity of these cells and supports their use as a model to study MSCs fate modulation. Besides, these findings should be also considered when studying fish bone structure and function, since at least in humans, there is a correlation between the appearance of bone marrow fat and the reduced bone forming capacity observed during diabetes and aging [75], [76]. Our data also suggest that fatty acids might be inducing adipogenesis potentially through different pathways, with fish oil-derived fatty acids such as EPA causing mainly formation of new adipocytes through activation of PPARγ, whereas vegetable fatty acids like LA appear to rather induce a process of fat accumulation in committed pre-adipocytes (Fig 7). These results advise that fatty acids from plant origin should be wisely used in aquafeeds, as they could induce the formation of less sensitive and functional hypertrophic adipocytes as previously suggested [12]. While we should be cautious because most of our data is based on a transcriptional level, and further studies are required to validate these observations; overall, this needs to be considered in feeds formulation to carefully find a balance according to the nature of the oil sources to ensure a healthy and high-quality fish.

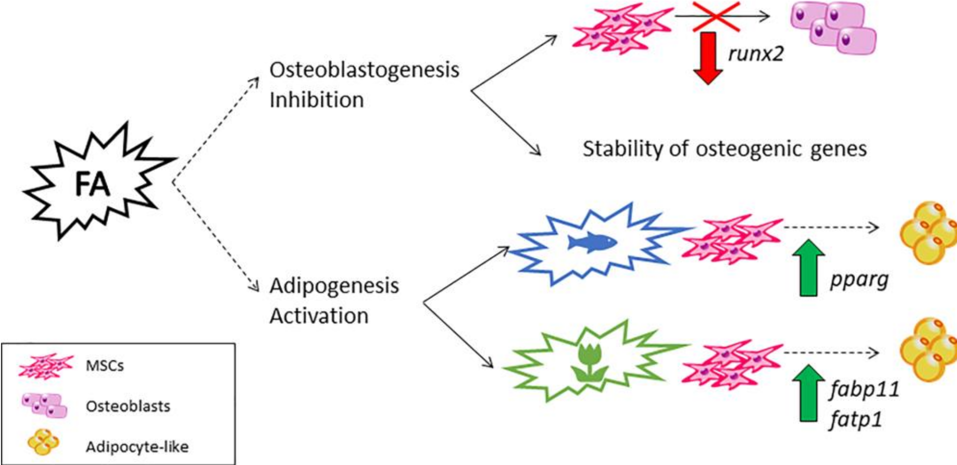



**Fig 7. Summary of fatty acids effects on gilthead sea bream bone-derived cells.** Schematic representation summarizing the effects of fatty acid (FA) treatments over bone-derived mesenchymal stem cells (MSCs) of gilthead sea bream. The fatty acids produce an inhibition of the osteogenic process through causing a down-regulation of runx2 and a stabilization of the osteogenic genes relative expression. On the other hand, fatty acids induce adipogenesis, with those fatty acids

characteristic from fish oils apparently via up-regulating pparg mRNA levels and in contrast, those typical from vegetable oils increasing the relative gene expression of fatty acid transporters (fabp11 and fatp1), thus potentially enhancing cell lipid accumulation.

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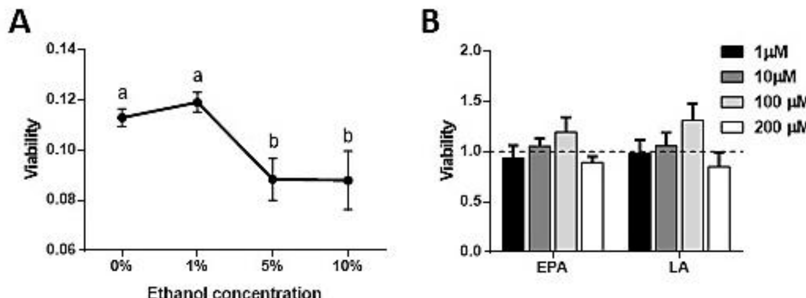
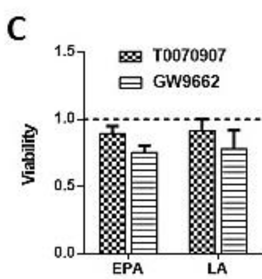
## Supporting information

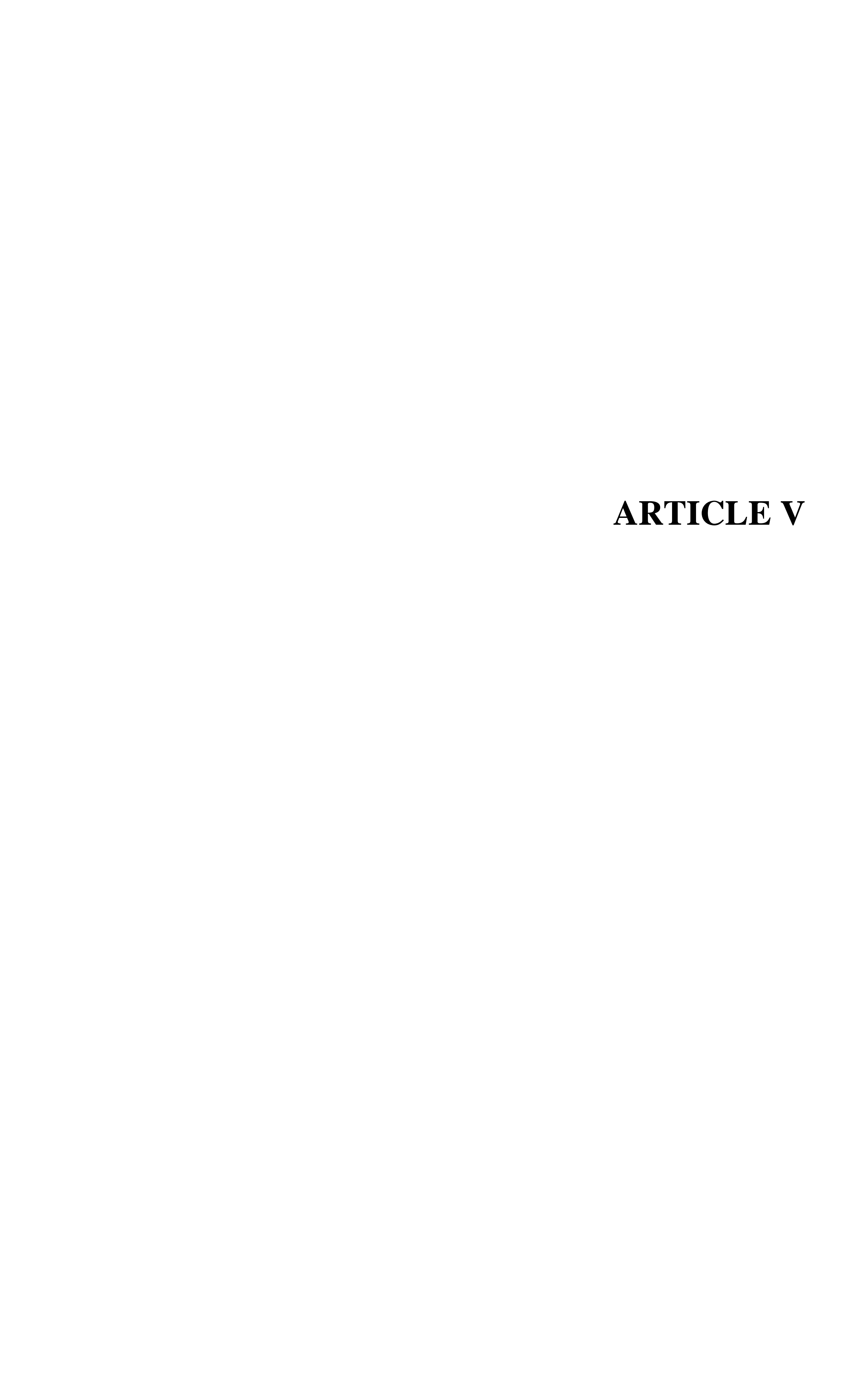
Fig. Supplementary 1




**S1 Fig. Effects of ethanol, fatty acids and antagonists treatments on cell viability.** Viability of gilthead sea bream bone-derived cells at day 4 determined by means of the MTT assay. Cells were treated (**A**) for 24 h with different concentrations of ethanol; or for 6 h (**B**) with different concentrations of selected fatty acids (EPA and LA) or were left untreated as control (dashed line), and (**C**) with the fatty acids EPA or LA in the absence (dashed line) or the presence of a PPARγ antagonist (T0070907 or GW9662). Data are shown as mean + SEM (n=3). Significant differences ($p<0.05$) among concentrations are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the corresponding control. EPA: eicosapentaenoic acid; LA: linoleic acid.

**Submit R1.zip contains the files with the complete raw data.**



# ARTICLE V

**Short-term responses to fatty acids on adipogenesis *in vitro* and lipid metabolism *in vivo* in rainbow trout (*Oncorhynchus mykiss*).**

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**Abstract**

Fish products are distinguished for being a rich source of omega 3 highly unsaturated fatty acids (HUFA) such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. The search for new sustainable oils to produce fish feeds has changed the fatty acid composition of the diets, lowering the proportion of HUFA; thus, not only influencing lipid metabolism but also affecting directly in the most valuable property of fish. In this study, rainbow trout cultured pre-adipocytes were treated with representative fatty acids commonly found in fish oils (EPA and DHA) or in vegetable oils (linoleic, LA and alpha-linolenic, ALA acids); and then EPA and LA were administered orally, to evaluate their effects on adipogenesis and adipose and liver tissues lipid metabolism. In the *in vitro* experiments, fatty acids increased lipid internalization, with ALA producing the highest effect; and the observed gene expression pattern of the transcription factors, enzymes and lipid transporters analysed suggested the activation of adipocyte metabolic turnover. Regarding the *in vivo* trial, in the adipose tissue, increased production of energy by β-oxidation could be suggested in response to the fatty acids because of the upregulated expression of peroxisome proliferator-activated receptors *ppara* and *pparb*, contrary to the results obtained in liver. Furthermore, increased lipogenesis *de novo* and lipid internalization were observed in adipose tissue with the increased expression of fatty acid synthase (*fas*) and the cluster of differentiation 36 (*cd36*) respectively, whereas in liver a decrease in lipogenesis and a stable expression of genes involved in lipid transport was found. Thus, the fish did not experience major differences depending on the fatty acid type, showing a similar metabolic response in front of a short-term fatty acid high disposal.

**Keywords:** eicosapentaenoic acid, linoleic acid, adipocyte, adipose tissue, liver.

## 1. Introduction

World population together with fish and seafood consumption have increased during the last years and will continue to rise in the future. Fish products are distinguished for their high content in polyunsaturated fatty acids (PUFA) and particularly, fish are attractive for being a rich source of omega 3 highly unsaturated fatty acids (HUFA) such as eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids (Pike & Jackson, 2010; Tocher, 2015). In aquaculture, the maintenance of these properties is crucial, and it can be achieved by providing quality diets assuring the necessary nutrients (Glencross, Booth & Allan, 2007; Rønnestad et al, 2013; Tocher, 2010). These diets are generally based on fish meal and oil obtained from small pelagic species; therefore, a relative low value protein is converted into a protein with a high value for human consumption (Bostock et al, 2010), but contrarily, this could be leading to an unsustainable cycle. Faced with this handicap, the aquaculture industry has inverted resources to find alternatives to achieve these dietary goals. The most economic and sustainable alternatives are meant to be oils and meals from plant origin. Nonetheless, the use of vegetable oils (VO) has some inconvenient such as their fatty acids (FA) profile differs from fish oils (FO), thus inducing metabolic alterations (Torstensen, Lie & Frøyland, 2000) and changes in fillet composition (Benedito-Palos et al, 2008, 2009, 2010; Sprague, Dick & Tocher, 2016).

In aquaculture production of rainbow trout (*Oncorhynchus mykiss*), a salmonid species with a valuable tasty meal, the feed is based mostly in fishmeal and FO, but the proportion of the former has been reduced in the last years with alternatives as soya flour (Cowx, 2005). Regarding the oils, soya oil has substituted almost 50% of the lipid portion; and mixtures of palm, rapeseed or linseed oils are currently used in commercial diets (FAO, 2018). VO are rich in PUFA n-6 and n-9, principally linoleic acid (LA; 18:2 n-6) and oleic acid (OA; 18:1 n-9) except for linseed oil, which is rich in α-linolenic acid (ALA; 18:3 n-3) but otherwise, they contain no n-3 HUFA. In marine species there is no transformation of typical FA from VO sources to HUFA as arachidonic acid (AA; 20:4 n-6), EPA or DHA (n-

3) due to the total non-functionality of the responsible enzymes of this pathway (Turchini, Torstensen & Ng, 2009). Contrarily, freshwater species and salmonids can perform these transformations to a certain degree, but not to ensure enough quantity of such HUFA to maintain the requirements of the species (Tocher, 2015).

Furthermore, dietary VO in excess can cause adipose tissue and hepatic metabolic alterations in fish (Bouraoui et al, 2011; Cruz-Garcia et al, 2011). Adipose tissue, as a metabolic organ, has an important role in the regulation of the organism's energy homeostasis (Choe et al, 2016). Adipocytes are the main cells of the adipose tissue that includes as well preadipocytes, endothelial cells, fibroblasts and other cell types (Kuda, Rossmeisl & Kopecky, 2018). Adipose tissue growth is reached by either hypertrophy or hyperplasia, through lipid accumulation that provokes an increase in cell volume or by formation of new adipocytes from precursor cells, respectively (Salmerón, 2018). Adipogenesis is the process by which mesenchymal cells are differentiated into mature adipocytes. During the first phase of adipogenesis transcription factors such as CCAAT/enhancer binding protein α (C/EBPα) and β (C/EBPβ) and peroxisome proliferator-activated receptor γ (PPARγ) and α (PPARα) are activated (Chen et al, 2016). Some of these transcription factors in turn activate specific genes related with lipid metabolism (fatty acid synthase, *fas* and hormone sensitive lipase, *hsl*) and transport, including fatty acid binding proteins, transporters or translocases (e.g. *fabp11*, *fatp1* or *cd36*, respectively), this way finishing the adipocyte maturation phase (Salmerón, 2018). This differentiation process is susceptible to be affected by the presence of FA by modifying the expression of the key transcription factors involved, as it has been observed in rodents, being EPA and DHA natural ligands activating PPARγ (Madsen Petersen & Kristiansen, 2005). The presence of FA also induces the differentiation of fish preadipocytes in culture (Cheng & Chen, 2015; Riera-Heredia et al, 2019; Todorčević et al, 2008). Lipid storage during adipogenesis can be specifically modified as showed in an *in vitro* study in cultured salmonid preadipocytes, where an increase in lipid droplets (LD) content by oleic acid, characteristic of VO, in comparison with the capacity of the FA typical of FO, EPA

and DHA was described (Todorčević et al, 2008). Furthermore, FO replacement by VO in gilthead sea bream and Atlantic salmon *in vivo* also alters adipose tissue growth enhancing hypertrophy instead of hyperplasia with the consequent dysregulation of lipid metabolism and causing an excess of fat deposition lowering the quality of the product (Cruz-Garcia et al, 2011; Torstensen et al, 2011).

These altered traits in adiposity are often accompanied by the presence of a fatty liver as seen in gilthead sea bream fed with a diet highly substituted by VO (Cruz-Garcia et al, 2011). The liver is known to be also an organ of fat accumulation but is responsible for the distribution of lipids to peripheral tissues thus, the connection between both liver and adipose tissue is tight. In this sense, the liver is an important organ for the evaluation of diets substitution due to its key role in fatty acid metabolism. For all that, expression of genes and proteins involved in adipogenesis, fat metabolism and lipid transport and accumulation from both adipose and liver tissues are considered indicators of fish metabolic status.

In order to study the potential consequences of dietary substitution by VOs on adipogenesis and the homeostasis of adipose tissue in fish, the present study aims first, to evaluate EPA, DHA, LA and ALA treatment in preadipocyte cells in terms of lipid accumulation and gene expression. Second, to analyse the effects of short-term oral administration of EPA and LA on plasma metabolites, and lipid metabolism-related genes and proteins expression in adipose and liver tissues of the rainbow trout.

## 2. Material and methods

### 2.1. *Animals and ethics statement*

Adult rainbow trout (*O. mykiss*) of approximately 250 g in weight were obtained from the fishery “Troutfactory S.L.” (Lleida, Spain) and were acclimated to the facilities in the Faculty of Biology at the University of Barcelona for two weeks. Fish were kept in 400 L fiberglass tanks under a 12 h light/12 h dark photoperiod and fed *ad libitum* twice daily with a commercial diet (Optiline-sf, Skretting, Burgos, Spain). Fish were fasted 24 h before experimental manipulations in order

to avoid contamination from the gastrointestinal tract during the adipose tissue extraction for cell culture and, to have the gastrointestinal tract empty during the gavage procedure. Before sacrifice by cranial concussion, fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS222, E10521, Sigma–Aldrich, Tres Cantos, Spain). All animal handling procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by Ethics and Animal Care Committee of the University of Barcelona, following the regulations and procedures established by the Spanish and Catalan governments (permit numbers OB35/17).

2.2. *Primary culture of adipocyte cells and FA treatment*

All cell culture reagents were purchased from Sigma–Aldrich (Tres Cantos, Spain) and Life Technologies (Alcobendas, Spain). All plastic materials were obtained from Nunc (LabClinics, Barcelona, Spain). Cells were isolated from rainbow trout adipose tissue and cultured according to the previously established procedure by Bouraoui, Gutiérrez and Navarro (2008) and were seeded at a density of $2 \times 10^4$ cells/cm$^2$. To perform the lipid quantification assay, the cells were seeded in 24 well plates and in 6 well plates for the gene expression analyses. The FA were applied in the same medium alone or in combination, once confluence was reached (day 7), and the duration of the treatments were of 6 h for gene expression analyses, and 48 h to quantify lipid accumulation. In all cases, two wells were used for each experimental condition. The FA (EPA, DHA, LA and ALA) were obtained from Cayman Chemical Company (Michigan, USA), were first dissolved in ethanol and used at a final concentration of 100 µM both, individually and in all tested combinations unless stated otherwise. A control condition without FA but vehicle (ethanol) was also performed. Final concentration of ethanol was below 1% to avoid causing negative effects in cell viability as tested in preliminary assays and in agreement with Riera-Heredia et al (2019) and Todorčević and Hodson (2015).

2.3. *Oil Red O staining*

To evaluate differentiation of precursor cells into mature adipocytes, intracellular neutral lipid accumulation was analysed by Oil red O (ORO) staining as explained in Salmerón et al (2013). Briefly, cells were fixed, rinsed in PBS, stained in ORO solution and lipid content was analysed by measuring absorbance at 490 nm (Infinite 200, Tecan, Switzerland). Afterwards, Comassie blue staining was performed and the dye extracted to determine protein levels. Thus, quantification of cell lipid content was calculated as the absorbance measured at 490 nm divided by the read at 630 nm corresponding to the protein content. Data are presented as fold change relative to the control (n=3-4). Pictures of the stained cells were obtained with a Zeiss Axiovert 40C inverted research grade microscope (Carl Zeiss Inc., Germany) equipped with a Canon EOS 1000D digital camera.

2.4. *In vivo FA treatment by gavage*

To evaluate the effect of selected FA by gavage, the following treatments: EPA, LA and control, were administered manually using 2 mL syringes coupled to a cannula large enough to reach the stomach as previously described (Librán-Pérez et al, 2013). The FA were obtained from Cayman Chemical Company (Michigan, USA) and were dissolved in ethanol as described in section 2.2. The administration dose was 20 mg of FA per kg of animal, force-feeding the fish with 1 mL of solution per 100 g. The stock solution was diluted with tank water (Librán-Pérez et al, 2012). Additionally, blue food coloring was added to the solution to confirm the correct administration. The solution for the control animals, without FA, contained the same ethanol concentration as the experimental solutions, tank water and food coloring.

After 6 h of the gavage procedure, fish were anesthetized with MS222 and blood was extracted from the caudal vein. Fish were sacrificed by cranial concussion, and samples of adipose tissue and liver were obtained, snap frozen in liquid nitrogen and stored at -80 °C.

2.5. *Plasma analyses*

Blood was centrifuged 10 min at 5000 rpm to extract plasma. Plasma samples (n=10) were analysed using commercial enzyme kits: glucose (Monlab, Barcelona, Spain), non-esterified fatty acids (NEFAs, Wako Chemicals GmbH, Neuss, Germany), and triglycerides and glycerol (Sigma-Aldrich, Tres Cantos, Spain).

2.6. *RNA extraction and cDNA synthesis*

In adipocyte cultures (n=6) the cells were lysed with a cell scraper and TRI Reagent (Applied Biosystems, Alcobendas, Spain) in a total volume of 1 mL per each two wells. For tissue samples, 100 mg of adipose tissue (n=9) and 50 mg of liver (n=8) were homogenated in 1 mL of TRI Reagent, using Precellys Evolution (Bertin Instruments, Montigny-le-Bretonneux, France). Total RNA was extracted according to the manufacturer's recommendations, dissolved in DEPC-treated water (RNase-free), quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and stored at −80°C. To eliminate any residual genomic DNA, total RNA (1 µg) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain), following the manufacturer's instructions.

2.7. *Quantitative PCR analyses*

Key genes implicated in adipogenesis and energy metabolism regulation were analysed by real-time quantitative PCR (qPCR) following the procedure previously described in Lutfi et al (2017). The genes evaluated comprise the following: The transcription factors or nuclear receptors*: cebpa, cebpb, ppara, pparb, pparg*, liver X receptor *(lxr)* and retinoid X receptor gamma (*rxr*); the enzymes: *fas*, lipoprotein lipase (*lpl*) and *hsl*; and lipid transporters: cluster of differentiation 36 (*cd36)*, fatty acid transport protein 1 (*fatp1*), fatty acid binding protein 11 (*fabp11*) and ATP-binding cassette transporter *(abca1*). Relative expression levels of the target genes were determined by the Pfaffl method (Pfaffl, 2001) using correction for primer efficiencies and normalizing the quantification

cycle (Cq) value of each gene registered during the annealing step to that of the most stable reference genes on each condition and determined using the CFX Manager Software (Bio-Rad). Reference genes for the *in vitro* experiment were elongation factor 1 alfa (*ef1a*) and ubiquitin (*ub*), for adipose tissue were beta-actin (*b-actin)* and *ef1a* and for liver were *18s* and *ef1a.*

**Table 1.** Primers used for real-time quantitative PCR. F, forward primer; R, reverse primer; Ta, annealing temperature; Acc. Num., GenBank accession number.

| Gene | Primer sequence (5'→3') | Ta (ºC) | Acc. Num. |
|---|---|---|---|
| *cebpa* | **F:** TGTGGCGATAAAGCAAGAGC | 57 | DQ423469.1 |
| | **R:** CTGGTGGGAATGGTGGTAGG | | |
| *cebpb* | **F:** CACAAAGTGCTGGAACTGGC | 60 | FR904306.1 |
| | **R:** TGGCACAGCGATAAATGGGT | | |
| *ppara* | **F:** CTGGAGCTGGATGACAGTGA | 54 | AY494835 |
| | **R:** GGCAAGTTTTTGCAGCAGAT | | |
| *pparb* | **F:** CTGGAGCTGGATGACAGTGA | 59 | AY356399.1 |
| | **R:** GTCAGCCATCTTGTTGAGCA | | |
| *pparg* | **F:** GCCAGTACTGTCGCTTTCAG | 60 | HM536192.1 |
| | **R:** TCCATAAACTCAGCCAGCAG | | |
| *lxr* | **F:** TGCAGCAGCCGTATGTGGA | 62 | NM_001159338 |
| | **R:** GCGGCGGGAGCTTCTTGTC | | |
| *rxr* | **F:** AAAGAGCGCAGTGAGAACGA | 55 | AJ969439.1 |
| | **R:** TGTAGGTCTCGGTCTTGGGT | | |
| *fas* | **F:** GAGACCTAGTGGAGGCTGTC | 54 | tcaa0001c.m.06_5.1.om.4 |
| | **R:** TCTTGTTGATGGTGAGCTGT | | |
| *lpl* | **F:** TAATTGGCTGCAGAAAACAC | 59 | AJ224693 |
| | **R:** CGTCAGCAAACTCAAAGGT | | |
| *hsl* | **F:** AGGGTCATGGTCATCGTCTC | 58 | TC172767 |
| | **R:** CTTGACGGAGGGACAGCTAC | | |
| *cd36* | **F:** CAAGTCAGCGACAAACCAGA | 62 | AY606034 |
| | **R:** ACTTCTGAGCCTCCACAGGA | | |
| *fatp1* | **F:** AGGAGAGAACGTCTCCACCA | 60 | CA373015 |
| | **R:** CGCATCACAGTCAAATGTCC | | |
| *fabp11* | **F:** CATTTGAGGAGACCACCGCT | 60 | NM_001124713.1 |
| | **R:** ACTTGAGTTTGGTGGTACGCT | | |
| *abca1* | **F:** CAGGAAAGACGAGCACCTTC | 58 | TC169876 |
| | **R:** TCTGCCACCTCACACACTTC | | |
| *18s* | **F:** GGCGCCCCCTCGATGCTCTTA | 65 | AF308735.1 |
| | **R:** CCCCCGGCCGTCCCTCTTAAT | | |
| *ef1a* | **F:** TCCTCTTGGTCGTTTCGCTG | 58 | AF498320 |
| | **R:** ACCCGAGGGACATCCTGTG | | |
| *b-actin* | **F:** ATCCTGACAGAGCGCGGTTACAGT | 61 | AJ438158 |
| | **R:** TGCCCATCTCCTGCTCAAAGTCAA | | |
| *ub* | **F:** ACAACATCCAGAAAGAGTCCAC | 58 | AB036060 |
| | **R:** AGGCGAGCGTAGCACTTG | | |

2.8. *Protein extraction and western blot analysis*

To perform protein extraction, 100 mg and 50 mg of adipose tissue and liver were used respectively (n=6). To homogenize the tissues 350 μL of RIPA (supplemented with proteases and phosphatases) were added to the samples and the Precellys Evolution coupled to a Cryolys cooling system (Bertin Instruments) was used. The supernatants were collected after a centrifuge of 30 min and stored at −80°C. Protein quantification was done by the Bradford method and, 12 μg of protein were subjected to electrophoresis (SDS-PAGE) on 15% polyacrylamide gels (125 V for 1 h 30 min). After overnight transfer to a PVDF-FL membrane, a staining with Revert™ Total Protein Stain (LI-COR, Inc., Biotechnology, Lincoln, USA) was performed to confirm that similar amounts of transferred proteins were on each lane. Subsequently, membranes were washed and blocked with Odyssey® Blocking Buffer (LI-COR, Inc., Biotechnology, Massatchussets, USA) and then incubated with the respective antibody: anti-CEBPα (#sc-61), anti-PPARγ (#sc-7196) and anti-LXR (#sc-1202) from Santa Cruz Biotechnology (California, USA) and anti-HSL (#4107S), anti-CD36 (#D8L9T), and anti-β-tubulin (#2146S) from Cell Signalling Technologies (Massatchussets, USA). After washing, the membranes were incubated with an IRDye secondary antibody (LI-COR, Inc., Biotechnology). The bands were visualised by infrared fluorescence using the Odyssey Imaging System (LI-COR, Inc. Biotechnology) and quantified using the Odyssey Infrared Imaging System software (version 1.2; Application Software). The signal band of each protein of interest was normalized to the signal of β-tubulin.

2.9. *Statistical analyses*

First, it was assessed data normality and homoscedasticity using Shapiro–Wilk and Levene's test, respectively. Comparison between two groups (each experimental treatment *versus* the control) was assessed by Independent samples' Student's t-test. For multiple mean comparisons (among FA treatments) of normal distributed data, one-way ANOVA was used followed by Tukey's or Dunnett's T3 *post hoc*

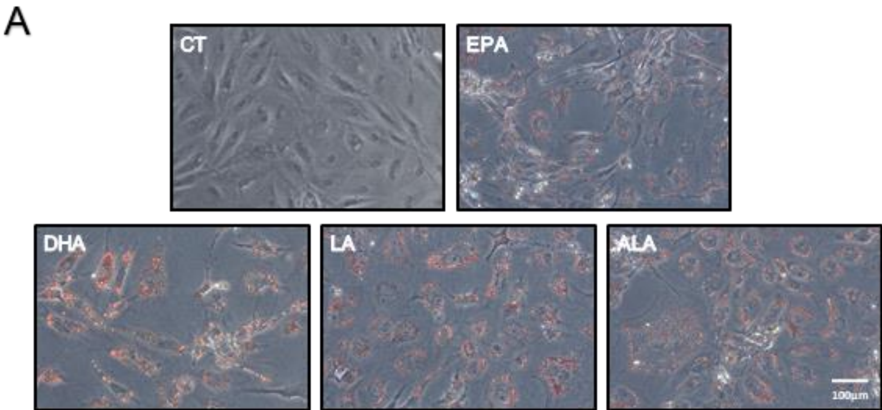
tests in case of homogeneous or heterogeneous variance data, respectively. When data did not fit normal distribution, the non-parametric Kruskal–Wallis test, followed by Mann–Whitney test, were used. Statistical analyses were performed using SPSS Statistics version 20 (IBM, Armonk, NY, USA). Results were presented as mean ± SEM. $p < 0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

## 3. Results

### 3.1. *Fatty acids effects in preadipocytes in vitro.*

The images obtained after ORO staining of the cells upon all treatments (EPA, DHA, LA or ALA 100 µM) showed a higher lipid accumulation than in control cells (Fig. 1A). In addition, we observed changes of cellular morphology in response to the treatments, becoming the cells more rounded with an enlarged cytoplasm while losing the fibroblastic shape, indicating the initiation of the differentiation process.

Lipid content analyses confirmed images observation, since cells treated with either one of the FA showed significantly higher intracellular lipid content compared to the control condition without FA (Fig. 1B). Among the treatments, ALA induced the highest accumulation of neutral lipids into the adipocytes, showing significant differences with EPA and LA, but not DHA.



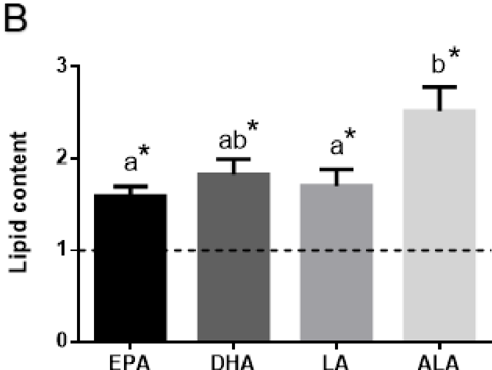



**Fig. 1. (A)** Representative phase-contrast images of rainbow trout preadipocyte cells after staining with Oil red O. Cells were treated at day 7 with different concentrations of individual fatty acids or were left untreated as control (dashed line in B) for 48 h and **(B)** quantification of lipid content. Data are shown as mean + SEM (n=3-4). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. CT: control; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

To further determine the effects of the FA on trout adipocytes, gene expression of the main genes involved in adipogenesis and lipid metabolism were analysed (Table 2A). The gene expression of *pparg* and *cebpa*, two key transcription factors of the adipogenic process, did not change with the treatments except after EPA incubation, which induced a significant downregulation compared to the control condition. Regarding other transcription factors and lipid metabolism-related genes, none presented significant differences either with respect to the control cells or among different FA treatments, except for the transporter *fatp1* that showed a significant increase in expression after incubation with the two FA typical of VO (LA and ALA) comparing with the control condition and DHA.

Moreover, the transcript levels of those genes were also studied in response to the presence of different FA combined in pairs (Table 2B). As in individual treatments, significant differences were not found for most of genes, except for *pparg*, *lxr* and *fatp1*. In this sense, *pparg* was significantly downregulated by all treatments compared to the control condition, as well as the combination of EPA and DHA caused a reduction in *lxr* expression. Finally, the combinations of LA with either

EPA or ALA showed a significant increase on *fatp1* expression when compared to the control cells and those with the combination of EPA plus DHA.

**Table 2.** Relative expression of transcription factors (cebpa, pparg and lxr), lipid metabolism-related genes (fas, lpl and hsl) and lipid transporters (cd36, fatp1, fabp11 and abca1) normalized to ef1a and ub in rainbow trout preadipocyte cells. Cells at day 7 were treated with different **(A)** individual or **(B)** combined fatty acids or were left untreated as control for 6 h. Data is expressed relative to control (value=1) and are shown as mean + SEM (n=4-6). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

A

| | EPA | DHA | LA | ALA |
|---|---|---|---|---|
| *cebpa* | 0,54 ± 0,144* | 0,63 ± 0,079 | 0,71 ± 0,077 | 0,73 ± 0,055 |
| *pparg* | 0,51 ± 0,109* | 0,66 ± 0,044 | 0,70 ± 0,128 | 0,81 ± 0,094 |
| *lxr* | 0,91 ± 0,112 | 0,96 ± 0,064 | 0,88 ± 0,068 | 0,95 ± 0,046 |
| *fas* | 0,69 ± 0,138 | 0,80 ± 0,109 | 0,74 ± 0,100 | 0,89 ± 0,107 |
| *lpl* | 0,97 ± 0,119 | 1,02 ± 0,066 | 0,98 ± 0,067 | 1,03 ± 0,059 |
| *hsl* | 1,12 ± 0,040 | 1,07 ± 0,059 | 0,94 ± 0,065 | 1,06 ± 0,051 |
| *cd36* | 0,70 ± 0,197 | 0,90 ± 0,091 | 0,83 ± 0,034 | 0,93 ± 0,067 |
| *fatp1* | 1,38 ± 0,158[ab] | 1,29 ± 0,029[b] | 1,64 ± 0,116*[a] | 1,78 ± 0,077*[a] |
| *fabp11a* | 1,27 ± 0,160 | 1,13 ± 0,155 | 1,14 ± 0,138 | 1,11 ± 0,106 |
| *abca1* | 0,85 ± 0,353 | 0,84 ± 0,057 | 0,80 ± 0,079 | 0,92 ± 0,093 |

B

| | EPA + DHA | EPA + LA | EPA + ALA | DHA + LA | DHA + ALA | LA + ALA |
|---|---|---|---|---|---|---|
| *cebpa* | 1,28 ± 0,208 | 0,78 ± 0,141 | 0,72 ± 0,091 | 0,80 ± 0,173 | 0,91 ± 0,079 | 0,84 ± 0,070 |
| *pparg* | 0,24 ± 0,027* | 0,31 ± 0,035* | 0,30 ± 0,036* | 0,41 ± 0,057* | 0,42 ± 0,046* | 0,42 ± 0,092* |
| *lxr* | 0,71 ± 0,040* | 1,06 ± 0,151 | 0,93 ± 0,126 | 0,94 ± 0,209 | 0,93 ± 0,078 | 0,87 ± 0,044 |
| *fas* | 1,23 ± 0,117 | 1,45 ± 0,200 | 1,29 ± 0,252 | 1,05 ± 0,113 | 1,28 ± 0,214 | 1,34 ± 0,222 |
| *lpl* | 0,95 ± 0,128 | 1,06 ±0,134 | 0,95 ± 0,106 | 1,09 ± 0,198 | 0,94 ± 0,118 | 0,79 ± 0,112 |
| *hsl* | 1,2 ± 0,159 | 1,21 ± 0,145 | 1,09 ± 0,116 | 1,13 ± 0,234 | 1,13 ± 0,051 | 1,02 ± 0,099 |
| *cd36* | 1,08 ± 0,051 | 1,46 ± 0,236 | 1,38 ± 0,197 | 1,02 ± 0,157 | 1,25 ± 0,122 | 1,33 ± 0,219 |
| *fatp1* | 1,04 ± 0,064[a] | 1,73 ± 0,178*[b] | 1,62 ± 0,160[ab] | 1,47 ± 0,169[ab] | 1,41 ± 0,105[ab] | 1,76 ± 0,180*[b] |
| *fabp11* | 1,02 ± 0,078 | 1,32 ± 0,152 | 1,09 ± 0,054 | 1,12 ± 0,176 | 1,05 ± 0,123 | 1,25 ± 0,129 |
| *abca1* | 1,32 ± 0,189 | 1,52 ± 0,296 | 1,25 ± 0,184 | 1,2 ± 0,337 | 1,1 ± 0,170 | 1,09 ± 0,203 |

3.2. *Fatty acids effects on plasma metabolites.*

Representative FA of the two oil sources fish or vegetables, EPA and LA respectively, were selected to study the effects *in vivo* in circulating metabolites and adipose tissue and liver gene expression of rainbow trout after the gavage procedure. Six hours after FA administration, plasma analyses showed significant

differences in glucose (Fig. 2A) and glycerol (Fig. 2C) but not in NEFAs (Fig. 2B) or triglycerides (Fig. 2D) levels. EPA treatment caused a diminution in glucose levels compared to the control condition, while LA increased the levels of glycerol when comparing to control and EPA treatment.

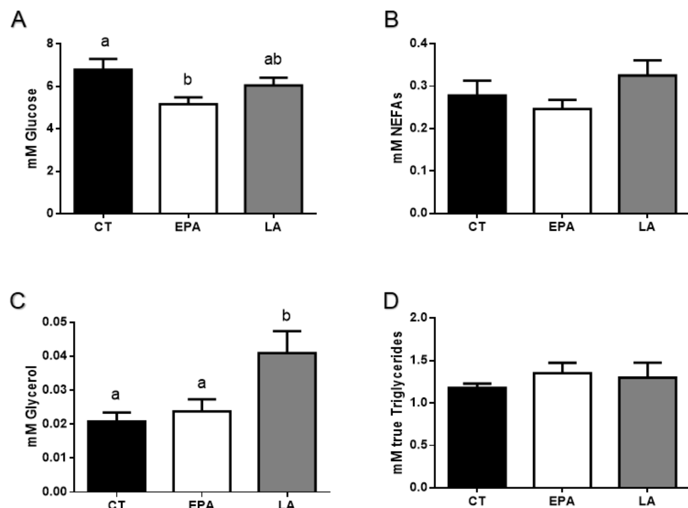



**Fig. 2.** Rainbow trout **(A)** Glucose, **(B)** NEFAs, **(C)** glycerol and **(D)** triglycerides plasma levels 6 h after the gavage with EPA, LA or vehicle without fatty acid as control. Data are shown as mean + SEM (n=10). Significant differences ($p<0.05$) among treatments are indicated by different letters. CT: control; EPA: eicosapentaenoic acid; LA: linoleic acid; NEFAs: non-esterified fatty acids; TGs: triglycerides.

### 3.3. *Fatty acids effects in adipose tissue.*

Gene expression of transcription factors, enzymes and FA transporters was analysed in adipose tissue. The transcription factors *ppara, pparb* and *rxr* were significantly upregulated by EPA and LA when comparing to the control, but differences between the two FA treatments were not found (Fig. 3A). Contrarily, expression of *cebpa, cebpb, pparg* and *lxr* was maintained very similar to the control. Regarding the lipid metabolism-related enzymes analysed, *fas* expression was increased in trout in response to the two FA, *hsl* was upregulated only by LA treatment, while *lpl* was contrarily downregulated but in this case after EPA treatment (Fig. 3B). Concerning FA transporters, *cd36* was significantly upregulated after the administration of both FA compared with the control

condition (Fig. 3C). Otherwise, *fabp11* showed a significant decrease with respect to the control, in the fish treated with EPA.

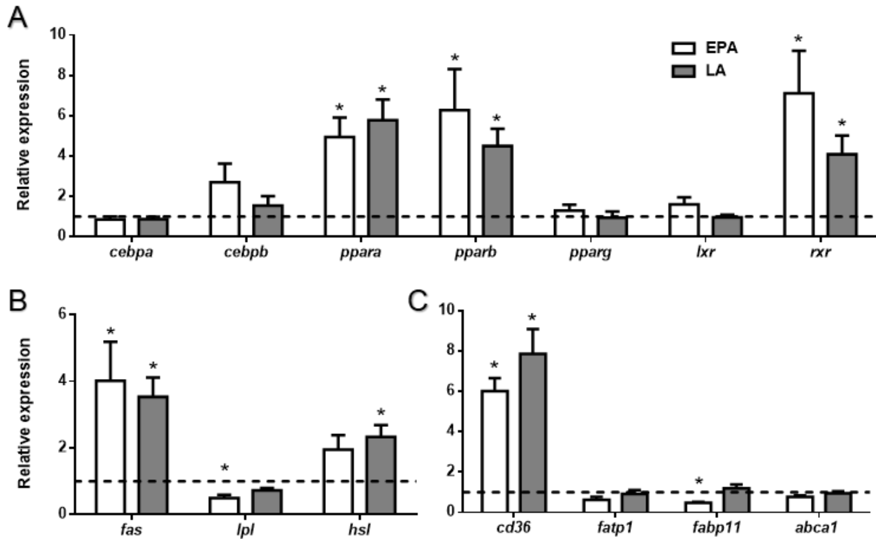



**Fig. 3.** Relative gene expression of **(A)** transcription factors, **(B)** lipid metabolism enzymes and **(C)** lipid transporters in adipose tissue from fish force-fed with EPA, LA or vehicle without fatty acid as control (dashed line). Data are shown as mean + SEM (n=4-8 for CT; n=7-8 for EPA; n=8-9 for LA). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; LA: linoleic acid.

Effects on proteins levels of representative members of each cluster of genes studied were also analysed (Fig. 4A). Differences were not found for the two transcription factors CEBPα (Fig. 4B) and PPARγ (Fig. 4C), neither for the enzyme HSL (Fig. 4D). Concerning the FA transporters, CD36 levels were increased in trout by LA treatment, but only significantly in comparison to the EPA-treated fish (Fig. 4E).

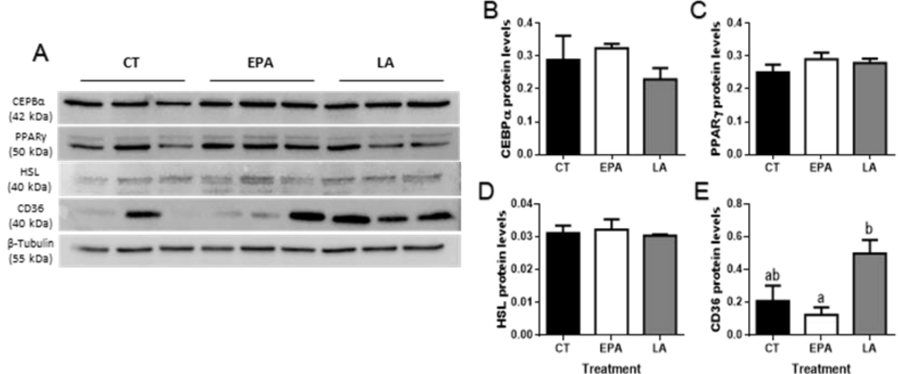



**Fig. 4.** Representative Western blots of the **(A)** transcription factors CEBPα and PPARγ, the enzyme HSL, the fatty acid transporter CD36 and β-tubulin in adipose tissue from fish force-fed with EPA, LA or vehicle without fatty acid as control. Quantification of protein levels of: **(B)** CEBPα, **(C)** PPARγ, **(D)** HSL and **(E)** CD36 normalized to β-tubulin. Data are shown as mean + SEM (n=6). Significant differences ($p<0.05$) among treatments are indicated by different letters. CT: control; EPA: eicosapentaenoic acid; LA: linoleic acid.

### 3.4. *Fatty acids effects in liver tissue.*

Expression of the same clusters of genes as in adipose tissue was analysed in liver. Regarding transcription factors, *ppara* was the only gene whose expression was significantly downregulated by both FA treatments. LA also significantly downregulated the relative expression of *pparb* and *lxr*, whereas EPA significantly diminished the expression of *cebpa* respect to the control and of *cebpb*, in this case also compared to LA treatment (Fig. 5A). Concerning the transcript levels of the enzymes related to lipid metabolism, *fas* and *hsl* were significantly downregulated in trout after the two FA treatments when compared to the control condition (Fig. 5B). Moreover, among the lipid transporters analysed, only *fatp1* expression was significantly reduced in fish after LA treatment while the rest of the genes remained unaltered (Fig. 5C).

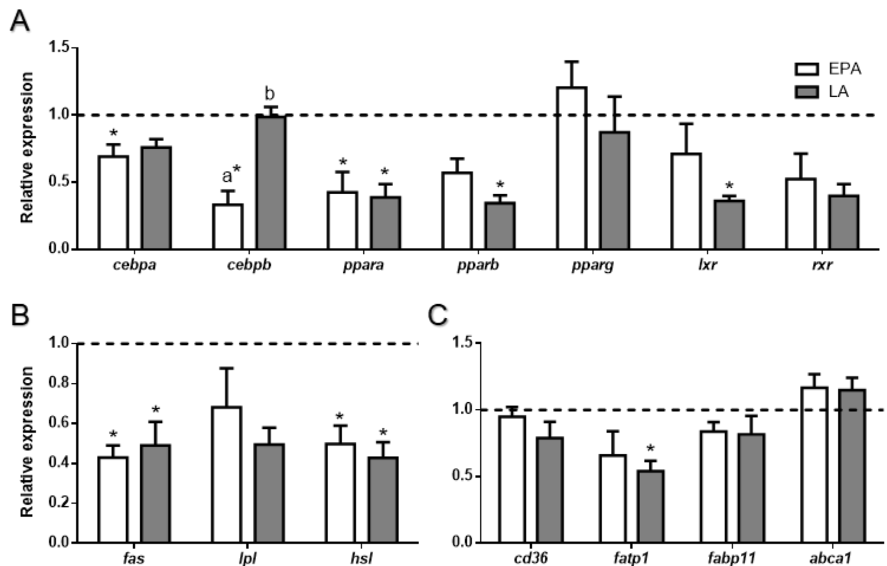



**Fig. 5.** Relative expression of genes related to **(A)** transcription factors, **(B)** enzymes and **(C)** lipid transporters in liver from fish forced-fed with EPA, LA or vehicle without fatty acid as control (dashed line). Data are shown as mean + SEM (n=7-8 for CT; n=6-7 for EPA; n=5-8 for LA). Significant differences (p<0.05) among treatments are indicated by different letters. Asterisks indicate significant differences (p<0.05) with the control. EPA: eicosapentaenoic acid; LA: linoleic acid.

Finally, differences were not found in the protein levels (Fig. 6A) of either liver X receptor (Figure 6B), HSL (Fig. 6C) or the fatty acid transporter CD36 (Fig. 6D).

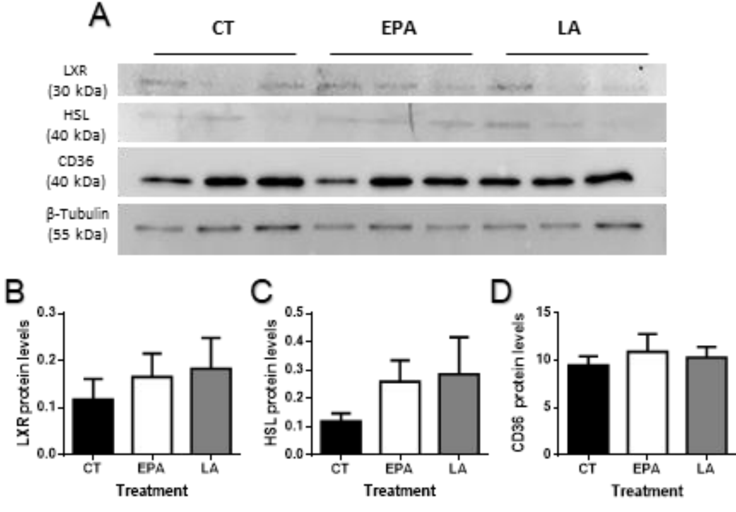


**Fig. 6.** Representative Western blots of the **(A)** transcription factor LXR, the enzyme HSL, the fatty acid transporter CD36 and β-tubulin in liver tissue from fish force-fed with EPA, LA or vehicle without fatty acid as control. Quantification of protein levels of: **(B)** LXR, **(C)** HSL and **(D)** CD36 normalized to β-tubulin. Data are shown as mean + SEM (n=6). Significant differences ($p<0.05$) among treatments are indicated by different letters. CT: control; EPA: eicosapentaenoic acid; LA: linoleic acid.

## 4. Discussion

Aquaculture has been looking for new sustainable strategies to face the lowered pelagic stocks to produce fish feeds, using VO to substitute FO. In this sense, the different dietary FA profiles in these oils have demonstrated to affect differentially fish lipid metabolism and adiposity. In this context, it must be considered the importance of testing the direct effects of specific FA *in vitro* as well as *in vivo* to obtain a general view of the importance of FO substitution in lipid metabolism of fish. To this end, this study has focused on the differential effects of FA typical from FO and those most commonly found in VO, on rainbow trout lipid metabolism, using as experimental models an *in vitro* culture of preadipocytes and an *in vivo* administration experiment by gavage. The main objective was to evaluate the potential effects caused by these FA in the process of adipogenesis and preadipocyte metabolism as well as to analyse the short-term response of key tissues involved in lipid homeostasis in front of an increase in FA disposal.

Cell morphology changes and increased lipid accumulation (both as indicative of adipocyte differentiation) were observed after incubation with the different FA demonstrating for the first time that the presence of a single FA, without any other lipid component or hormones, is able to induce adipogenesis in rainbow trout adipocyte precursor cells. Indeed, it has been reported that the presence of FA in the media is critical to induce the adipogenic process specifically in fish cells (Bouraoui et al, 2008; Oku et al, 2006; Salmerón et al, 2013; Vegusdal et al, 2003), and this has been also observed in avian adipocyte precursor cells (Matsubara et al, 2005). Accordingly, in the present study, the cells began to accumulate lipids stimulating adipocyte differentiation in response to all the FA treatments after 48

h, although ALA produced the greatest effect; overall, suggesting that this FA may stimulate, more than the others, uptake and fat depot in these cells. Similarly, Todorcevic et al (2008) observed in mature salmon adipocytes that FA from VO such as oleic acid, were able to induce more lipid accumulation than FA characteristic of FO; and this lower capacity to be stored of FO-derived FA was also previously reported in mammalian 3T3-L1 adipocytes (Kim et al, 2006). Moreover, the same enhanced lipogenic capacity of FA abundant in plant oils (e.g. LA) compared to those from FO in another cellular fish model (i.e. gilthead sea bream bone-derived mesenchymal stem cells), has been recently reported (Riera-Heredia et al, 2019); thus, demonstrating the potential of such FA inducing both, adipogenesis as well as mature adipocytes hypertrophy.

Next, to further evaluate the effects of the different treatments in cultured preadipocytes, the expression of relevant adipogenic driving genes was analysed. In rainbow trout, it has been previously observed that a standard procedure incubating preadipocytes with a differentiation medium containing a hormonal cocktail and lipid mixture induces adipocyte maturation (Bouraoui et al, 2008), and an increase in adipogenic genes expression (Bou et al, 2017), with some of them showing a transient upregulation after 24 h induction (Riera-Heredia et al, *unpublished*). Nonetheless, almost no information is available on the early activation of preadipocytes in response to the presence of single FA or their combinations.

Concerning transcription factors, in the present study *cebpa* and *pparg* expression was downregulated by EPA, and *pparg* was also decreased by all the paired FA combinations. This result is in accordance with the anti-adipogenic effect of EPA and DHA observed in several *in vitro* experiments performed in adipocytes from mammals, fish or cell lines; although some others have also demonstrated no effect or a pro-adipogenic effect of these two FA in the same cells, despite incubation times or the precise moment of cell differentiation vary among experiments (Reviewed in Tordocevic & Hodson, 2015). Concretely, Huang et al (2010) also showed a significant reduction in the expression of *cebpa* when EPA at high

concentrations was applied during cell differentiation and transcript levels were measured at mature adipocytes stage in Atlantic salmon. On the other hand, in bone cell-derived from gilthead sea bream, EPA in the culture media increased expression of *pparg* after 6 h when compared to LA and ALA (Riera-Heredia et al, 2019). Salmon preadipocytes have demonstrated that possibly they are early fated to be adipocytes because they already express *pparg* before the induction of differentiation (Todorčević et al, 2010) and similarly this has been also observed in rainbow trout adipocytes (Riera-Heredia et al, *unpublished*). This fact could explain the decrease (most probably transient) of expression at 6 h, of this transcriptional factor in our cell model. Nevertheless, the effect of FA modulating adipogenesis and lipid accumulation in mammals, as well as in fish is very complex and not completely understood yet and differential effects observed between reported *in vitro* studies could be consequence at least in part of the different conditions among experiments (Todorcevic & Hodson, 2015).

Regarding the expression of maturing adipocyte markers, specifically genes that codify for key enzymes such as FAS, involved in the synthesis of FA, remained stable or low, as observed after adipogenesis induction in gilthead sea bream (Salmerón et al, 2016). Therefore, FA in the culture media seems to provoke a downregulated expression of *fas*, possibly through the inhibition of this enzyme by a negative feedback (Riera-Heredia et al, 2019; Salmerón et al, 2016). Similarly, differences could not be found in *lpl* or *hsl* gene expression during early differentiation into adipocytes of rainbow trout cultured preadipocytes when the whole transcriptional profile of this process was analysed (Bou et al, 2017). These results could be also suggesting a balanced metabolic turnover characteristic of the adipocyte to avoid lipotoxicity of FA (Saponaro et al, 2015).

Concerning the genes involved in the uptake and transport of FA, only the mRNA levels of *fatp1* increased, especially in cells treated with LA, ALA or the combinations containing either one of these FA. These data indicated that this transporter is rapidly activated in response to potential ligands and should have an important role in the uptake of FA from cell environment in fish preadipocytes in

agreement with previous studies (Sánchez-Gurmaches et al, 2012; Todorcevic et al, 2008). All in all, FA characteristic of VO (LA and ALA) apparently demonstrated more potential to upregulate this FA transporter. This is accompanied by a higher potency stimulating lipid uptake and accumulation inside the preadipocytes as observed; as well as was previously reported in Atlantic salmon preadipocytes and in bone-derived cells from gilthead sea bream (Huang et al, 2010; Riera-Heredia et al, 2019). Considering the results obtained in this *in vitro* approach, and for their abundance in FO and VO respectively, EPA and LA, appeared to be good candidates, to obtain more information after a short-term *in vivo* challenge in the same species. However, there are very few studies about the effects of these oils or specific FA in the short term or during the postprandial period *in vivo* (Librán-Pérez et al. 2012).

In this context, Librán-Pérez et al (2012, 2013) showed that the oral administration of FO or its main FA (EPA and DHA) by gavage or intraperitoneally improve the control of glycemia in trout. In mammals, some studies demonstrated that n-3 PUFA improve insulin resistance and the secretion of this hormone (Riccardi, Giacco & Rivellese, 2004; Wang & Chan, 2014). In our study, only EPA administration showed a decrease in glucose plasma levels indicating that this possible protective role could be depending on the specific FA. Furthermore, LA force-fed fish presented increased glycerol plasma levels when compared to those administered vehicle and EPA solutions. In mammals, increased plasma glycerol has been associated with a mild hypoglycaemia besides being a possible sign of adipose tissue lipolysis (Flattem et al, 2001). Nevertheless, no effect was observed in circulating triglycerides after the different FA treatments. Despite being a short-term study, this result is in concordance with a long-term VO substitution trial in gilthead sea bream where changes in triglycerides and glucose levels were not found (Cruz-Garcia et al, 2011). On the contrary, triglycerides levels in plasma were lower in mice fed with n-3 PUFA supplemented diets than with n-6 PUFA (Gnoni & Giudetti, 2016). Thus, the impact of the increase in FA disposal in trout plasma parameters was relatively small.

These observed slight changes in circulating metabolites in trout could be reflecting quick adjustments in adipose tissue and liver. In adipose tissue, lipid metabolism homeostasis and adipogenesis are partially controlled by PPARs co-ordinately with coactivators/corepressors. PPARs can act as lipid sensors contributing to adipose tissue homeostasis in circumstantial physiological challenges (Corrales et al, 2018). When PPARs are activated by PUFA and eicosanoids, there is a recruitment of RXR to form a heterodimer and facilitate the union to promotors that will activate different key genes (Nakamura et al, 2004). In our study, the administration of EPA and LA induced an increase in the expression of three transcription factors (*ppara*, *pparb* and *rxr*), suggesting an activation of FA oxidation to provide energy to the adipocyte. On the other hand, differences were not found in gene or protein expression of PPARγ that besides regulating adipogenesis also controls genes involved in lipogenesis (Bouraoui et al, 2008). Nevertheless, *fas* showed a clear upregulated expression in adipose tissue from force-fed rainbow trout, indicating increased lipogenesis *de novo*, suggesting a stimulation of lipid anabolism with the FA treatments. In our study, the expression of *lpl* was reduced significantly by EPA, but not by LA. The regulation of *lpl* could be depending on the FA used as showed by Liang, Ogata & Oku (2002), where diets supplemented with oleic acid and with EPA provoked a diminished expression of *lpl*, but not after the supplementation with oleic acid and LA, concluding that the type of FA, rather than the degree of unsaturation influences adipose tissue *lpl* expression, as it occurs in mammals (Amri et al, 1996; Raclot et al, 1997; Takahashi & Ide, 2000).

Regarding the last enzyme studied, *hsl* presented upregulation after LA administration, although this result was not correlated with its protein levels, where no differences were found. These changes in expression are in concordance with the study of Cruz-Garcia et al (2011) that in isolated adipocytes from gilthead sea bream fed with substituted diets, showed increased expression of *hsl* together with an increased HSL activity. Therefore, suggesting enhanced intracellular lipolysis as demonstrated also in mammals (Berger & Barnard, 1999; Farnier et al, 2003;

Gregor & Hotamisligil, 2007). Altogether, the global response of adipose tissue to the increase in absorbed FA seems to be a general activation of lipid metabolism.

On the other hand, the reduction observed in *hsl* expression in liver, despite what was found in adipose tissue, could demonstrate a diminution of lipolysis thus reduced fat reserves degradation in liver, being in agreement with the fact that the expression of genes related to lipid metabolism are known to be regulated in a tissue specific manner (Feingold & Grundfel, 2018). In fact, the liver also plays a key role coordinating lipid oxidation, FA, cholesterol and lipoprotein biosynthesis. In liver, the expression of *cebpa* and *cebpb* was downregulated significantly by EPA treatment, as well as a decreased expression in *ppara* and *pparb* was observed with both FA. These results could be explained by a reduction of FA metabolism in liver due to treatments, as shown in Librán-Pérez et al (2012; 2013) in similar experimental conditions. Expression of *lxr*, a transcription factor related to the lipogenic pathway in liver (Pégorir, May & Girard, 2004), was decreased in our study by LA treatment, together with a significant downregulation of *fas* by the two FA, which could be indicative of a decrease in hepatic lipogenesis, contrary to what was found in adipose tissue. In fact, several studies showed *fas* decreased expression using FA incubations in trout hepatic cells (Alvarez et al, 2000), diets with increased content of n-3 and n-6 PUFA in mice (Gnoni & Guidetti, 2016) or after an intraperitoneal injection of oleic acid in trout (Librán-Pérez et al, 2013).

Regarding FA transporters and binding proteins in adipose tissue, the upregulation observed in *cd36* gene expression with the two FA treatments suggested that in front of an increase of fat availability there is an augmented FA internalization into the adipocyte (Salmerón, 2018). These changes were not observed in our *in vitro* model suggesting that other systemic factors might be involved, although *fatp1* expression indeed increased after LA and ALA adipocyte incubations but was not modified after *in vivo* FA administration. These responses suggest a possible differential role of the two transporters, that also have been described to present different expression patterns among tissues (Torstensen et al, 2009; Weil, Lefèvre & Bugeon, 2012). In any case, only LA succeeded in increasing CD36 protein

levels when compared to EPA, which could evidence a probably different post-transcriptional regulation of this transporter depending on the FA present and reinforcing the higher potency of FA from vegetal origin to promote lipid uptake. Finally, only EPA caused a significant decrease in the expression of the intracellular FA-binding protein *fabp11*.

On the contrary, differences were not found in FA transporters in the liver, except for the downregulation of *fatp1* by LA treatment. This result is in concordance with Librán-Pérez et al (2013), where the hepatic transporters analysed remained unchanged after treatment. All this could indicate a different FA dynamic between tissues and thus lipid internalization could have finished in the liver at the selected time of sampling. This would help to explain the different observed patterns in gene expression in both tissues, suggesting a general activation of lipid metabolism in adipose tissue and a depression in liver.

In summary, FA increased lipid accumulation in *in vitro* adipocytes from rainbow trout, especially ALA. Gene expression patterns of transcription factors, enzymes and lipid transporters, characteristic of the mature adipocyte, indicate little differences between the specific FA, suggesting a general activation of the metabolic turnover that finally results in an increased cellular lipid storage. Similarly, adipose tissue from FA-treated animals apparently activated the production of energy by β-oxidation with the increased expression of *ppara* and *pparb*, probably, directed to the *de novo* lipogenesis (upregulation of *fas*), although at the same time, the presence of FA activates the expression of *cd36*, involved in their uptake, which in turn would stimulate lipid depot. All this could mean that the administration of EPA or LA activates lipolytic and lipogenic pathways of the adipocyte simultaneously, although with no clear differences between the two FA. On the contrary, the liver tissue presented a decrease in fat metabolism suggested by downregulated expression of related transcription factors, and lipogenic and β-oxidation markers. Altogether indicating that tissue specific FA dynamics could be responsible for the differential responses.

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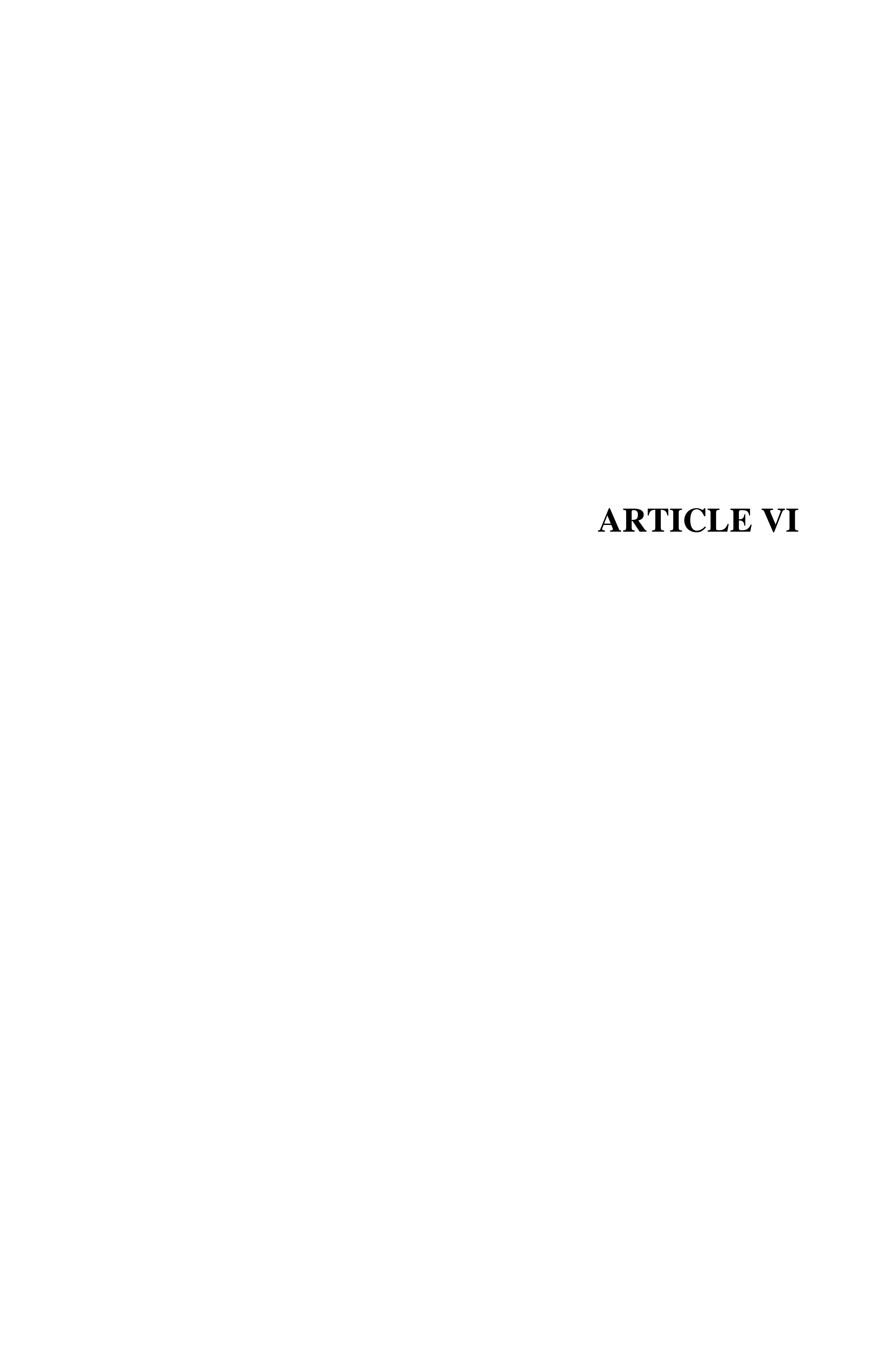

# ARTICLE VI

# Vegetable oils as a good alternative for optimal growth of gilthead sea bream under ocean warming

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## Abstract

Feeding an increasing world population is leading to a major demand of fish products due to their high nutritional value; therefore, the aquaculture sector needs to develop new tools to increase production and face future environmental conditions. One of the improvements in this sector has been to reduce the usage of fishmeal and fish oil (FO) in diet formulations to promote a more sustainable production. In the present study, gilthead sea bream has been fed a FO substituted diet containing palm oil (P) while being maintained at 21 ºC or at 28 ºC to mimic the future water warming due to climate change. Furthermore, in the condition of elevated temperature fish have been fed in addition with two other diets containing rapeseed oil (R) or a combination of both vegetable oils (PR). Elevated temperature induced a significant increase in growth, but a reduction in the hepatosomatic and viscerosomatic indexes, and in circulating triglycerides (TGs), although significant differences were not found among the fish fed the three distinct diets at 28 ºC for any of the somatic or plasma parameters analysed. In the adipose tissue, the smaller adipocytes size together with the downregulated expression of cluster of differentiation 36 (*cd36*) in fish maintained at 28 ºC compared to fish maintained at 21 ºC suggested an activation of adipogenesis or a depletion in TGs cell content. Similarly, lipid droplets of smaller area and downregulation of *cd36* and fatty acid binding protein 11 (*fabp11*) were found in the liver of fish when reared at 28 ºC compared to those at 21 ºC. Concerning differences among dietary groups, fish fed R diet showed more hepatic lipid droplets, but fish fed PR diet had smaller adipocytes as well as less area occupied by smaller lipid droplets in the liver added to the upregulation of peroxisome proliferator-activated receptors (*ppara, pparb* and *pparg*) and genes related to lipid transport; altogether demonstrating that this diet could be the most equilibrated for gilthead sea bream to grow at a high rearing temperature. Thus, this study highlights the need of fine-tuning the substitution of FO by vegetable oils in the elaboration of feeds for this species to assure proper growth and physiological status in a future global warming scenario.



**Keywords:** fish oil substitution, vegetable oils, adipose tissue, liver.

## Introduction

Fish production must face the handicap of feeding an increasing world population, which is leading to a major demand of these kind of products due also to their high nutritional value (United Nations, 2016; Godfray et al, 2010). Furthermore, the aquaculture sector has to impulse new strategies to increase production, assuring a high-quality product, also considering the future environmental challenging conditions.

One of the strategies promoted in the aquaculture industry in the last decades has been to reduce the usage of fishmeal and fish oil (FO) in diet formulations to promote a more sustainable production of fish feeds (Tacon & Metian, 2015). The major approach has been to substitute these limiting products by similar ingredients from vegetable sources (i.e. vegetable oils, VO), always adjusting the substitution according to the nutritional requirements of each fish species (Kaushik et al, 1995; Rosenlund et al, 2001; Torstensen et al, 2005). Unfortunately, fewer studies have been performed about FO substitution in a scenario of altered environmental conditions. Global climate change will increase the mean sea surface temperature more than 2 ºC by the end of this century (IPCC. Climate change, 2014); consequently, temperature is an important aspect that aquaculture must face on. In this sense, the combined influence of different alternative plant dietary lipid sources and temperature have been reported on Senegalese sole (*Solea senegalensis*) (Guerreiro et al, 2012), European sea bass (*Dicentrarchus labrax*) (Vagner et al, 2007) and in salmonids, where changes in fillet fatty acid composition have been also described (Jobling & Bendiksen, 2003; Norambuena et al, 2015, 2016; Tocher et al, 2004; Wijekoon, Parrish & Mansour, 2014). Therefore, temperature is an important factor to consider when a VO is selected for a substitution.

Several studies have proved that the partial substitution of FO by VO can guarantee proper growth and fish feed utilization (Benedito-Palos et al, 2008; Betancor et al, 2016; Izquierdo et al, 2000; 2003). Nevertheless, adverse effects have been

described as well in fish fed with highly substituted VO diets, affecting digestion, absorption, hepatic metabolism, lipid storage, fatty acid profile and adipose tissue growth (Bell et al, 2001; Bouraoui et al, 2011; Caballero et al, 2002; Cruz-García et al, 2011; Lopes et al, 2017; Montero et al, 2003). Adipose tissue as liver plays an important role in lipid metabolism and energetics homeostasis regulation (Choe et al, 2016). In adipose tissue, two types of growth have been described: hyperplasia that implies the production of new adipocytes via precursor cells, a process called adipogenesis, and hypertrophy where lipid accumulation increases the cell volume (Otto & Lane, 2005), and both processes can be affected by VO substitution. In this context, Cruz-Garcia et al (2011) and Torstensen et al (2011) demonstrated that highly substituted VO diets could lead to hypertrophic adipose tissue growth therefore causing abnormal fat deposition because of a dysregulation of lipolysis and lipid accumulation. Besides, these alterations frequently go hand in hand with the presence of a fatty liver (Cruz-Garcia et al, 2011) thus, due to its function in tissue lipids distribution through lipoproteins, the liver is also a key indicator of the effects caused by FO substitution in fish.

Metabolic control of both tissues is tightly connected in fish (Guillaume et al, 2004; Halver, 1989) despite of the tissue specific regulation of the expression of some transcription factors and genes related to lipid metabolism. Among these, CCAAT/enhancer binding proteins (C/EBPs) are activated with adipogenesis thus increasing the expression of *cebpa*, *cebpb* and peroxisome proliferator-activated receptor γ (*pparg*) (Chen et al, 2016). Other specific genes related to β-oxidation of fatty acids (*ppara* and *pparb*), lipid turnover such as fatty acid synthase (*fas*), hormone sensitive lipase (*hsl*), lipoprotein lipase (*lpl*) or fatty acid transport like *fatp1* or the FAT translocase *cd36*, are considered indicators of the metabolic status both, in hepatic and adipose tissues in fish (Grygiel-Górniak, 2014; Oku & Umino, 2008; Salmerón et al, 2016; Tocher, 2003).

Regarding the oils fatty acid composition, FO is rich in n-3 highly unsaturated fatty acids (HUFA) such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which optimal proportion in gilthead sea bream (*Sparus aurata*) has been

estimated at 2:1 (Ibeas et al, 1997). In fact, the optimal ratio of these fatty acids is key given that an absence or a disproportion of these nutrients are known to affect growth and lipid metabolism (Caballero et al, 2003; Montero et al, 2003; Tocher, 2003). On the other hand, the most commonly used VO in feeds formulation are palm (PO), soybean (SO), rapeseed (RO), linseed and sunflower oils, which are composed by different fatty acids although most of them having n-6 and n-9 long-chain polyunsaturated (PUFA) in abundance (Turchini, Ng & Tocher, 2010). Otherwise, VO have almost no n-3 HUFA therefore, in fish fed diets with a high replacement of FO by VO has been observed a reduction in the profile and nutritional qualities of the final product (Rosenlund et al, 2010). Among the VO, RO is generally cheaper and available at greater amounts than FO and is rich in monounsaturated fatty acids (MUFA) (Turchini, Torstensen & Ng, 2009), which are known to be readily catabolized by β-oxidation (Bell et al, 2001). While PO, that contains about 50% of saturated fatty acids (SFA), is the most produced oil in the world and one of the cheapest oils in the market, and it has demonstrated to be a potential alternative to FO in salmonids (Ng, Tocher & Bell, 2007).

In a recent study by our group in gilthead sea bream fed different diets with variations in VO sources, it has been observed that the fish fed a diet with an elevated PO content, presented higher growth when compared to other diets with blends of several VO, but showed an excess of adipose tissue (Sánchez-Moya et al, *under revision*). Moreover, in some warm-water species, PO also showed some advantages compared to other VO (Ng, Lim & Sidek, 2001; Ng, Sigholt & Bell, 2004), by apparently enhancing flesh quality (Bell et al, 2000, Scaife et al, 2000) due to the bioaccumulation in the fillet of antioxidants present in this oil (Lim, Boey & Ng, 2001). Added to these interesting properties, both SFA and MUFA, present in PO and RO respectively, are known to be preferred over PUFA for energy production in fish (Henderson, 1996). With all that, the aims of the present study are to analyse the effects of a high respect to usual rearing temperature in gilthead sea bream fed with PO, and to investigate the combined effects of diet and temperature in fish maintained at 28 ºC fed three different substituted FO diets

with PO, RO or a blend of both VO. Growth parameters and several somatic indexes, plasma metabolites, tissue histology as well as quantitative expression of genes involved in lipid metabolism in liver and adipose tissue were evaluated.

## 2. Material and methods

### 2.1. *Animals, experimental design and ethics statement.*

Juvenile gilthead sea bream (*S. aurata*) were obtained from the fishery "Piscimar" (Burriana, Spain) and were acclimated to the facilities in the Faculty of Biology at the University of Barcelona for one month. Fish were kept in 400 or 200 L fiberglass tanks under a 12 h light/12 h dark photoperiod, maintained either at 21 or 28 ºC in two different rooms and fed three times a day a commercial diet (Optibream, Skretting, Burgos, Spain).

For the experimental trial, three different partially substituted FO diets (Table 1) manufactured by Skretting ARC (Stavanger, Norway) were administered to four groups of fish for 2 months, from the end of October to the end of December. Two groups of fish were fed with a diet containing PO (P) and were reared at the 2 different temperatures, 21 and 28 ºC; while the remaining groups maintained at 28 ºC were fed with a diet containing either RO (R) or a diet with a combination of PO and RO (PR). All diets were formulated with an equal basal content of LO. Each group of fish was distributed in 3 replicate tanks (1x400L and 2x200L) at the corresponding 21 or 28 ºC conditioned rooms. Fish were fed with constant rate of 2.5% body weight adjusted each week. At the end of the trial, fish were fasted for 24 h before sacrifice in order to avoid contamination of the tissues with contents from the gastrointestinal tract during sampling. Nine fish per group (three fish per tank) were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS222) (Sigma–Aldrich, Tres Cantos, Spain) and blood was extracted from the caudal vein. The blood was centrifuged 10 min at 5000 rpm to separate the plasma, which was stored at -80 ºC until metabolites concentrations were determined. Then, fish were sacrificed by cranial concussion and samples of adipose tissue and liver were snap frozen in liquid nitrogen and stored at -80 ºC until performing gene expression

analyses. Furthermore, samples of liver and adipose tissue from four extra fish per condition were taken and fixed in 10% buffered formalin.

All animal handling procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the regulations and procedures established by the Spanish and Catalan governments.

**Table 1.** Composition of experimental diets (%). Palm (P); Rapeseed (R); Palm + Rapeseed (PR)

| **Ingredients (%)** | **P** | **R** | **PR** |
|---|---|---|---|
| Wheat | 6,83 | 6,83 | 6,83 |
| Corn meal | 10 | 10 | 10 |
| Wheat gluten | 14,79 | 14,78 | 14,78 |
| Soya protein | 25 | 25 | 25 |
| Broad beans | 10 | 10 | 10 |
| Fish meal | 20 | 20 | 20 |
| Fish oil | 5,34 | 5,34 | 4,45 |
| Linseed oil | 1,33 | 2 | 1,33 |
| Rapeseed oil | 0 | 6,67 | 3,34 |
| Palm oil | 6,67 | 0 | 3,55 |
| Phosphate | 0,03 | 0,03 | 0,03 |
| **Composition (%)** | | | |
| Moisture | 7,6 | 7,7 | 7,5 |
| Protein | 51,9 | 51,8 | 51,7 |
| Fat | 19,2 | 18,9 | 19,4 |
| Ash | 5,7 | 5,7 | 5,6 |

2.2. *Growth parameters and plasma analyses*

The following parameters and indexes concerning somatic growth were determined or calculated using the indicated formulas: initial body weight (IBW),

final body weight (FBW), weight gain (WG) [(FBW-IBW)x100], somatic growth rate (SGR) [(ln (FBW)−ln (IBW))/t×100], body length (BL), condition factor (CF) [(FBW)/(BL)3×100], hepatosomatic index (HSI) [(Liver weight/FBW)x100], viscerosomatic index (VSI) [(Viscera weight)/FBW)x100] and mesenteric fat index (MFI) [(Mesenteric fat weight/FBW)x100].

Plasma samples were analysed for metabolites using commercial enzyme kits: non-esterified fatty acids (NEFA-HR2, Wako Chemicals GmbH, Neuss, Germany), and TGs and glycerol (Serum Triglyceride Determination Kit, Sigma-Aldrich, Tres Cantos, Spain), following the manufacturers' indications.

2.3. *Histology analyses*

Fixed adipose tissue samples were dehydrated in a graded ethanol series and embedded in paraffin. Sections of 4 µm obtained with a microtome (Leica RM2125, Leica Microsystems, Wetzlar, Germany) were stained with haematoxylin and eosin (H&E) and observed under a light microscope (Olympus PM10SP Automatic Photomicrography System).

In the case of liver, pieces of tissue previously fixed from the same fish were transferred to optimal cutting temperature (OCT) compound and frozen. Sections (10 µm) were cut with a cryostat (Leica CM3050, Leica Microsystems, Wetzlar, Germany) stained with either 0.7% Oil red O (ORO) or 0.5% periodic acid-Shiff (PAS) following previously established protocols (Hui et al, 2017; Mehlem et al, 2013) with little adaptations. Briefly, for ORO staining, slides with 2-4 sections of the tissue were dried at room temperature, rinsed in PBS, then placed in warmed propylene glycol and finally in ORO staining solution at 60 ºC. The slides were washed, and haematoxylin staining was performed, followed by washes and were consequently mounted with Kaiser Gelatin'. For PAS staining, slides with also 2-4 tissue sections were dried at room temperature, rinsed in PBS, placed in PAS solution, washed and finally submerged in Schiffs' reactive. The slides were then washed, and haematoxylin staining was performed followed by washes, dehydration and mounting with Entellan. Afterwards, preparations were observed

at light microscopy (Olympus PM10SP Automatic Photomicrography System). For the quantification of the lipid or glycogen content 4 images from different sections of the tissues from each fish were used and analysed with ImageJ (National Institutes of Health, United States). All reagents for histology staining were purchased from Sigma–Aldrich (Tres Cantos, Spain).

2.4. *RNA extraction and cDNA synthesis*

Frozen pieces of adipose tissue (100 mg) and liver (50 mg) were homogenated in 1 mL of TRI Reagent (Invitrogen, Alcobendas, Spain), using the Precellys Evolution technology (Bertin Instruments, Montigny-le-Bretonneux, France). Total RNA was extracted according to the manufacturer's recommendations, was dissolved in DEPC-treated water (RNase-free), quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and stored at −80°C. To eliminate any residual genomic DNA, total RNA (1 µg) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain), following the manufacturer's instructions.

2.5. *Real-time quantitative PCR (qPCR) analyses*

The key genes implicated in adipogenesis and energy metabolism regulation and analysed by qPCR comprise the following (Table 2): The transcription factors or nuclear receptors: *ppara, pparb*, *pparg* and liver X receptor (*lxr*); the enzymes: *fas*, *lpl* and *hsl*; fatty acid transporters: *cd36*, fatty acid transport protein 1 (*fatp1*) and fatty acid binding protein 11 (*fabp11*). Reference genes analysed were *b-actin*, elongation factor 1 alfa (*ef1a*) and ribosomal protein S18 (*rps18*). qPCR analyses and preliminary validation assays were performed as described in Riera-Heredia et al (2018, 2019). The stability of the reference genes as well as the mRNA levels of expression of the genes of interest calculated relative to the most stable reference genes (geometric mean of *ef1a* and *rps18*) according to the Pfaffl method (Pfaffl, 2001) were determined using the CFX Manager Software implemented in the CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain).

**Table 2.** Primers used for real-time quantitative PCR. F, forward primer; R, reverse primer; Ta, annealing temperature; Acc. Num., accession number.

| Gene | Primer sequence (5'→3') | Ta (ºC) | Acc. Num. |
|---|---|---|---|
| *ppara* | **F:** TCTCTTCAGCCCACCATCCC | 62 | AY590299 |
| | **R:** ATCCCAGCGTGTCGTCTCC | | |
| *pparb* | **F:** AGGCGAGGGAGAGTGAGGATGAGGAG | 69 | AY590301 |
| | **R:** CTGTTCTGAAAGCGAGGGTGACGATGTTTG | | |
| *pparg* | **F:** CGCCGTGGACCTGTCAGAGC | 66 | AY590304 |
| | **R:** GGAATGGATGGAGGAGGAGGAGATGG | | |
| *lxr* | **F:** GCACTTCGCCTCCAGGACAAG | 62 | FJ502320 |
| | **R:** CAGTCTTCACACAGCCACATCAGG | | |
| *fas* | **F:** TGGCAGCATACACACAGACC | 60 | AM952430 |
| | **R:** CACACAGGGCTTCAGTTTCA | | |
| *lpl* | **F:** GAGCACGCAGACAACCAGAA | 60 | AY495672 |
| | **R:** GGGGTAGATGTCGATGTCGC | | |
| *hsl* | **F:** GCTTTGCTTCAGTTTACCACCATTTC | 60 | EU254478 |
| | **R:** GATGTAGCGACCCTTCTGGATGATGTG | | |
| *cd36* | **F:** GTCGTGGCTCAAGTCTTCCA | 60 | ERR12611_isotig20793 |
| | **R:** TTTCCCGTGGCCTGTATTCC | | |
| *fatp1* | **F:** CAACAGAGGTGGAGGGCATT | 60 | ERR12611_isotig43042 |
| | **R:** GGGGAGATACGCAGGAACAC | | |
| *fabp11* | **F:** CATTTGAGGAGACCACCGCT | 60 | ERR12611_isotig32312 |
| | **R:** ACTTGAGTTTGGTGGTACGCT | | |
| *b-actin* | **F:** TCCTGCGGAATCCATGAGA | 60 | X89920 |
| | **R:** GACGTCGCACTTCATGATGCT | | |
| *ef1a* | **F:** CTTCAACGCTCAGGTCATCAT | 60 | AF184170 |
| | **R:** GCACAGCGAAACGACCAAGGGGA | | |
| *rps18* | **F:** AGGGTGTTGGCAGACGTTAC | 60 | AM490061 |
| | **R:** CTTCTGCCTGTTGAGGAACC | | |

## 2.6. *Statistical analyses*

Data normality and homoscedasticity were assessed using Shapiro–Wilk and Levene’s test, respectively. Independent samples’ Student’s t-test was used for comparison between two groups (fish fed diet P at different temperatures). For multiple mean comparisons (among the three dietary groups at the same temperature) of normal distributed data, one-way ANOVA was used followed by Tukey’s or Dunnett’s T3 *post hoc* tests in case of homogeneous or heterogeneous variance data, respectively. When data did not fit normal distribution, the non-parametric Kruskal–Wallis test, followed by Mann–Whitney test, were used.

Statistical analyses were performed using SPSS Statistics version 22 (IBM, Armonk, NY, USA). Results were presented as mean ± SEM, and $P < 0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

## 3. Results

### 3.1. *Growth parameters, somatic indexes and plasma metabolites.*

The fish fed the P diet presented significant differences in growth parameters and somatic indexes when the animals reared at the two different temperatures were compared (Table 3). Fish fed with P diet at 28 ºC showed significantly higher FBW, WG, SGR and BL, and significantly lower HSI and VSI values when compared to fish fed diet P at 21 ºC. On the contrary, significant differences were not observed in CF or MFI, despite lower values were found in fish reared at high temperature regarding mesenteric fat.

When comparing the fish reared at 28 ºC and fed the three different experimental diets, significant differences were not found, although for FBW, WG and HSI slightly higher values were observed in gilthead sea bream fed diet P in comparison with the fish fed the R or PR diets. Contrarily, VSI and MFI values where lower in fish fed with diet P than in those fed the other two diets.

**Table. 3**. Growth parameters and somatic indexes from fish fed with the experimental diets P at 21 ºC or P, R and PR at 28 ºC for 2 months. Data are shown as mean ± SEM (n=3 tanks). Asterisks indicate significant differences between fish fed with palm diet at different temperatures. No differences were observed between diets at elevated temperature ($p<0,05$). Initial body weight (IBW); Final body weight (FBW); Weight gain (WG) [(FBW-IBW)x100]; Somatic growth rate (SGR) [(ln (FBW)−ln (IBW))/time×100] (time: 53 days); Body length (BL); Condition factor (CF) [(FBW)/(BL)3×100]; Hepatosomatic index (HSI) [(Liver weight/FBW)x100]; Viscerosomatic index (VSI) [(Viscera weight)/FBW)x100]; Mesenteric fat index (MFI) [(Mesenteric fat weight/FBW)x100]. Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

| | 21 ºC | 28 ºC | | |
|---|---|---|---|---|
| | **P** | **P** | **R** | **PR** |
| IBW (g) | 24,21 ± 0,57 | 22,89 ± 0,40 | 22,80 ± 0,75 | 22,24 ± 0,77 |
| FBW (g) | 54,32 ± 1,78* | 68,02 ± 2,34 | 63,33 ± 2,09 | 62,21 ± 3,80 |
| WG (%) | 124,3 ± 5,19* | 197,5 ± 13,27 | 177,8 ± 2,33 | 179,2 ± 7,62 |
| SGR (%) | 1,52 ± 0,04* | 2,05 ± 0,08 | 1,93 ± 0,02 | 1,94 ± 0,05 |
| BL (cm) | 15,06 ± 0,02* | 16,55 ± 0,11 | 16,37 ± 0,07 | 16,23 ± 0,22 |
| CF (%) | 1,59 ± 0,06 | 1,5 ± 0,04 | 1,44 ± 0,03 | 1,45 ± 0,03 |
| HSI (%) | 1,62 ± 0,05* | 1,05 ± 0,12 | 0,90 ± 0,03 | 0,90 ± 0,05 |
| VSI (%) | 7,79 ± 0,51* | 5,94 ± 0,17 | 6,33 ± 0,2 | 6,34 ± 0,19 |
| MFI (%) | 1,64 ± 0,16 | 1,17 ± 0,15 | 1,49 ± 0,03 | 1,43 ± 0,13 |

In relation to metabolites' plasma levels, the effects of temperature in fish fed with the P diet showed significant differences in TGs being lower in animals maintained at 28 ºC (Fig. 1A), while non-esterified fatty acids presented the same tendency, but the change was not significant (Fig. 1B). Moreover, significant differences were not found in glycerol levels between the fish fed with diet P at the two temperatures tested (Fig. 1C), and neither between the fish fed the different experimental diets and reared at 28 ºC for none of the plasma parameters analysed (Fig. 1A-C).

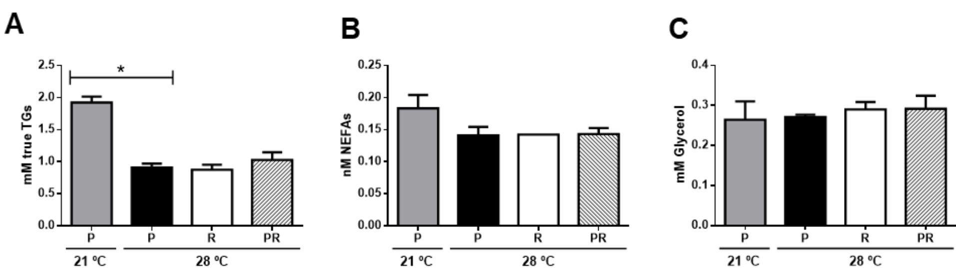
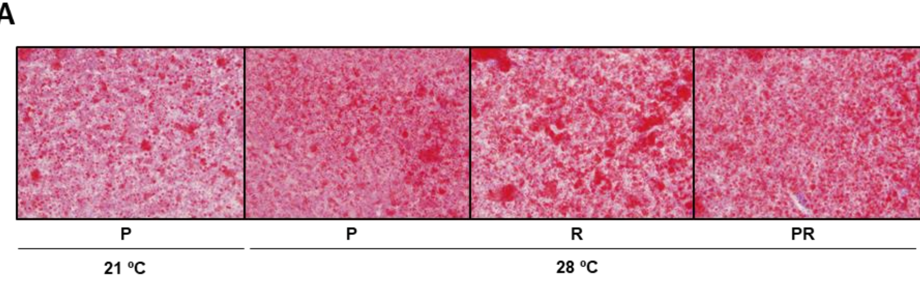



**Fig. 1**. Plasma metabolites levels in fish fed with the experimental diets P at 21 ºC or P, R or PR at 28 ºC for 2 months: (A) TGs (B) NEFAs, and (C) Glycerol. Asterisks indicate significant differences between fish feed with palm diet at different temperatures. Data are shown as mean ± SEM (n=7-10). No differences were observed between diets at elevated temperature ($p<0,05$). Triglycerides (TGs); Non-esterified fatty acids (NEFAs); Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

3.2. *Liver histological changes in response to diet and temperature.*

The images obtained after ORO staining of the liver sections of fish upon all treatments (P at 21 ºC and P, R and PR at 28 ºC) showed visual differences in lipid

accumulation (Fig. 2A). In addition, significant differences in the number and size of lipid droplets (LD) and the total lipid content were determined. Specifically, when comparing livers from fish fed with the P diet at the two temperatures, those from fish maintained at 28 ºC presented a significant bigger area occupied by lipids (Fig. 2B), with a higher number of LD (Fig. 2C) but, of smaller size (with less than $10^{-2}$ $mm^2$) than those presented in fish reared at 21 ºC (Fig. 2E). Regarding livers from fish fed with the P, R or PR diets at 28 ºC differences in the surface area occupied by LD were observed, being the R diet-fed fish the group with significantly greater area containing lipid content, then the P group and finally the PR group (Fig. 2B). Nevertheless, the number of LD was significantly higher in the PR diet-fed group than in the P one, although being again the fish fed with the R diet the one that significantly presented the greatest number of LD (Fig. 2C). Considering the individual average area of LD, all conditions showed similar results (Fig. 2D). When analysing the size distribution of LD (Fig. 2E), the PR diet-fed group presented significantly higher number of LD of smaller size than the fish fed the monosubstituted diets, but a lower percentage of larger LD; while the fish fed the diet R, presented significantly more LD of bigger size compared to the other groups of fish at 28 ºC. Contrarily, PAS staining did not show significant differences among groups, although fish at 28 ºC fed the R diet seemed to accumulate less glycogen when compared with gilthead sea bream fed the P or PR diets (Figs. 2F and 2G).



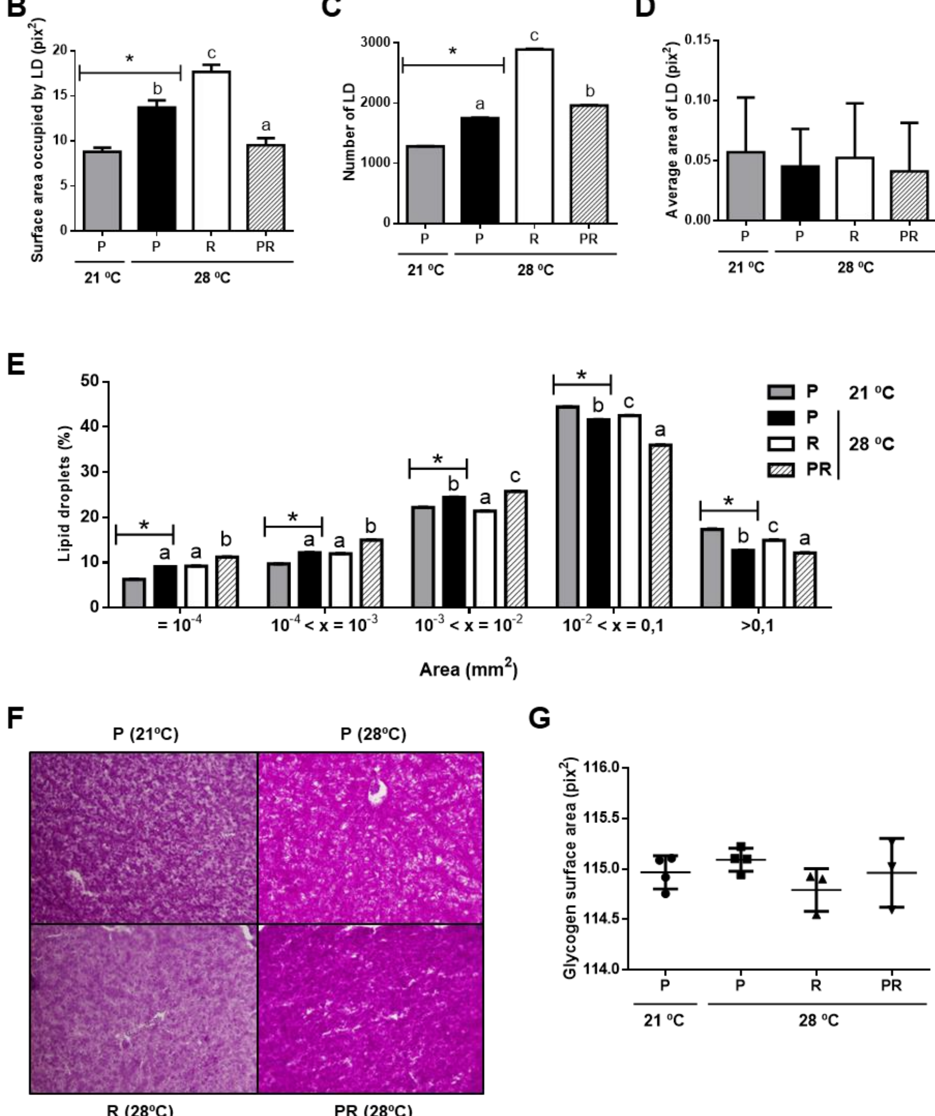
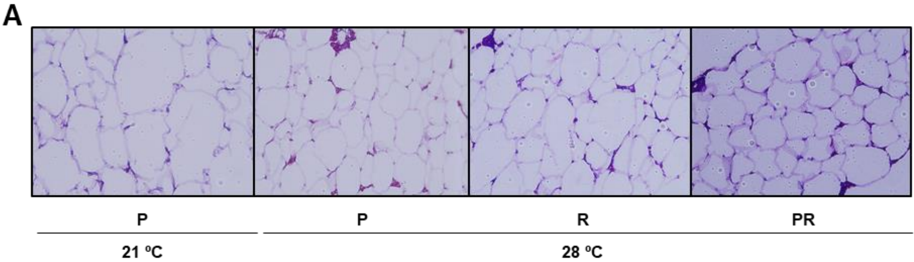



**Fig. 2. (A)** Representative images of Oil red O (ORO) staining of liver sections from fish fed with the experimental diets P at 21 ºC or P, R or PR at 28 ºC for 2 months. **(B)** Surface area occupied by lipid droplets (LD), **(C)** number of LD, **(D)** average area of the LD and **(E)** LD size distribution in the liver. Data are shown as mean ± SEM (n=3-4). Asterisks indicate significant differences between fish fed with P diet at different temperatures. Different letters indicate significant differences between fish fed the different diets at elevated temperature ($p<0{,}05$). **(F)** Representative images of Periodic acid–Schiff (PAS) staining of liver sections from fish fed with the experimental diets P at 21 ºC or P, R or PR at 28 ºC for 2 months. **(G)** Area occupied by glycogen. Data are shown as mean ± SEM (n=3-4). Asterisks indicate significant differences between fish fed with P diet at different

temperatures. Different letters indicate significant differences between fish fed the different diets at elevated temperature (p<0,05). Images magnification 20X. Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

### 3.3. *Adipose tissue histological changes in response to diet and temperature.*

H&E staining of adipose tissue slices (Fig. 3A) showed differences due to rearing temperature in fish fed diet P. Gilthead sea bream at 28 ºC showed a significantly higher percentage of smaller adipocytes compared with fish at 21 ºC, and despite not being significant, also presented a reduced percentage of bigger adipocytes (Fig. 3B). Concerning to the differences observed in response to diet in fish reared at 28 ºC, again fish fed the PR diet presented significantly more smaller adipocytes than those animals fed the diet R, showing the fish fed the P diet an intermediate value (Fig. 3B).



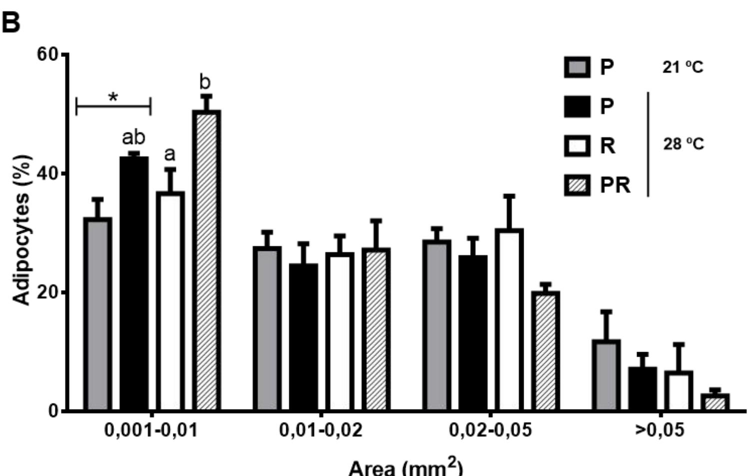



**Fig. 3.** **(A)** Representative images of hematoxylin and eosin (H&E) staining of adipose tissue sections and **(B)** adipocytes size distribution from fish fed with the experimental diets P at 21 ºC or P, R or PR at 28 ºC for 2 months. Magnification 20X. Data are shown as mean ± SEM (n=3-4). Asterisks indicate significant differences between fish fed with P diet at different temperatures. Different letters indicate significant differences between fish fed the different diets at elevated temperature ($p<0,05$). Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

### 3.4. *Gene expression changes in liver and adipose tissue in response to temperature*.

Gene expression of transcription factors, enzymes and fatty acid transporters was analysed in liver and adipose tissue samples from fish fed the P diet and reared at 21 or 28 ºC. The transcription factors *pparb, pparg* and *lxr* were downregulated in both tissues in the high-temperature group, although only *pparg* was significantly reduced in the adipose tissue (Figs. 4A and 4D). Regarding the enzymes related with lipid metabolism analysed, *fas*, *lpl* and *hsl*, the expression showed no significant differences in either tissue (Figs. 4B and 4E). Concerning fatty acid transporters, similar tendencies of reduced expression were found in both tissues with the increase in temperature, although only *cd36* was significantly downregulated in the two tissues and *fabp11a* in the liver (Figs. 4C and 4F).

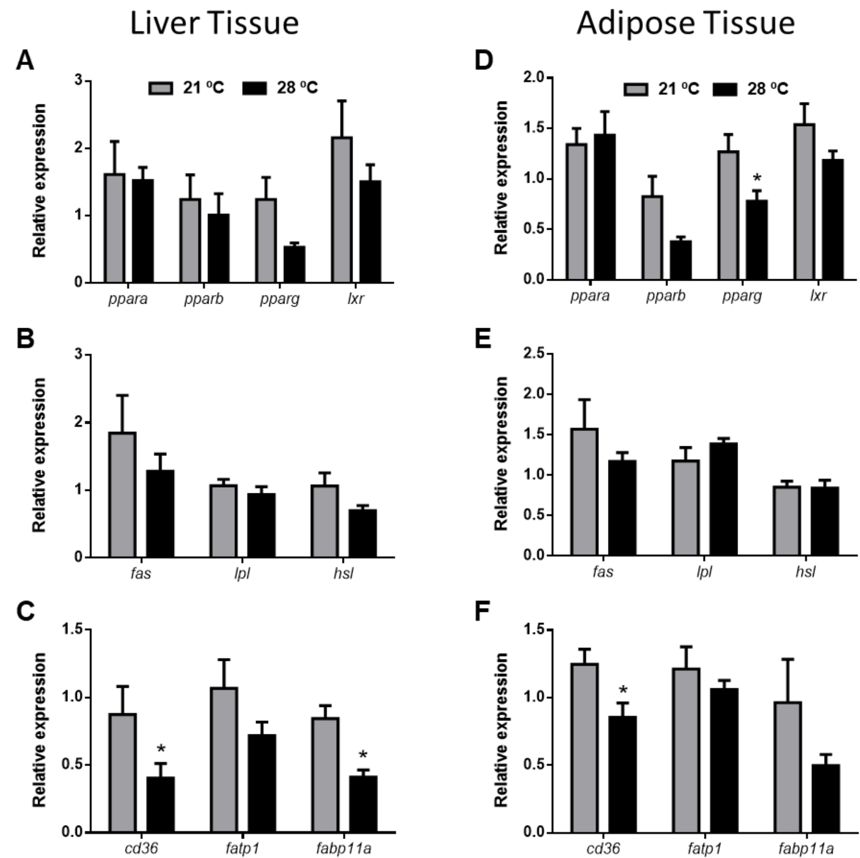



**Fig. 4.** Relative expression of transcription factors **(A, D)**, lipid metabolism-related genes **(B, E)** and fatty acid transporters **(C, F)** in the liver **(A-C)** and adipose tissue **(D-F)** from fish fed with P diet at 21 ºC and 28 ºC for 2 months. Data are shown as mean ± SEM (n=7). Asterisks indicate significant differences between fish reared at different temperatures (p<0,05). Palm (P).

### 3.5. *Gene expression changes in liver and adipose tissue in response to diet at 28 ºC.*

The same clusters of genes were analysed in the fish fed the R and PR diets and compared with the animals fed the P diet at 28 ºC. About transcription factors, gene expression of *ppara, pparb* and *lxr* was increased significantly in liver from gilthead sea bream fed PR diet, and *pparb* and *pparg* in adipose tissue compared with the P diet-fed fish (Figs. 5A and 5D). Moreover, in liver, *pparg* and *lxr* transcript levels, as well as those of *pparb* and *pparg* in adipose tissue, were also

significantly higher in fish fed the PR diet when compared with the R diet-fed fish. Concerning the expression of lipid metabolism-related enzymes, differences were not observed among the three dietary groups in the liver (Fig. 5B). On the other hand, in adipose tissue an upregulation of *fas, lpl* and *hsl* expression caused by diet PR was found compared to fish fed the P or R diets, although the levels were only significantly higher for *hsl* (Fig. 5E). With respect to fatty acid transporters' expression, PR diet-fed fish presented a significant upregulation in *cd36* and *fatp1* expression in the liver when compared to the other two groups of fish; while R diet-fed fish showed a significant downregulation compared to fish fed P diet in *cd36* expression (Fig. 5C). In adipose tissue, only PR diet-fed fish presented a significant upregulation of *fabp11* expression when compared to fish fed P and R diets (Fig. 5F).

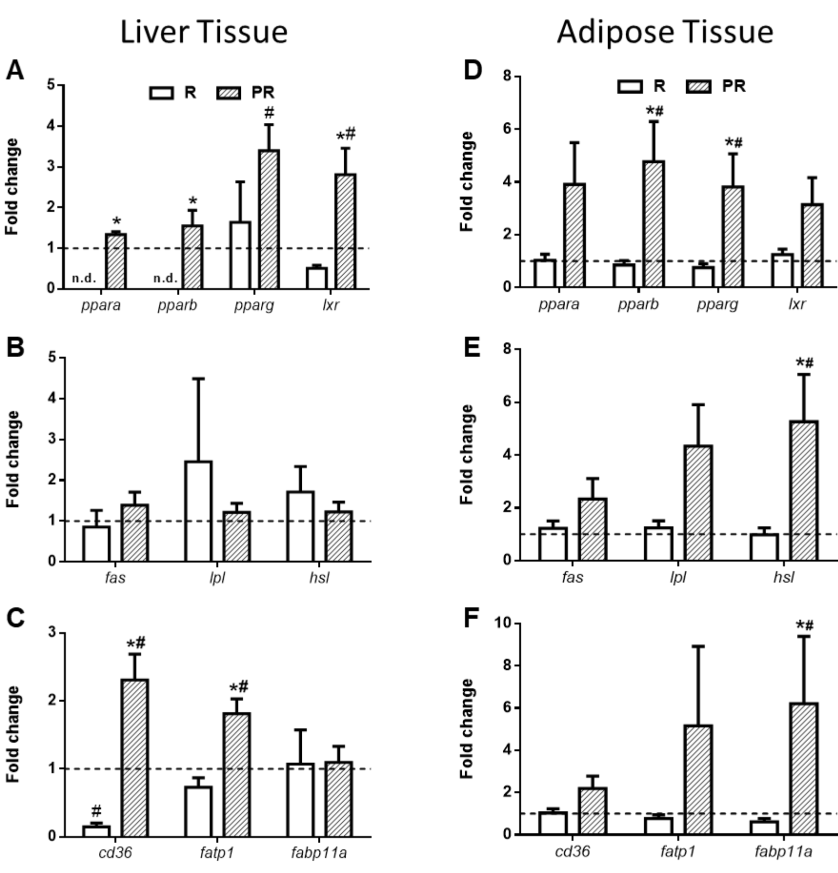


**Fig. 5.** Fold change expression of transcription factors **(A, D)**, lipid metabolism-related genes **(B, E)** and fatty acid transporters **(C, F)** in the liver **(A-C)** and adipose tissue **(D-F)** of fish fed with the experimental diets R or PR respect to fish fed diet P (dotted line) and reared at 28 ºC for 2 months. Data are shown as mean ± SEM (n=7). Asterisks indicate significant differences between fish feed with R or PR compared to P. Hash indicate significant differences between R and PR diets (p<0,05). Palm (P); Rapeseed (R); Palm + Rapeseed (PR).

## 4. Discussion

The present study aimed to elucidate the possible effects of FO substitution by different VO in a future scenario of increased water temperature, since further knowledge is required to take preventive measures at the level of aquafeeds formulation to optimize sustainable aquaculture production of gilthead sea bream assuring fish welfare and product quality.

In this context, elevated temperature (28 ºC) significantly increased somatic growth, while decreased VSI, HSI and MFI in comparison with fish reared at 21 ºC independently of the administered diet. According to this, studies on Atlantic salmon (Handeland et al, 2000; 2003; Handeland, Imsland & Stefansson, 2008) and gilthead sea bream (Guillaume et al, 2004) previously demonstrated that growth is increased by elevated temperatures, as expected in agreement with a boosted fish metabolism. Nevertheless, other factors can be involved in pushing this growth enhancement since temperature is known to be key in the digestibility of fatty acids; being lower with increasing saturation and chain length as seen in salmonids (Caballero et al, 2002; Guillaume et al, 2004; Ng et al, 2004; Olsen & Ringø, 1998; Torstensen, Lie & Frøyland, 2000), as well as especially at a reduced temperature (Ng et al, 2004; Olsen & Ringø, 1998). Thus, the importance of VO substitution at different temperatures remains partly in the melting point of the fatty acids present in each oil (Guillaume et al, 2004). This is in concordance with the results found in our study, in which fish reared at elevated temperatures presented higher growth rates, possibly as an indicative of greater feed efficiency due to the higher digestibility of dietary lipids at 28 ºC even when fish are feeding the P diet, containing a high content of SFA (i.e. with reduced digestibility). To

confirm this hypothesis, data on feed intake or diet digestibility would have been useful, but these parameters were not possibly analysed in our experimental conditions. Regarding HSI, this parameter was demonstrated to be also influenced, increasing with low temperatures and decreasing at high temperatures in largemouth bass (*Micropterus salmoides*) and in Kumgang Fat Minnow (*Rhynchocypris kumgangensis*), respectively (Heidinger & Crawford, 1977; Im, Kong & Ghil, 2016) according to our findings, where fish at 28 ºC presented lower HSI when compared to fish at 21 ºC.

Besides, fish fed with diet P at 28 ºC presented lower plasma levels of TGs in comparison with fish maintained at 21 ºC. Ongoing with the considerations exposed above, Ng, Lim and Boey (2003) described in rainbow trout fed a PO diet at a low temperature an increased presence of undigested TGs in feces, therefore supporting that a high temperature appears to improve lipid digestibility. Another aspect to take into account is that, at high temperatures, stomach evacuation had been showed to be faster than at low temperatures in salmon (Handeland et al, 2008). In this last study, salmon where reared at 6, 10, 14 and 18 ºC and stomach evacuation after 24 hours in fish at the highest temperature was almost 100 percent whereas in fish at the lowest temperature was only 50 percent. Thus, the differences observed in our experiment in metabolites plasma levels could have been consequence of the faster time of stomach evacuation (and thus nutrient absorption) in the gilthead sea bream maintained at 28 ºC. Different conditions between studies could have also produced these substantial differences on temperature effects. In fact, parameters as animal size can affect the response in front of temperature changes, as seen in smaller juvenile Atlantic cod (*Ghadus morua* L.) in which warm temperatures were shown to seem favourable whereas in larger specimens were not (Tirsgaard, Behrens & Steffensen, 2015). In this context, our study demonstrated that high temperature decreased TGs and slightly decreased NEFAs levels in gilthead sea bream plasma suggesting better metabolic performance.

Respecting the effects of temperature in adipose tissue and liver at a histological

level, fish maintained at 28 ºC presented more smaller adipocytes as well as LD of smaller area in the liver than those fish reared at 21 ºC, suggesting induced adipogenesis with increased formation of new adipocytes, and enhanced lipid degradation and disminished lipid uptake in the liver. In fact, the increase in water temperature in salmon has been observed to decrease (Hevrøy et al, 2012) or increase (Frøyland et al, 2000) β-oxidation in liver but more concretely, in juvenile Atlantic salmon at 20 or 10 ºC fed three different oil-blend diets, high water temperature led to a significant increase in total fatty acid β-oxidation (Norambuena et al, 2015). Moreover, in darkbarbel catfish (*Pelteobagrus vachellii*) liver has been described a reduction in cholesterol and TGs levels at elevated temperatures, suggesting an increase of hepatic lipid metabolism, thereby accelerating the absorption and utilization of lipids and leading to decreased fat deposition and a low HSI (Qiang et al, 2017) in concordance with our data.

Nevertheless, although hepatic LD were small in size at 28 ºC the total surface occupied by LD was higher at this temperature. These observed histological features are not completelly explained by the changes in relative gene expression in liver and adipose tissue induced by temperature. In fish fed the P diet and maintained at 28 ºC, contrarily to the possible hyperplasia observed by histology, gene expression of the key regulator of adipogenesis, *pparg*, was downregulated in adipose tissue when compared to fish at 21 ºC. In fact, the pattern of expression of this transcription factor in cultured preadipocytes from different fish species in the presence of lipids in the medium, has showed a transient increase at shorter times without clear changes in the long term during the adipogenic process (Salmerón et al, 2016; Riera-Heredia et al, *unpublished*). On the other hand, elevated temperature downregulated also almost all genes implied in fatty acid transport either in liver or adipose tissue when compared to fish maintained at low temperature. This result, seemed to be in accordance with the reduced MFI at 28 ºC and with the results observed in adipose and liver histology where hypertrophy and high lipid accumulation respectively, were avoided at high temperature, confirming the possible induction of the usage of TGs in storage in adipose tissue

and fatty acid oxidation in liver while reducing lipid mobilization between tissues. All this suggests that high temperatures even in fish fed lipid content from VO in the diet seem to be beneficial to obtain good growth rates and proper metabolic function as well as fish with healthy adipose tissue through enhanced adipogenesis as shown in humans (Choe et al, 2016).

With respect to the feeding with the different substituted diets P, R or PR in fish reared at 28 ºC, differences in growth were not observed despite of fish fed P diet presented slightly increased WG but decreased VSI and MFI. Similarly, a significant increased growth in fish fed with PO was seen in a recent study where 10 experimental diets with different VO substitutions were tested in gilthead sea bream under normal farming conditions (Sánchez-Moya et al, 2019). Actually, several studies have proved in different fish species that PO can improve growth performance or at least not to affect growth negatively (Fonseca-Madrigal et al, 2005; Lim et al, 2001; Ng et al, 2003, 2004; Rosenlund et al, 2001; Torstensen et al, 2000). Nevertheless, in our study, this result was not significant, in concordance with other studies in Atlantic salmon, sea bass and sea bream as well (Rosenlund et al, 2001; Izquierdo et al, 2003, 2005; Fountoulaki et al, 2009), where fish fed diets with different VO substitution (up to 69%) presented similar or poorer growth performance, feed and protein utilization when feeding PO diets compared to diets with RO or SO. The differences found between studies could be the result of different fish size (Tirsgaard et al, 2015), percentatge of substitution used (Lim et al, 2001), challenge time or rearing conditions of the fish.

As mentioned above, at elevated temperatures the digestibility of fatty acids present in the different substituted diets could increase. Even so, the tissue lipid composition is known to be affected by the FO substitution with VO (Francis et al, 2006; Izquierdo et al, 2005; Montero et al, 2005). In Atlantic salmon, it has been observed that the effect of different VO substitution was reflected in the total lipid present in the liver being the diet with RO inclusion the responsible of increased lipid accumulation (Bell et al, 2001; Karalazos et al, 2007; Ruyter et al, 2006; Torstensen, Frøyland & Lie, 2004). In the present study, liver histology

demonstrated that fish fed with R diet presented more LD, which is in concordance with those studies. The possible explanations of this increased lipid accumulation in hepatocytes could be associated to impaired lipoprotein synthesis (Caballero et al, 2006), or an imbalance between hepatic lipid synthesis, oxidation or their export to other tissues (Postic and Girard, 2008). Another explanation could be the unbalanced n-3/n-6 ratio (Robaina et al, 1998; Wassef, Wahby & Sakr, 2007) derived from the n-6 fatty acids present in the VO used in detriment of n-3 from FO, together with the increased amount of n-9 oleic acid present in some VO. In this context, Fountoulaki et al (2009) concluded that the high amount of oleic acid was responsible for the observed excessive hepatic lipid accumulation in *Sparus aurata*, over fish fed soya or RO diets. Plasma levels and liver histology results are consistent with those found in Atlantic salmon, where VO increased liver TGs stores whereas in plasma the lipid content diminished (Jordal, Lie & Torstensen, 2007). Otherwise, smaller adipocytes as well as less area occupied by also smaller LD in the liver was found in fish fed PR diet compared to the other dietary groups at 28 ºC. As discussed before, a higher presence of smaller adipocytes reflects the possible increased capacity of the tissue to recruit preadipocytes and to develop new adipocytes avoiding a damaging hypertrophy (Medina-Gomez & Vidal-Puig, 2005). Contrarily in gilthead sea bream fed diets with 66% of FO substitution (6% more than in the present study) by a mixture of VO with LO as principal VO (28% more than in the present study), it was observed an enlargement of the adipocytes thus impairing the storage capacity of this tissue (Cruz-García et al, 2011). On the other hand, the reduced hepatic area occupied by also smaller LD in those fish fed diet PR, could possibly be because of increased FA oxidation (Kolditz et al, 2008).

In line with this, fish fed the PR diet showed an activation of lipid metabolism in liver and adipose tissue as deduced from the qPCR results. Upregulated expression of *pparg*, *cd36* and *fatp1* in liver and *pparb, pparg, hsl* and *fabp11* in adipose tissue indicated increased fatty acid mobilization and use. In this sense, contradictory results about the effects of dietary lipids in liver gene expression have been reported, with *pparg* not showing nutritional regulation in salmon (Jordal et al,

2007; Morais et al, 2011). Indeed, in Jordal et al (2007) an upregulation of *pparg* expression was observed in a time point that coincided with high lipid storage levels in the Atlantic salmon liver.

In a recent study (Torrecillas et al, 2017), a blend of linseed oil, RO and PO was produced as a FO substitution for being a combination assuring a balance of SFA and MUFA in sea bass tissues. Up to 60% FO substitution in European sea bass by two mixtures of these three VO did not cause a marked effect on growth, lipogenesis and tissue lipid uptake when compared to a 100% FO diet (Richard et al, 2006). In gilthead seabream no impairment of lipogenic activity and lipid content in fish liver was detected when using a blend of PO and LO (4:1) to replace FO (Bouraoui et al, 2011). Recently in the same species, fish fed with different oil blends demonstrated to avoid damaging effects of individual VO for substitution, as RO, by lowering its proportion by mixing it with LO or PO (Sánchez-Moya et al, 2019).

Overall, the combination of fatty acids from PO and RO in the formulation of the PR diet seems to be the most equilibrated regarding histological data and metabolic status, supporting the gilthead sea bream to grow and have balanced lipid accumulation even in a scenario of a water temperature rise. Thus, the present study has demonstrated that dietary lipid sources have an important impact on adipose tissue and liver lipid metabolism, indicating that it is necessary to take care and measure of the substitution by raw materials in the elaboration of feeds for this species for the sustainability of aquaculture in the future.

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# ARTICLE VII

**Gene expression analyses in malformed skeletal structures of gilthead sea bream (*Sparus aurata*) (2019).**

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# Gene expression analyses in malformed skeletal structures of gilthead sea bream (*Sparus aurata*)

**Running title: Gene expression in malformed fish bone**

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**Abstract**

The incidence of skeletal anomalies in reared fish has been translated for years in important economic loses for the aquaculture industry. In the present study, we have analysed the gene expression of extracellular matrix components and transcription factors involved in bone development in gilthead sea bream presenting different skeletal anomalies: lordosis (LD), lordosis-scoliosis-kyphosis (LSK) or opercular, dental or jaw malformations in comparison to control (CT) specimens. Results showed a possible link between the presence of LD and LSK and the significant down-regulation of genes involved in osteoblasts maturation and matrix mineralization (collagen type 1-alpha, osteopontin, osteocalcin, matrix Gla protein and tissue non-specific alkaline phosphatase), as well as in bone resorption (cathepsin K and matrix metalloproteinase 9) compared to CT animals. Contrarily, the key osteogenic transcription factor *runx2* was up-regulated in the malformed vertebra suggesting impaired determination of mesenchymal stem cells towards the osteoblastic lineage. Despite the gene expression patterns of the other malformed structures were not affected in comparison to CT fish, the results of the present study may contribute in the long term to identify potential candidate gene profiles associated to column deformities that may help reducing the incidence of appearance of skeletal anomalies in this important aquaculture species.

**Keywords:** osteogenesis, skeletal anomalies, lordosis-scoliosis-kyphosis, lack of operculum.

## 1. Introduction

Aquaculture research is focused on obtaining the most sustainable and profitable production, minimizing the cost, but assuring a good high-quality product. Notwithstanding, one of the major problems being faced in the last decades is the elevated presence of skeletal anomalies, leading to important economic loses. Those fish that are severely affected either die, have to be early discarded during the selection process or downgraded because they are not suitable for the market. The incidence varies greatly among farms, species and batches, and even depending on the accuracy of the different detection methods used (for a review see Boglione *et al.,* 2013). Nevertheless, in Spanish aquaculture, up to 30% of marine fish larvae have on average, at least one type of malformation (Afonso *et al.,* 2000; Boglione *et al.,* 2001). Gilthead sea bream (*Sparus aurata*) is a valuable Mediterranean species, for which a 27% incidence of skeletal anomalies, with a survival rate of only 5% of these malformed specimens, was reported 3 decades ago (Andrades *et al.,* 1996). Nonetheless, 15 to 50% of *Sparidae* juveniles with severe anomalies are nowadays culled out of the productive cycle at the end of the hatchery phase (Boglione & Costa, 2011); indicating this is still an important bottleneck.

Deformities can appear elsewhere, causing serious problems to the individuals (e.g. affecting feeding and growth), compromising their welfare and survival, as reported for European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (Matsusato *et al.,* 1986; Divanach *et al.,* 1996; Koumoundouros *et al.,* 2002). Some of the most common affectations described are in the cranial region, affecting the operculum (OPC), jaw (JW) or dentary bone (DNT), as well as in the vertebral centra, where lordosis (LD), scoliosis, kyphosis or a combination of these three anomalies leading to a more severe malformation referred to as LSK could be observed. Among the skull anomalies, the gill cover has been recorded to affect up to 80% of gilthead sea bream individuals (Koumoundouros, 2010) and pugheadness to be the more frequent among the DNT and JW anomalies also in this species. Even so, in Mediterranean marine species including gilthead sea

bream, LD is one of the best-studied malformations. In that species, haemal LD for example can be found in about 13% of the specimens (Georgakopoulou *et al*., 2010), whereas kyphosis or scoliosis are rarely observed. Among the non-genetic factors discussed as causative of LD (along the entire vertebral centra), there are the non-inflation of the swim bladder, inappropriate tank hydrodynamism or feeding behaviour, although discrepancies exist in the literature about their relative importance (reviewed in Boglione *et al*., 2013). In Atlantic salmon (*Salmo salar*), temperature seems to be the most potent causative factor of spinal anomalies (Wargelius *et al*., 2010); however, genetic factors have also been postulated as responsible in non-salmonid fish. In gilthead sea bream, development of the LSK syndrome has been associated with family structure (Afonso *et al*., 2000), and consanguinity has been shown to increase the incidence of LD and lack of an OPC, having both characters high heritability (Afonso *et al*., 2009; Navarro *et al*., 2009). More recently, García-Celdrán *et al*., (2015) and Negrín-Báez *et al*., (2015) also reported a significant relationship between the prevalence of LD, LSK and lack of OPC and the mating of broodstock of different origins suffering from the same deformity, thus confirming the importance of inheritance in the presence of these malformations in gilthead sea bream.

Despite both, simple Mendelian genetics and polygenic determinants could explain at least in part the incidence of skeletal anomalies; studies evaluating alterations in specific genes are limited in fish. In this sense, zebrafish (*Danio rerio*) carrying a mutation in the collagen type 1 alpha-1 (*col1a1*) has been identified as a model of osteogenesis imperfecta, a type of skeletal dysplasia (Fisher *et al*., 2003). Moreover, several mutants for genes involved in arch development have also been reported in the same species presenting craniofacial abnormalities (Schilling *et al*., 1996; Piotrowski *et al*., 1996). In gilthead sea bream, transcripts characteristic of bone cells have been recently identified and their expression associated with the different processes that occur during osteogenesis (Vieira *et al.,* 2013; Riera-Heredia *et al.,* 2018); although studies potentially relating dysregulation of these genes with the emergence of malformations are, as far as we know, absent in this

species. The principal transcription factor involved in the control of osteoblast differentiation is Runt-related transcription factor 2 (Runx2) (Vieira *et al.,* 2013). Then, COL1A1 plays an important role in structuring and stablishing the production of the extracellular matrix (ECM), while the tissue non-specific alkaline phosphatase (TNAP) and the non-collagenous proteins, osteonectin (ON), osteopontin (OP) and osteocalcin (OCN), are required for osteoblast maturation and ECM mineralization during bone development (Riera-Heredia *et al.,* 2018). Besides, considering abnormalities are more frequent in larval and juvenile fish as they exhibit increased bone growth and turnover rates, factors controlling bone resorption should be also considered. Characteristic markers of osteoclasts involved in bone matrix degradation, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK) and matrix metalloproteinase 9 (MMP9) have been also identified in gilthead sea bream (Vieira *et al.,* 2013).

The main objective of this study is to increase knowledge about the involvement of gene factors in the onset of skeletal anomalies in gilthead sea bream. Special attention is paid to the relationship between the expression of potential candidate genes and the occurrence of a specific deformity. The ultimate goal is to further use this information to optimize the aquaculture production of high-quality fish with reduced incidence of bone malformations.

## 2. Materials and Methods

### *2.1. Animals and ethics statement*

Gilthead sea bream (*Sparus aurata*) were obtained from the hatchery Tinamenor S.L. (Pesués, Cantabria, Spain). Fish were reared on standard hatchery conditions at 20-22ºC, 90-110% $O_2$ saturation, 15:9 daily photoperiod, and were fed throughout the day on a diet containing 56% protein and 18% lipid (Perla, Skretting). Animal handling procedures complied with the guidelines of the European Union Council (86/609/EU), Spanish and Catalan Governments legislation and were approved by the Ethics and Animal Care Committee of the University of Barcelona. Forty-seven animals from the same batch of production

with or without skeletal anomalies and an average weight of 2.7±0.1 g, were pre-selected by the hatchery personnel trained to do the usual discarding of malformed animals and transferred to the University of Barcelona. Transportation was done by road with the fish in sealed cubitainers with saturated oxygen at very low density to minimize stress. The fish were sampled at arrival, and the anomalies identification was done according to visual inspection by all the researchers taking part in the sampling. Figure 1 shows representative images of the different malformed animals, and the sampling was performed as schematically illustrated in Figure 2. Samples (n=6-10 per group) were taken from control (CT) normal specimens or animals with lordosis (LD), lordosis-scoliosis-kyphosis (LSK), or malformations in the operculum (OPC), dental bone (DNT) or the jaw (JW). For the column malformations, once the cleaning of the vertebrae was performed, the phenotype was confirmed as the one identified during the external visualization. Samples of vertebrae from the LD specimens were taken both, from affected (LDA) and normal non-affected (LDN) portions of similar size, although the location along the column for each sample may have varied from one fish to another depending on the specific position of the malformation. All bone samples were immediately frozen using liquid nitrogen and stored in the freezer at -80ºC until RNA extraction.

*2.2. RNA extraction and cDNA synthesis*

Total RNA was extracted using Tri Reagent (Life Technologies, Alcobendas, Spain) following the manufacturer's instructions, quantified using a NanoDrop2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and, the quality analysed by 1% (w/v) agarose gel electrophoresis. One µg of total RNA per sample was DNase treated (Life Technologies, Alcobendas, Spain) and used to synthesise first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Vallès, Spain) following the manufacturer's recommendations.

*2.3. Quantitative real-time PCR (qPCR)*

The qPCR assays were conducted according to the requirements of the MIQE guidelines (Bustin *et al.,* 2009) using a CFX384$^{TM}$ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). Prior to the analyses, specificity of the reaction, absence of primer-dimers formation, as well as identification of the appropriate cDNA working dilution for each assay was determined by running a dilution curve with a pool of samples. Reactions were performed in triplicate and contained cDNA, iQ SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain) and 250 nM (final concentration) of forward and reverse primers (Table 1). The protocol consisted on 1 cycle of 3 min at 95ºC and 40 cycles of 10 s at 95ºC and 30 s at 60-68ºC (primer dependent, see Table 1), followed by an amplicon dissociation analysis from 55 to 95ºC with a 0.5ºC increase every 30 s. SYBR Green fluorescence was recorded during the annealing-extending phase of cycling. Expression results were normalized to the geometric mean of ribosomal protein L27a (*rpl27a*) and elongation factor 1α (*ef1a*) as they were the most stable of the reference genes tested according to the GeNorm algorithm implemented in the CFX Manager Software (Bio-Rad, El Prat de Llobregat, Spain). Target transcript abundance was analysed using the delta-delta method (Pfaffl, 2001) with the same CFX Manager Software (Bio-Rad, El Prat de Llobregat, Spain). In addition to showing the quantitative gene expression results of each malformation with the corresponding CT samples, in order to compare the overall changes in gene expression among malformed tissues, a heat map was created. The data from the deformities in the different structures: vertebral column (VC; combining LD and LSK), OPC, DNT and JW were analysed relative to their corresponding CT samples and standardized (i.e. standard score normalization), and a heat map with these values was then generated using PermutMatrix version 1.9.3 (Caraux and Pinloche, 2005).

*2.4. Statistical analyses*

Statistical analyses of all parameters were performed in SPSS Statistics version 20 (IBM, Armonk, NY, USA). Normality was analysed according to the Shapiro-Wilk test and homogeneity of variance according to Levene’s test. Statistical

differences among vertebral column groups (CT, LD and LSK) were assessed by one-way ANOVA, followed by Tukey's *post hoc* test. Statistical differences between malformed OPC, DNT and JW with their respective CT group and between the LDA and LDN regions and each one of them with the CT column were assessed using a student T-test. Significant differences were considered at $p < 0.05$ for all statistical tests performed. Data are presented as mean ± standard error of the mean (s.e.m.). Significant differences are indicated with different letters or asterisks.

## 3. Results

Relative gene expression from vertebra bone was analysed in CT, LD and LSK fish. As indicated in the material and methods section, LDN samples from non-affected column regions of LD animals were extracted in addition to the malformed LDA fragments and evaluated separately. Despite that tissue was visually normal, the pattern of expression of the different genes analysed in LDN samples was similar to that of the LDA samples and significantly different from that of the CT animals (Table 2). Therefore, expression data from both LDN and LDA column samples of the lordotic specimens were combined and presented together (named as LD) in Figure 3, where comparisons were made with the CT and LSK specimens.

Regarding ECM components, a decrease in their expression was found in the vertebral column along with the increased severity of the malformation types for all the genes studied (Fig. 3A). *col1a1* and Matrix Gla protein (*mgp*), presented a significantly lower expression in LSK when compared to CT fish, with LD values being intermediate; while *ctsk* showed only significantly lower levels of expression in LD compared to CT animals. In the case of *op*, *tnap* and *mmp9*, transcript expression was significantly lower in both deformed bones than in CT ones. Finally, *ocn* presented the clearest differences, since the three conditions were significantly different; being this gene more expressed in CT animals than in LD,

and in those more than in the fish presenting the LSK syndrome that showed the lowest levels.

On the other hand, the mRNA levels of most of the transcription factors analysed were similar among the three animal groups (Fig. 3B). Only LD fish showed a significantly higher expression of *runx2* compared with the CT specimens, but those had a significantly higher expression of distal-less homeobox 5a (*dlx5a*) compared to both LD and LSK fish.

When the relative gene expression of all these molecules was tested in OPC-affected fish compared to CT animals, significant differences were not seen but some tendencies were observed (Fig. 4A and 4B). Thus, fibronectin 1a (*fib1a*), *runx2* and distal-less homeobox 2a (*dlx2a*) appeared to increase in deformed OPC samples.

In the DNT tissue (Fig. 5A and 5B), *dlx2a* was the only gene differentially expressed, being lower in the malformed fish compared to the CT one, while the rest of the genes analysed only showed tendencies, like *on* and distal-less homeobox 6 (*dlx6*), which were less expressed in malformed than in CT fish.

Considering the JW (Fig. 6A and 6B), great variability was observed among samples and significant differences were not found. Nevertheless, the most expressed genes in the malformed tissue compared to CT samples were *fib1a, runx2* and *dlx6*.

Finally, a comparative study was carried out analysing all tissue deformities respect to their corresponding CT samples (Fig. 7). Different behaviours were seen depending on the origin of the tissue and the type of molecule evaluated. Regarding ECM components, whereas in the JW most genes were up-regulated (red), in the vertebral column (VC), OPC and DNT almost all genes were down-regulated (green) with the exception of *fib1a* (Fig. 7A). On the other hand, transcription factors were mainly up-regulated (red) in the VC, OPC and JW deformed tissues in comparison to the CT samples, especially *runx2*, which also appeared increased in the DNT bone (Fig. 7B).

## 4. Discussion

In the present study, for the first time in gilthead sea bream, the gene expression profile of osteogenic ECM molecules and transcription factors in fish presenting different skeletal malformations (LD, LSK, OPC, DNT and JW) was evaluated in comparison to CT specimens, with the objective of trying to link specific gene patterns with anomalies. Although the data was not so outstanding for the cranial malformations, in the case of the vertebral centra, the changes in expression of several genes appeared to be related to the type of anomaly (LD or LSK).

The coordinated and sequential expression of specific osteogenic molecules is required for the proper development of the skeleton in *Sparus aurata* (Riera-Heredia *et al.,* 2018). This expression pattern is susceptible to be altered by genetic factors, and heritability of certain types of malformations has been reported in this species (Navarro *et al*., 2009; Negrín-Báez *et al.,* 2015). In our current findings, LD and LSK fish had up-regulated *runx2* expression but otherwise, *col1a1, op, ocn*, *mgp*, *tnap* and *mmp9* were down-regulated compared to the CT specimens, supporting that altered expression of these genes might be linked to the appearance of the skeletal anomaly. Moreover, abiotic factors such as nutrition and temperature (Gisbert *et al.,* 2008), or a combination of factors (genotype+environment) can also modify gene expression, leading to increased incidence of skeletal malformations. For example, a temperature drop challenge has been demonstrated in gilthead sea bream to have an impact impairing bone responsiveness (Mateus *et al.,* 2017; Riera-Heredia *et al.,* 2018). In Atlantic salmon, embryonic rearing at an elevated water temperature modifies the expression of several osteogenic genes and is associated to a higher number of vertebral deformities once the fish reach the juvenile state (Ytteborg *et al.,* 2010b).

Furthermore, lordotic animals had visually an affected as well as a non-affected region in their vertebral column; although using other analytical methods (i.e. radiography or histology) it is plausible considering that other tissue defects along the whole column might have been identified. In Atlantic salmon, up to four

vertebral regions have been described with different morphology and gene expression profiles during normal development (Kacem *et al*., 1998); and also, that site-specific deformities could develop differentially within a particular life stage or environmental condition (i.e. seawater *versus* freshwater), maybe due to this patterning (revised by Fjelldal *et al*., 2012). In our study, recording of the specific location of the malformation in lordotic gilthead sea bream was not performed, but in all cases, the gene expression pattern of LDN samples was similar to the LDA. This result demonstrated that the presence of a vertebral anomaly affects at a transcriptional level not only locally the malformed part of the tissue, but also the expression pattern of the entire column. Similarly, Boursiaki *et al*., (2019) observed recently that the presence of scoliosis in gilthead sea bream is more frequent in the caudal region, but in those malformed fish, both the caudal and abdominal vertebrae were significantly shorter when compared to the vertebrae of a normal fish. In other studies, with *Salmonidae* species, malformed vertebrae have been shown to have comparable mineral content to normal vertebrae, even if differences existed at an earlier stage of the malformation process (Fjelldal *et al*., 2012). Overall, these data support our findings on gene expression in LD animals despite the location of the malformation in the column. Therefore, although we cannot know if the altered expression observed is the cause of the malformation and not a consequence; our results suggest that the genes identified (or at least their combined expression profiles) are potential candidates as markers to tag and identify possible defects in bone maturation resulting in malformed ossified tissue in gilthead sea bream.

The relevance of the specific ECM genes that were found down-regulated in our malformed fish has been demonstrated in mammalian studies. For example, the human genetic disorder osteogenesis imperfecta, associated with a mutation in *col1a1*, induces severe skeletal deformities or mobility problems (Steiner *et al.,* 2005). In zebrafish, a similar mutation causes defects in bone growth (Fisher *et al*., 2003). Taken into account that 90% of the bone ECM is composed of collagen, specifically type I, the fact that in our study the most severe skeletal malformation

(SLK) showed significantly the lowest transcript levels of *col1a1*, indicates a likely possible problem for that tissue to produce a normal ECM. Together with COL1A1, among the non-collagenous ECM proteins required for bone matrix mineralization, the down-regulation of *on*, *op* and *ocn* upon temperature treatments, has been associated with a restrained differentiation of Atlantic salmon cultured osteoblasts (Ytteborg *et al.,* 2010a), and a defect in the late maturation of osteoblasts in mammals (Termine *et al.,* 1981; Boskey, 1992). In fact, Delany *et al.,* (2000) reported that *on*-null mice display decreased trabecular bone volume and stiffness than wild type mice. Similarly, in *ocn*-KO mouse maturation of mineral crystals in the bones is compromised (Boskey *et al.,* 1998). Furthermore, inappropriate calcification, osteomalacia or soft bones can be caused by alterations in the function of the phosphatases system in mice (Millán *et al.,* 2013). This could be also speculated as causative of vertebral malformations in gilthead sea bream, since a significant down-regulated expression of *tnap* was also found in LD and LSK fish in comparison to CT specimens.

Skeletal growth is controlled by facilitating bone formation and limiting bone degradation, which is mediated by osteoclasts through the action of CTSK, TRAP and MMP9. *ctsk* and *trap* gene expression in goldfish (*Carassius auratus*) scales correlated with the need of calcium during the reproductive season (Azuma *et al.,* 2007), while *mmp9* was up-regulated during tissue remodelling in the regenerating fin of the teleost fish *Poecilia latipinna* (Rajaram *et al.,* 2016). Altered expression of these osteoclast markers have not been studied in fish yet in relation to skeletal anomalies, but in humans and in mice, a mutation in *ctsk* has been reported to cause supernumerary teeth due to reduced osteoclast activity (Helfrich, 2005). In our study, in CT fish, the significantly higher levels of *ctsk* expression found compared to LD, and those of *mmp9* compared to LD and LSK specimens, could thus suggest a balanced bone formation and resorption ratio in these non-affected fish, although more research would be required to confirm this hypothesis.

In addition to well-adjusted bone turnover for proper skeletal development, recruitment of new osteoblast cells is also important. Runx2 is one of the key

factors that has been identified in zebrafish during skeletal tissue development (Flores *et al.,* 2006), and more recently also in gilthead sea bream (Vieira *et al.,* 2013). As in mammals (Liu & Lee, 2012), up-regulation of *runx2* gene expression in fish with a column deformity supposes a mismatch between the developmental stage and the bone status leading to a fragile tissue due to higher presence of non-mature osteoblasts (Ytteborg *et al.,* 2010a). To this end, the expression of *runx2* has been demonstrated to be clue in the lineage determination of mesenchymal stem cells (MSCs) towards osteoblasts *in vitro*. In this sense, in bone-derived MSCs from gilthead sea bream, cell fate was modulated down-regulating *runx2* (Riera-Heredia *et al.*, 2019) thus, compromising the bone tissue integrity, as previously seen in mammals (Beredsen & Olsen, 2014). Similarly, in lordo-kyphotic Senegalese sole (*Solea senegalensis*), condensation of MSCs or trans-differentiation of pre-osteoblastic cells to the chondrocyte linage has been hypothesized (Cardeira *et al*., 2012).

Other factors involved in osteoblasts differentiation and proliferation belong to the family of the distal-less homeobox proteins (DLX) (Bendall & Abate-Shen, 2000; Komori, 2006; Marie, 2008). In zebrafish, DLX5 is present in the developing visceral skeleton and during scale regeneration (Tamamongood *et al.,* 2012, Verreijdt *et al.,* 2006). The present results reflected a significant down-regulation of this gene in LD and LSK specimens compared to CT fish, indicating again an association between the presence of these deformities and changes in the gene expression of tissues formed at the onset of development. Regarding this, morpholino-mediated knock-down of *dlx5a/6a* in zebrafish showed a failure of cleithrum formation (Heude *et al.,* 2014). Moreover, DLX2a has been related with dental development in mammals (Zhao *et al.,* 2000) and zebrafish (Borday-Birraux *et al.,* 2006). Despite being an early developmental gene in the latter species (Verreijdt *et al.,* 2006), our results in gilthead sea bream showed a significant decrease in the expression of *dlx2a* in malformed DNT specimens compared to their corresponding CT fish, suggesting a possible imprinted failure effect during development.

Overall, the major differences in gene expression associated to a skeletal malformation in the gilthead sea bream of the present study have been found in the vertebral column. Contrarily the other malformed structures analysed (OPC, DNT and JW) did not shown significant changes in gene expression patterns. Development of skeletal anomalies is a complex process that may be explained not only in terms of gene transcription alteration, but also, due to other types of factors, which can also interact with each other. In fact, although in gilthead sea bream, skeletal deformities like LD, LSK, lack of OPC or vertebral fusion, have been related to genetic components and inheritance (García-Celdrán *et al*., 2015; Navarro *et al*., 2009; Negrín-Báez *et al*., 2015), some other authors have proved that rearing conditions, stress or inadequate feeding can also be leading to deformities in skeletal structures (Koumoundouros *et al.,* 1997; Prestinicola *et al.*, 2013). In this sense, Prestinicola *et al*., (2013) demonstrated that it is possible to lower the severe skeletal anomaly incidence and the meristic count variability of dermal bones of reared gilthead sea bream juveniles by lowering stocking densities. Furthermore, synchronicity between bone and muscle growth is required for proper musculoskeletal development (Vélez *et al.,* 2018; Ytteborg *et al*., 2012). Hence, enhanced muscular growth induced by hyperthermia or activity from too intense water currents was significantly associated with increased vertebral deformities in Atlantic salmon (Ytteborg *et al*., 2010b) and European sea bass (Divanach *et al*., 1997), respectively. Furthermore, in mammals, the bone-produced OCN has been reported as crucial for the adaptation of the skeletal muscle to exercise (Mera *et al.,* 2016), suggesting that the crosstalk between bone and muscle tissues is as a factor to be taken into account to fully understand the causes behind fish skeletal malformations.

To sum up, in our study, for vertebral column deformities (LD and LSK), the pattern of expression of genes related to ECM maturation and mineralization as *col1a1*, *op, ocn, mgp* and *tnap,* as well as those involved in bone resorption like *ctsk* and *mmp9,* seem to be key to discern between normal and potentially malformed fish. Contrarily, in OPC, DNT and JW malformations, different

expression profiles had not been observed, making difficult to point to possible gene markers for these anomalies. Notwithstanding, besides having observed clear affectations in specific bones concomitantly to alterations in the relative expression of some genes involved in bone turnover, it would be necessary to analyse other batches of fish from different hatcheries, before drawing final conclusions, in addition to performing more detailed analyses to further characterize the different pathologies. Thus, more studies are necessary to confirm if the genes expression profiles proposed can be markers of bone development to detect possible malformations to assure optimized production of fish, which would be of real interest for the aquaculture industry.

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# 6. Tables

**Table 1: Primers used for real-time quantitative PCR.**

Tm: melting temperature, Acc. Num.: Gen Bank accession number. Beta-actin (*β-actin*); Collagen type 1 alpha-1 (*col1a1*); Cathepsin K (*ctsk*); Distal-less homeobox 2a, 3b, 5a and 6 (*dlx2a, dlx3b, dlx5a* and *dlx6*); Elongation factor 1-alpha (*ef1α*); Fibronectin 1a (*fib1a*); Matrix Gla protein (*mgp*); Matrix Metalloproteinase 9 (*mmp9*); Osteocalcin (*ocn*); Osteonectin (*on*), Osteopontin (*op*); Osterix (*osx*); Ribosomal protein L-27 (*rpl27*); Runt-related transcription factor (*runx2*); Tissue non-specific alkaline phosphatase (*tnap*); Tartrate-resistant acid phosphatase (*trap*).

| *Gene* | *Primer sequence (5'-3')* | *Tm (°C)* | *Acc. Num.* | *Reference* |
|---|---|---|---|---|
| ***β-actin*** | F: TCCTGCGGAATCCATGAGA<br>R: GACGTCGCACTTCATGATGCT | 60 | X89920 | Vélez *et al.*, 2014 |
| ***col1a1*** | F: GAGATGGCGGTGATGTGGCGGAGTC<br>R: GCCTGGTTTGGCTGGATGAAGAGGG | 68 | DQ324363 | Rosa *et al.*, 2010 |
| ***ctsk*** | F: AGCGAGCAGAACCTGGTGGAC<br>R: GCAGAGTTGTAGTTGGGGTCGTAG | 60 | DQ875329 | Salmerón *et al.*, 2015 |
| ***dlx2a*** | F: CGGAGCGATCTCGTATAGCC<br>R: ACACGGGGATGCTTTTCGAT | 60 | SRR278741_ isotig04863 | Present study |
| ***dlx3b*** | F: CTGCCACAAGTGATCAGGAA<br>R: CTGCAGAGCAAACAGTCGAG | 60 | SRR278741_ isotig06751 | Present study |
| ***dlx5a*** | F: CTCCAAGAGCCAACGAGTGT<br>R: AGCCTACAGCCACAAAGCAT | 60 | SRR278741_ isotig08598 | Present study |
| ***dlx6*** | F: GCTCTGTGCCGTCTAACCTT<br>R: TACCCTACCCCTACGTGAGC | 60 | SRR278741_ isotig14107 | Present study |
| ***ef1α*** | F: CTTCAACGCTCAGGTCATCAT<br>R: GCACAGCGAAACGACCAAGGGGA | 60 | AF184170 | Salmerón *et al.*, 2013 |
| ***fib1a*** | F: CGGTAATAACTACAGAATCGGTGAG<br>R: CGCATTTGAACTCGCCCTTG | 60 | FG262933 | Vieira *et al.*, 2012 |
| ***mgp*** | F: TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC<br>R: CGGGCGGATAGTGTGAAAAATGGTTAGTG | 68 | AY065652 | Rosa *et al.*, 2010 |
| ***mmp9*** | F: ATTCAGAAGGTGGAGGGAGCG<br>R: CATTGGGGACACCACCGAAGA | 60 | AM905938 | Vieira *et al.*, 2013 |
| ***ocn*** | F: TCCGCAGTGGTGAGACAGAAG<br>R: CGGTCCGTAGTAGGCCGTGTAG | 60 | AF048703 | Pinto *et al.*, 2001 |
| ***on*** | F: AGGAGGAGGTCATCGTGGAAGAGCC<br>R: GTGGTGGTTCAGGCAGGGATTCTCA | 68 | AY239014 | Rosa *et al.*, 2010 |
| ***op*** | F: AAAACCCAGGAGATAAACTCAAGACAACCCA<br>R: AGAACCGTGGCAAAGAGCAGAACGAA | 68 | AY651247 | Rosa *et al.*, 2010 |
| ***osx*** | F: CAGTCAGGGATTCAGCAACA<br>R: GGTGAAGGAGCCAGTGTAGG | 60 | ERR22591_ isotig06993 | Present study |
| ***rpl27*** | F: AAGAGGAACACAACTCACTGCCCCAC<br>R: GCTTGCCTTTGCCCAGAACTTTGTAG | 68 | AY188520 | Rosa *et al.*, 2010 |
| ***runx2*** | F: ACCCGTCCTACCTGAGTCC<br>R: AGAAGAACCTGGCAATCGTC | 60 | JX232063 | Vieira *et al.*, 2012 |
| ***tnap*** | F: CATCGCAACCCTTTTCACAGTCACCCG<br>R: AACAGTGCCCAAACAGTGGTCCCATTAGC | 68 | AY266359 | Rosa *et al.*, 2010 |
| ***trap*** | F: CTTAATCGTTGCCATCCCTGTG<br>R: CTCCCATCTGCTCTGCTACTTTG | 60 | FM147928 | Vieira *et al.*, 2013 |

**Table 2:** Relative gene expression of ECM components and transcription factors in vertebral column samples of gilthead sea bream. The samples correspond to a region of the vertebral column without visual anomaly (LDN, n=7-8) or with evident lordosis (LDA, n=8). Results are shown as the mean ± s.e.m normalized by the control (CT) specimens. Asterisks indicate significant differences with CT normal fish ($p < 0.05$). Significant differences were not found between the LDN and LDA groups ($p < 0.05$).

| Gene | LDN | LDA | | Gene | LDN | LDA |
|---|---|---|---|---|---|---|
| *col1a1* | 0,91 ± 0,07 | 0,89 ± 0,07 | | *ctsk* | 0,68 ± 0,10* | 0,72 ± 0,11 |
| *fib1a* | 1,06 ± 0,14 | 1,46 ± 0,19* | | *mmp9* | 0,48 ± 0,07* | 0,52 ± 0,08* |
| *on* | 0,77 ± 0,04* | 0,90 ± 0,08 | | *runx2* | 1,60 ± 0,21* | 1,57 ± 0,13* |
| *op* | 0,25 ± 0,03* | 0,38 ± 0,07* | | *osx* | 1,04 ± 0,11 | 1,02 ± 0,18 |
| *ocn* | 0,59 ± 0,10 | 0,73 ± 0,09 | | *dlx2a* | 1,68 ± 0,34* | 1,98 ± 0,34* |
| *mgp* | 0,77 ± 0,07 | 0,63 ± 0,10* | | *dlx3b* | 0,89 ± 0,10 | 0,95 ± 0,15 |
| *tnap* | 0,60 ± 0,10* | 0,57 ± 0,06* | | *dlx5a* | 0,78 ± 0,10* | 0,65 ± 0,08* |
| *trap* | 0,55 ± 0,10* | 0,51 ± 0,06* | | *dlx6* | 1,46 ± 0,18* | 1,77 ± 0,32* |

## 7. Figure legends

**Figure 1:** Representative images of the specimens sampled with: lordosis (LD), lordosis-scoliosis-kyphosis (LSK), operculum (OPC), dental (DNT) or jaw (JW) malformations. Scale bar 1 cm.

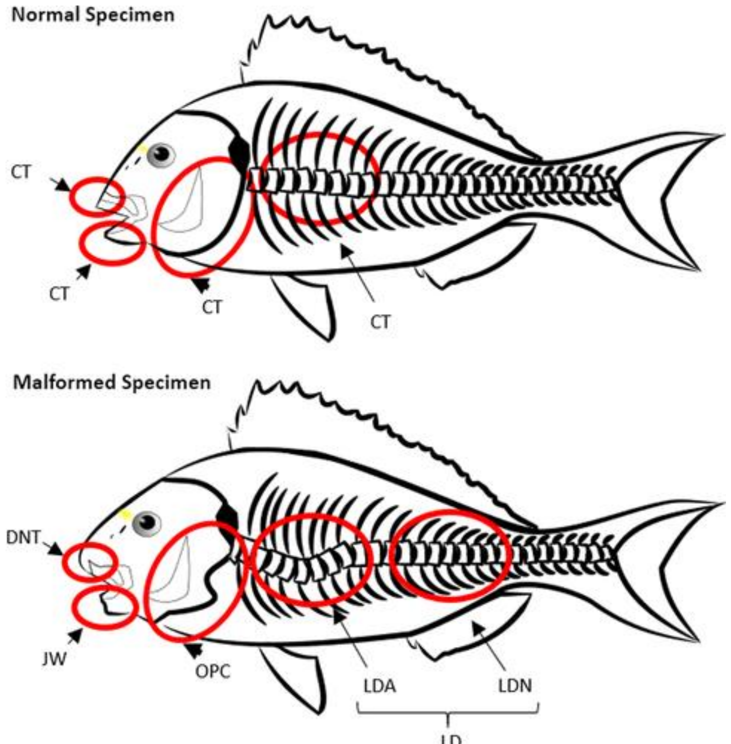



**Figure 2:** Schematic representation of the bone samples extracted from a normal and a malformed specimen. To simplify all the different anomalies evaluated have been indicated in a single animal, although none specimen had more than one malformation at a time. CT: control, LD: lordosis, OPC: operculum, DNT: dental and JW: jaw. The LDA and LDN samples in the malformed animal correspond to a region of the vertebral column with or without visual anomaly, respectively in an LD animal. In the case of the animals showing a lordosis-scoliosis-kyphosis (LSK) malformation, the vertebral column sample was taken from the affected area.

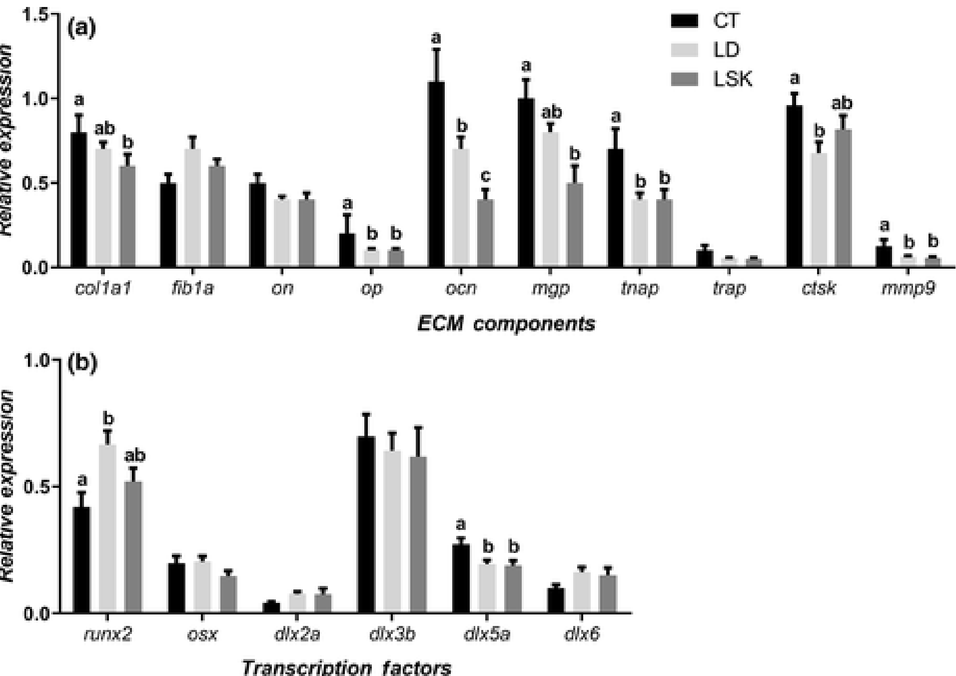



**Figure 3:** Relative gene expression of (**A**) ECM components and (**B**) transcription factors in gilthead sea bream vertebral column fragments of control animals (CT, n=7-8) or specimens with lordosis (LD, n=14-16) or lordosis-scoliosis-kyphosis (LSK, n=10-12). Results are shown as the mean ± s.e.m. Different letters indicate significant differences among groups ($p < 0.05$).

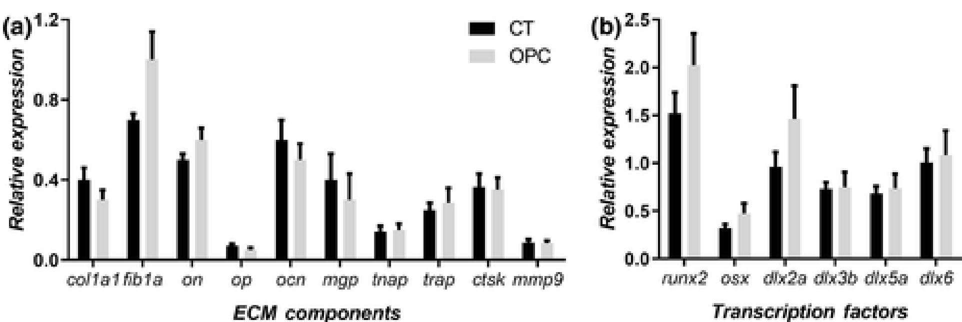



**Figure 4:** Relative gene expression of (**A**) ECM components and (**B**) transcription factors in gilthead sea bream control (CT, n=10-11) or specimens with an operculum deformity (OPC, n=6-7). Results are shown as the mean ± s.e.m. Different letters indicate significant differences between groups ($p < 0.05$).

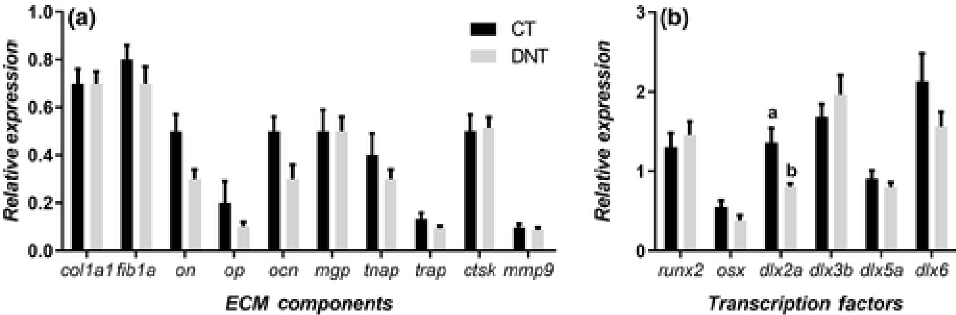



**Figure 5:** Relative gene expression of (**A**) ECM components and (**B**) transcription factors in gilthead sea bream control (CT, n=9-11) or specimens with a dental deformity (DNT, n=8). Results are shown as the mean ± s.e.m. Different letters indicate significant differences between groups ($p < 0.05$).

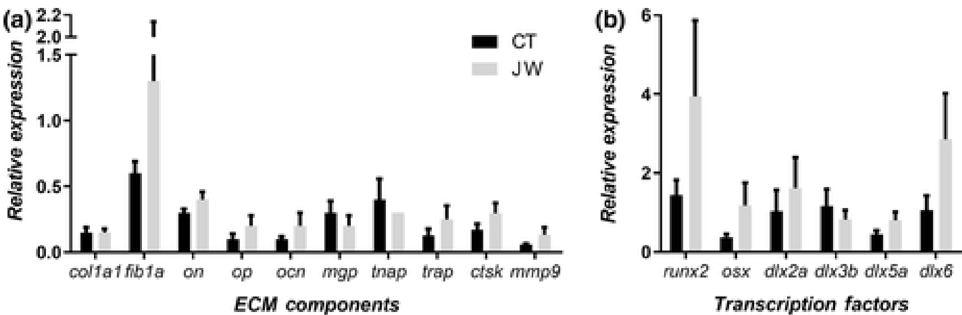



**Figure 6:** Relative gene expression of (**A**) ECM components and (**B**) transcription factors in gilthead sea bream control (CT, n=4-9) or specimens with a jaw deformity (JW, n=2-6). Results are shown as the mean ± s.e.m. Different letters indicate significant differences between groups ($p < 0.05$).

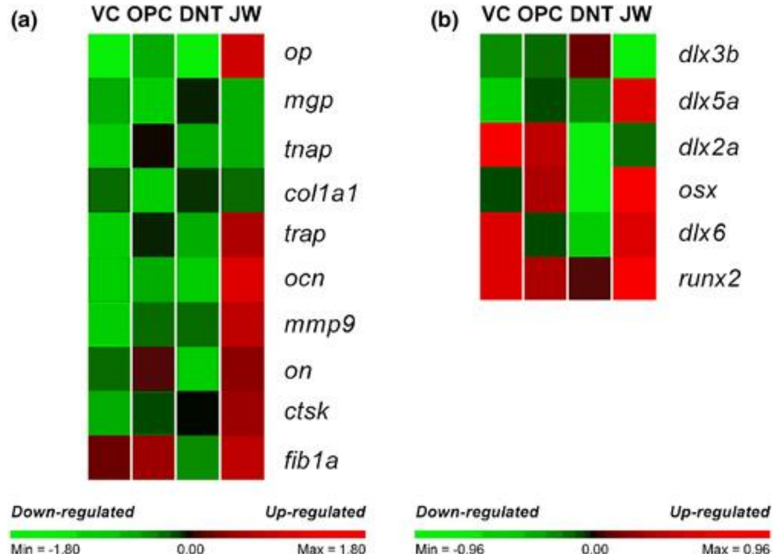
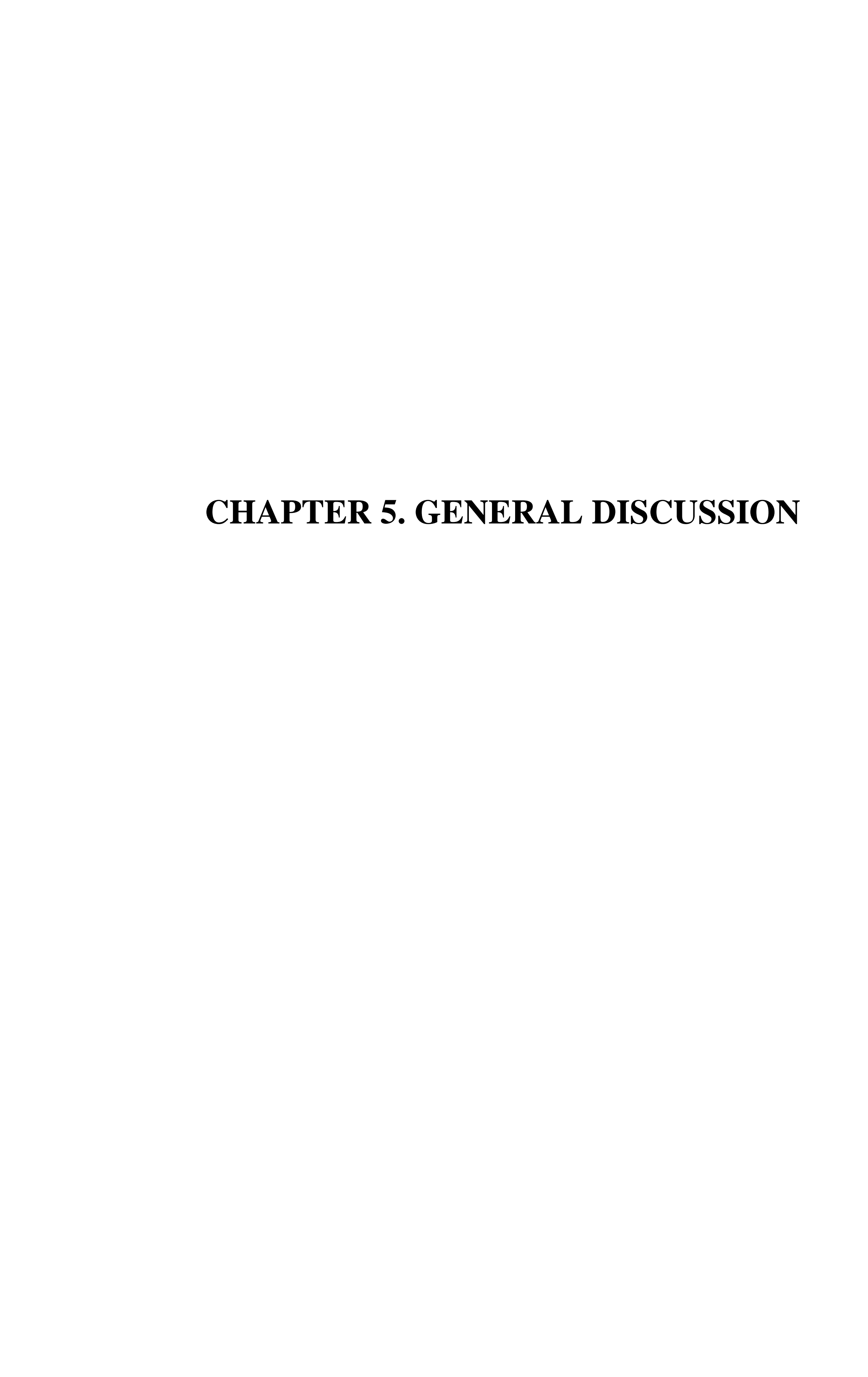



**Figure 7:** Heat maps presenting the changes in (**A**) ECM components and (**B**) transcription factors gene expression in gilthead sea bream with different malformations. Gene expression was first calculated relative to the geometric mean of ribosomal protein L27a (*rpl27a*) and elongation factor 1 alpha (*ef1a*) and then was standardized following a standard score normalization against the corresponding control (CT) samples. Red and green shading, respectively, indicate the highest and lowest expression levels, as specified in the scale bar at the bottom of the figure. For simplicity and because the changes were always in the same direction, the vertebral column data includes combined the LD and LSK fish (VC, n=12-16). Operculum (OPC, n=6-7), dental (DNT, n=8) and jaw (JW, n=2-6).

## Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.



# CHAPTER 5. GENERAL DISCUSSION

Health and welfare in farmed fish are important aspects that aquaculture must preserve (Jennings et al, 2016), hence the importance of developing tools to meet these requirements and achieve adequate growth of the fish. Aquaculture is being challenged by climate change, the poor sustainability of raw materials used in diets formulation, the impact on fish metabolism of the alternatives used in these diets and, by the high prevalence of malformations in farmed fish. In this sense, several factors such as fatty acids, elevated temperatures and highly substituted diets with different protein sources or oils from plant origin have been described to alter the proper growth of fish, finally compromising skeletal or adipose tissue development.

In this context, and in order to evaluate the influence of nutritional and environmental factors on skeletal and adipose tissues biology and lipid homeostasis in fish, mainly at a transcriptional level, seven different experimental trials have been conducted in two different species. First, we described the gene expression pattern during differentiation of cultured preadipocytes into mature adipocytes from rainbow trout (Article **I**) and gilthead sea bream (Article **II**). Second, we provided the gene expression profile along *in vitro* MSCs osteoblastogenesis and *in vivo* skeletal development in gilthead sea bream (Article **III**). Next, we examined the plasticity of MSCs derived from gilthead sea bream bone to differentiate into adipocyte-like cells after different interventions (Articles **II** and **IV**). Furthermore, we also investigated the effect of fatty acids from different oil sources in cultured gilthead sea bream bone-derived MSCs (Article **IV**) and rainbow trout cultured preadipocytes, as well as in force-fed rainbow trout (Article **V**). In addition, we analysed the effect of temperature in gilthead sea bream cultured MSCs from bone and during *in vivo* skeletal development (Article **III**), and in adipose tissue and lipid homeostasis in fish fed substituted diets (Article **VI**). Finally, we provided a gene expression analysis of several skeletal malformations in gilthead sea bream (Article **VII**).

### 5.1. Morphology and gene expression changes during *in vitro* adipogenesis

Adipogenesis in fish is known as the differentiation process in which precursor cells become adipocytes. This process requires *in vitro* an induction medium to start the activation of a complex transcriptional network, to finally the cells express genes characteristic of the mature adipocyte such as those controlling lipid uptake, transport and synthesis among others, involving the integration of many different signalling pathways and transcription factors to become the cells fully differentiated and functional (Salmerón, 2018).

In this context, MSCs isolated from adipose tissue of adult rainbow trout or gilthead sea bream can be differentiated into mature adipocytes by supplementing the growth medium with dexamethasone, IBMX, insulin and lipid mixture (Bouraoui et al, 2008; Salmerón et al, 2013). The morphological changes of the cell from a fibroblastic to a more rounded form indicate the start of adipogenesis with the subsequent lipid accumulation inside the cell as in mammalian models (Mor-Youssef Moldovan et al, 2019). Insulin is the most potent adipogenic inducer in mammals, activating *pparg* (Klemm et al, 2001; Nadeau et al, 2004), and in fish, it was demonstrated also that favours cultured adipocyte differentiation in rainbow trout, gilthead sea bream and other fish species but without being indispensable (Bouraoui et al, 2008; Oku et al, 2006; Salmerón et al, 2013). CCAAT enhancer-binding proteins *cebpb* and *cebpd* expression is respectively activated by IBMX and dexamethasone (Chen et al, 2016), consequently inducing the gene expression of the master regulators of adipogenesis, *cebpa* and *pparg*. Notwithstanding, the addition of lipids, in particular, DHA or EPA, as natural ligands of PPARγ (Grygiel-Górniak, 2014) in the differentiation medium, is in some fish species required to obtain full mature adipocytes from preadipocyte primary cultures. In this sense, several mixtures containing cholesterol and cod liver oil fatty acids as lipid cocktails or the addition of LA or OA at high concentrations, demonstrated successful maturation of adipocytes in different fish species (Bouraoui et al, 2008;

Li, 2012; Ljubojevic et al, 2014; Oku et al, 2006; Salmerón et al, 2013; Vegusdal et al, 2003; Wang et al, 2012).

As mentioned above, in mammals, the first actors starting this process are the C/EBPs and PPARγ. In the study with cultured preadipocytes from rainbow trout, similar patterns of expression of *cebpa, cebpb* and *pparg* during differentiation were observed, with a transient upregulation after the induction with the corresponding medium, turning on the machinery to obtain mature adipocytes and then decreasing the expression to return to basal levels during the remaining of the process (Article **I**). This increased expression in *pparg* after induction of differentiation and the posterior decrease to basal levels was also found in Atlantic salmon (Todorčević et al, 2008). Contrarily, in our study with gilthead sea bream preadipocytes, *pparg* presented higher levels of expression in subconfluent cells while decreased after induction (Article **II**), similarly as observed in another study in Atlantic salmon (Todorčević et al, 2010). On the other hand, transcriptional expression of *pparg* was found to increase gradually during cell differentiation in both grass carp (Li, 2012) and large yellow croaker (Wang et al, 2012), whereas in red sea bream the expression remained unaffected during the whole process (Oku & Umino, 2008). Thus, these results suggest that species and/or characteristics of the initial culture, or the induction cocktail, may influence the response of such factors expression at the onset of adipogenic differentiation.

Otherwise, in our study in gilthead sea bream, it was found that PPARγ protein expression increased from preadipocytes to early-differentiated adipocytes, thus showing a different pattern to that observed at the gene level, suggesting the existence of a possible specific posttranscriptional control (Article **II**). Higher levels of PPARγ in mature cells when compared with proliferating cells were also observed previously in rainbow trout cultured preadipocytes (Bouraoui et al, 2008), therefore referring a possible function as a transcription factor not only during the first steps of the adipogenic process but also in the mature adipocyte. Furthermore, other nuclear receptors as RXR evidenced their relationship with transcriptional factors such as PPARγ with which heterodimerize, presenting a

significant upregulated expression after differentiation as observed in preadipocytes from rainbow trout (Article **I**).

Ongoing with the PPARs family, PPARα and PPARβ have been described to be involved in fatty acid oxidation in mature adipocytes (Torstensen et al, 2009) and present different gene profiles during differentiation depending on the species. In gilthead sea bream, *ppara* decreased early and *pparb* at a later stage of preadipocyte differentiation (Article **II**), whereas in Atlantic salmon the expression of both genes remained unchanged (Huang et al, 2010), contrary to what was found in red sea bream, where *ppara* increased during 7 days after induction and then decreased (Oku & Umino, 2008). Afterwards, all these transcription factors modulate the expression of other genes involved in the regulation of lipid metabolism in the adipocyte. These genes are associated to lipogenesis (e.g. *fas* and *lpl*), lipolysis (e.g. *hsl*) or lipid transport (e.g. *cd36, fatp1, fabp11* and *plin2*), and showed different expression patterns during adipocyte differentiation and maturation thus, it cannot be discarded that their regulation could be species-specific or modulated at activity level. In cultured adipocytes from rainbow trout and gilthead sea bream, *hsl* and *fas* presented similar tendencies with a downregulation at the onset of differentiation, and *lpl* showed the same response in the latter, although in rainbow trout the expression significantly increased abruptly after induction to subsequently decrease (Articles **I** and **II**). In this sense, previous studies in large yellow croaker (Wang et al, 2012), rainbow trout (Bouraoui et al, 2012), and red sea bream (Oku et al, 2006; Oku & Umino, 2008) showed also increased *lpl* expression after induction of differentiation, suggesting that this enzyme is involved in the control of lipid accumulation in these species during adipogenesis. Next, as an example of the importance and the role they play in adipocyte maturation, genes analysed in rainbow trout including *fatp1, fabp11, cd36* and *plin2* showed significant increased expression at the late stages of differentiation due to their implication in lipid storage (Article **I**). Specifically, the significant increase of *plin2* was gradual, according to its function coating lipid droplets, and the results previously obtained in the same species at transcript and

protein levels (Bou et al, 2017). This increased lipid accumulation during adipogenesis has been also corroborated through intracellular lipid staining in both species (Articles **I** and **II**), being in agreement with previous reports in rainbow trout (Bouraroui et al, 2008), gilthead sea bream (Salmerón et al, 2013), Atlantic salmon (Todorčević et al, 2010) and grass carp (Li, 2012).

In summary, *in vitro* studies in different fish species revealed that adipogenesis is a complex process as it is in mammals (Rosen & MacDougald, 2006; Rutkowski et al, 2015). Cells change the morphology from a fibroblastic to a more rounded shape together with lipid internalization and accumulation. This process starts with a specific differentiation medium that induces the expression of the principal transcription factors that in turn, enhance the expression of many genes that altogether regulate the synthesis, uptake and mobilization of lipids in the adipocyte to control fat metabolism with some differences in the expression profiles between fish species.

## 5.2. Gene expression pattern during osteogenesis *in vitro* and *in vivo*

The process of osteogenesis is known to comprise three stages, cell commitment, production of ECM and mineralization. As seen in adipogenesis, induction of this differentiation *in vitro* requires the addition of an osteogenic medium, which contains ascorbic acid and a source of minerals (i.e. calcium and phosphate) and then, cells change its fibroblast-like form into a geometrical cobblestone structure with the appearance in addition, of mineralized hydroxyapatite nodules in the ECM at the final stages of differentiation. As in mammals, this same pattern has been also described in gilthead sea bream bone-derived primary cultured cells (Capilla et al, 2011) and in two cell lines (VSa13 and VSa16) of the same species (Pombinho et al, 2004).

In the present work, we have reported the gene expression profile of the molecules involved in the osteogenic process *in vitro* and during embryonic and larval

skeletal development in gilthead sea bream (Article **III**). Like *pparg* in adipogenesis, *runx2* is well known as a key transcription factor in osteoblast differentiation in mammals (Aguello & De Bari, 2010; Ducy et al, 1997). In fish, *runx2* has demonstrated to be an excellent indicator of the osteogenic capacity of cells because of its increased expression at the early stages of osteogenesis (Flores et al, 2006; Karsenty, 2008; Kawasaki, Suzuki & Weiss, 2004; Marie, 2008; Ytteborg et al, 2012). This transcription factor is also known to regulate the expression of other genes related to matrix production and mineralization like *fib1a, on, op, mgp* among others (Komori, 2009a, 2019; Lian & Stein, 2003). In gilthead sea bream (Article **III**), we did not observe differences in *runx2* expression during culture development, probably because of the selected sampling times. In fact, in Article **IV**, bone-derived primary cultured cells from the same species where exposed to fatty acids for 6 hours at days 3-4 of culture with the consequent downregulated expression of *runx2*, thus confirming its role also in gilthead sea bream as an early inductor of osteoblastogenesis at that time point. Besides *runx2,* BMP2 is a secreted factor that has been used as indicative of cell commitment, although similarly, increased expression was not observed in the first steps of differentiation whereas an upregulation of *bmp2* expression was found in a later step (Article **III**), in agreement to mammals (Rosen, 2009; Yamaguchi et al, 1996). Next, the peaks of expression of other components of the ECM with different functions were observed distributed along osteoblast differentiation. In gilthead sea bream, both *in vivo* and *in vitro*, *fib1a* expression increased significantly early during bone development being downregulated later (Article **III**), as seen in MC3T3-E1 osteoblasts (Ribeiro, Sousa & Monteiro, 2010), and in rats and chicken (Owen et al, 1990; Stein, Lian & Owen, 1990; Winnard et al, 1995), in agreement with the role of this glycoprotein in cell adhesion and the initial phases of osteogenesis. On the other hand, other molecules such as *op, on* and *mgp* are known to be important for the calcification of the ECM (Chakatun et al, 2014; Lu et al, 2013). Thus, in gilthead sea bream osteoblast culture as well as *in vivo*, *op* and *on* showed significantly increased expression during this end phase (Article **III**), according to the data found in Atlantic salmon cells (Ytteborg et al,

2012). Moreover, the results in expression obtained during *in vivo* embryogenesis (Article **III**) concur with the expression profiles observed in other fishes (Estêvão et al, 2005; Fonseca et al, 2007; Pinto, Ohresser & Cancela, 2001; Rafael, Laizé & Cancela, 2006; Redruello et al, 2005). Hereby, the corroboration of the potential role of all these molecules during the osteogenic process given by the gene expression profiles observed together with the concordance to *in vivo* results, demonstrates that this *in vitro* model is a valuable tool to study osteogenesis in fish.

### 5.3. *In vitro* plasticity of MSCs derived from bone to become adipocyte-like cells

The bone-derived cells from gilthead sea bream demonstrated their ability to differentiate into other linages depending on the differentiation media applied to the culture (Capilla et al, 2011). In this sense, one aim of this thesis was to study in more detail the capacity of these cells to become adipocyte-like cells (Article **II**) and, to investigate how some specific elements (i.e. fatty acids) usually present in the diet can modify such fate (Article **IV**).

As described in preceding sections, during adipogenesis as well as osteogenesis, several genes were described as major markers of the different developmental steps for each process. In particular, transcription factors are key to change the characteristic gene expression of cells from the pre-committed lineage to become a different cell type. In this regard, we reported in Articles **II** and **IV** that the incubation of gilthead sea bream MSC derived from bone with adipogenic media or even specific fatty acids affects the gene expression in those cells turning them into round cells filled with lipid. First, in our experiments, the expression of osteogenic genes (i.e. *fib1a*, *mgp*, *op*) remained low or without alterations thus, indicating the change in the transcriptomic profile and the possible switch into adipocyte-like cells. Afterwards, our results showed that the bone-derived cells presented increased expression of genes related to adipogenesis in the first days of

differentiation to then decrease. As seen in cultured preadipocytes from rainbow trout (Article **I**), in these modified bone-derived cells, *ppars*, *fas*, *lpl* and *hsl* among others, were in general significantly upregulated when adipogenic medium was applied to then decrease to lower levels during differentiation (Article **II**). Nevertheless, in this particular study (Article **II**) the expected decrease in expression of the principal transcription factor of the osteoblastic linage (i.e. *runx2*) was not observed probably again because of the transient nature of the change, as it was seen in Article **IV**. *runx2* is known to be responsible of promoting cell differentiation of immature osteoblasts and also, of inhibiting the commitment to the adipocyte linage in mammals, as confirmed by an adenovirus infection carrying the *runx2* gene, that reduced *lpl* and *pparg* expression and the formation of lipid droplets (Komori et al, 2009b; Zhang et al, 2006). In addition, a mutual inhibition between *runx2* and *pparg* when adipogenesis or osteogenesis are activated was suggested not only in mammals (Ge et al, 2016), but also in precursor cells from Atlantic salmon visceral fat (Ytteborg et al, 2015). In this regard, the significant increase observed in *runx2* expression at a late stage of differentiation of bone-derived MSC into adipocyte-like cells could be provoked by the decrease in *pparg* expression during the same interval (Article **II**). In a similar way, in Article **IV**, this mutual inhibition was observed, with the induction of adipogenesis via fatty acids, with *pparg* being upregulated while *runx2* was downregulated, indicating the change into the adipocyte linage.

Furthermore, it was recently described in mice using *in vitro* and *in vivo* approaches that OP inhibits adipogenic *versus* osteogenic differentiation by regulating C/EBPs expression, revealing the critical role of this bone ECM molecule along development (Chen et al, 2014). Similarly, in murine models of osteogenesis imperfecta (Gioia et al, 2012), it was demonstrated that a mutation in the gene coding for collagen, supposes a decreased expression of early (e.g. *runx2*) and late (e.g. *col1a1*) osteoblastic markers, but also increases the expression of genes related to adipogenesis (e.g. *pparg, lpl, fabp4*). Therefore, evidence for the existence of a tight connection between the adipogenic and the osteogenic

pathways makes clear the need of further research in fish to avoid undesired differentiation of MSCs *in vivo*, since this ability could negatively impact on health as it can be observed in mammalian diseases (Gao et al, 2014).

## 5.4. Fatty acids effects on osteogenesis, adipogenesis and lipid homeostasis

Fatty acids typical from FO (EPA and DHA) and those most commonly found in VO (LA and ALA) are present in fish diets formulations. Initially, these diets were essentially composed by FO, but since some years ago, in order to reduce the negative marine ecological impact, this oil was substituted more than a 50% with VO for some species (Benedito-Palos et al, 2008; Izquierdo et al, 2003; Torstensen et al, 2008). In several cases, these substitutions have implied imbalances in the n-3/n-6 PUFA ratio, leading to a reduction of EPA and DHA, diminishing fish quality and its beneficial contribution with n-3 HUFA to human nutrition (Karalazos et al, 2007). Thus, although *in vivo* the influence in fish seems obvious, these components must suppose an impact at a cellular level, also considering the ability of linage commitment interconversion of MSCs. As mentioned above, fatty acids could be considered in the group of chemical factors that modulate MSCs differentiation; therefore, we studied their effects in gilthead sea bream bone-derived cells (Article **IV**), and in rainbow trout on *in vitro* preadipocytes and *in vivo* on lipid metabolism in force-fed fish (Article **V**).

First, toxicity was evaluated, and we found that the incubation of preadipocytes and MSCs from bone with the fatty acids did not suppose any effect on viability as it was already demonstrated on the skeletal VSa16 cell line of gilthead sea bream (Viegas et al, 2012). Then, together with the cellular morphological changes taking place, lipid accumulation (determined by means of ORO staining as a differentiation sign), was increased significantly when fatty acids were applied in culture (Articles **IV** and **V**). In fact, a similar behaviour in bone-derived MSCs

from gilthead sea bream was initially demonstrated in Capilla et al (2011) and in our previous experiment with the same cell system (Article **II**), adding in both cases adipogenic media to the cells, thus confirming their multipotentiality. In agreement, several other studies suggested that the presence of fatty acids in differentiation media are critical to launch the adipogenic process in fish MSCs (Bouraoui et al, 2008; Oku et al, 2006; Salmerón et al, 2013; Vegusdal et al, 2003), as also observed in avian adipocyte precursor cells (Matsubara et al, 2005). Subsequently, concerning the different specific fatty acid treatments, whereas LA and the combinations containing this fatty acid supposed a higher fat accumulation in bone-derived MSCs compared to the other fatty acids (Article **IV**), ALA was responsible for a higher significant effect in preadipocytes from rainbow trout (Article **V**). This difference could be attributed to either the cellular model or the fish species, but it is interesting to note that in both cases the fatty acid with increased adipogenic potential belongs to the group of those mostly present in VO sources. Likewise, some studies previously demonstrated a reduced potential of fatty acids from FO to be stored in 3T3-L1 preadipocytes (Kim et al, 2006), while fatty acids from VO (i.e. OA) were observed to induce more TG accumulation in mature salmon adipocytes (Todorčević et al, 2008). In general, our results also suggest that fatty acids characteristic of VO may stimulate the uptake and depot of extracellular fats in differentiating adipocytes from different origin, more than the other treatments tested. In this sense, similar differences between dietary oil sources were reported in gilthead sea bream, showing that VO produce cellular hypertrophy and alteration of lipid metabolism in the adipose tissue (Cruz-García et al, 2011). Therefore, the fact that bone MSCs have the ability to become adipocyte-like cells could suppose a major problem for fish bone development *in vivo*, putting the MSCs in the spotlight, together with the factors that may be involved in cell fate decision, as observed in mammals, with the increasing prevalence of both, obesity and osteoporosis nowadays (Chen et al, 2016).

Regarding the effects of fatty acids at a transcriptional level, genes related with osteogenic and adipogenic processes were observed to be modulated by these

components *in vitro* and *in vivo.* In bone-derived MSCs from gilthead sea bream, all fatty acid treatments induced a decrease in *runx2* expression, and constant or unaltered expression of genes involved in osteoblastogenesis (*fib1a, mgp, op*, etc.) (Article **IV**) as previously observed with the addition of adipogenic medium in the same type of cells (Article **II**). Our results suggest that even though these cells are predestined to become osteoblasts, the fatty acids action stops osteoblast differentiation and enhances the differentiation into adipocyte-like cells. This dysregulation in linage determination has been associated, either in mammals (Choi et al, 2002; Nuttall & Gimble, 2004) or in fish, with the appearance of different developmental disorders or diseases. In this sense, compressed vertebrae were observed in Atlantic salmon fed high levels of SO (Gil Martens et al, 2010), supporting the fact of being the diet one of the major causative factors of this dysregulation (Helland et al, 2006). Thus, in Articles **IV** and **V**, we demonstrated that fatty acids enhance adipogenic differentiation of cells independently of their original lineage. Starting from the beginning, the key actor involved in this process, PPARγ, can be activated by LC-PUFA (Grygiel-Górniak, 2014), and EPA and DHA are known to display more potency on activating this transcription factor, compared to the n-6 PUFA (i.e. LA) (Schmidt & Ecker, 2008). *pparg* in bone MSCs showed increased gene expression levels with EPA incubation (Article **IV**), accordingly to what was found in humans (Casado-Díaz et al, 2013); and, in the gilthead sea bream osteoblast-like VSa16 cell line, EPA induced a decrease in the mRNA levels of *runx2* (Viegas et al, 2012). Altogether suggesting that in these cells, *pparg* expression can not only *per se* induce adipogenesis, but also result in the inhibition of differentiation towards osteoblasts by suppressing *runx2* (Article **IV**). This was hypothesized in mammals by other authors (Beredsen and Olsen, 2014; Jeon et al, 2003; Lecka-Czernik et al, 1999). Furthermore, in our results (Article **IV**), both *cebpb* (Lefterova et al, 2009; Moreno-Navarrete & Fernández-Real, 2012) and the nuclear receptor *rxr*, showed a significant upregulated expression in response to EPA and EPA+DHA combination, altogether leading to adipocyte differentiation, as expected according to the expression profile during adipogenesis of rainbow trout preadipocytes (Article **I**). On the contrary, the

expression of *pparg* in *in vitro* preadipocytes from rainbow trout was reduced by EPA; while in force-fed animals, effects on PPARγ protein and gene expression were not observed in adipose tissue (Article **V**). Another adipogenic transcription factor, *cebpa*, showed as well a significant downregulation in rainbow trout preadipocytes after EPA incubation (Article **V**), according to observations in Atlantic salmon but only in mature adipocytes incubated with high concentrations of EPA, overall suggesting that the moment of cell development and species characteristics can modulate the response to fatty acids (Huang et al, 2010). Further transcription factors from the PPARs family were studied in the experiment of oral administration of EPA and LA in rainbow trout, showing a significant increased expression of *ppara*, *pparb* as well as *rxr* in adipose tissue (Article **V**). Therefore, the upregulation of these three transcription factors either with EPA or LA could suggest an activation of fatty acid oxidation (Torstensen et al, 2009), thus, supplying energy to the adipocyte. Regarding the liver tissue from these force-fed trout, EPA downregulated *cebpa* and *cebpb* and LA *ppara* and *pparb* in general indicating a possible reduction of fatty acid metabolism in this tissue by fatty acids (Bauer et al, 2015; Librán-Pérez et al, 2012; 2013).

With regards to key enzymes involved in lipid metabolism, our results demonstrated different patterns of expression depending on the experiment. In both *in vitro* models, *fas* showed stable or low gene expression levels probably as a consequence of the inhibition of *de novo* fatty acid synthesis by the addition of the fatty acids into the culture medium (Article **IV** and **V**). While in the *in vivo* approach, *fas* expression was upregulated by both fatty acids in adipose tissue (Article **V**). *In vitro*, *lpl* and *hsl* gene expression presented no differences (Article **V**) according to the observations made in a recent study, also in rainbow trout preadipocytes (Bou et al, 2017). On the contrary, in orally administered rainbow trout, EPA but not LA significantly decreased *lpl* expression (Article **V**). This differential effect in adipose tissue, depending on the fatty acid, was also reported in *Pagrus major* fed with diets supplemented with OA plus EPA or LA being the first combination responsible for the downregulated expression of *lpl* (Liang et al,

2002). Otherwise, HSL did not show differences at protein level with LA whereas gene expression was upregulated (Article **V**), in concordance with findings in isolated adipocytes from gilthead sea bream fed diets highly substituted with VO (Cruz-García et al, 2011) that demonstrated an increase of *hsl* expression and consequently enhanced intracellular lipolysis. In the liver of these force-fed fish with EPA and LA, both fatty acids decreased significantly *fas* expression, therefore decreasing lipogenesis, contrary to what was found in adipose tissue, and accordingly to the fast inhibition of *fas* transcription observed when PUFA were added *in vivo* or to cultured rat hepatocytes (Ren et al, 1997). In fact, *fas* decreased expression was reported in trout hepatocytes using fatty acids incubations (Alvarez et al, 2000), hepatic tissue after an intraperitoneal injection of OA (Librán-Pérez et al, 2012, 2013), and even in mice fed diets with increased content of n-3 and n-6 PUFA (Gnoni & Guidetti, 2016). Similarly, as in the *in vitro* approaches, *lpl* expression showed no differences between treatments, but the decrease in *hsl* expression was contrary to the results found in the adipose tissue of the same fish, suggesting a reduction in hepatic lipolysis and showing again different metabolic responses between both tissues (Article **V**).

Concerning the genes involved in the uptake and transport of fatty acids, gilthead sea bream bone-derived MSCs and preadipocytes from rainbow trout presented similar results. Expression of *fatp1* increased significantly especially in response to fatty acids commonly found in VO (LA and ALA) (Articles **IV** and **V**). Thus, demonstrating the fast activation of this transporter and its key role in the stimulation of the adipogenic process and uptake of fatty acids from the surroundings as previously seen in salmonids (Huang et al, 2010; Sánchez-Gurmaches et al, 2012). Moreover, *fabp11* expression was also increased by these two fatty acids in bone-derived MSCs (Article **IV**), which together with the results of *fatp1*, suggested that fatty acids commonly found in VO utilize this stimulated fatty acid uptake as a mechanism of action to enhance adipogenesis. In fact, this increase in fatty acid uptake in gilthead sea bream was also suggested to induce adipocyte size increase (Cruz-García et al, 2011). Otherwise, with the oral

administration of EPA and LA in rainbow trout (Article **V**), *cd36* expression was significantly upregulated in adipose tissue supporting the increased internalization of fatty acids due to higher availability. On the other hand, at protein level, LA was the fatty acid that significantly increased CD36 when compared to EPA, suggesting a different posttranscriptional regulation depending on the fatty acid (Article **V**). Although in our study, changes in *fatp1* expression were not found, EPA decreased the expression of *fabp11* contrary to the results obtained in salmon, where the fish fed with a diet containing VO and protein presented decreased transcript levels of this transporter (Torstensen et al, 2011). Regarding fatty acid transporters in the liver from fish orally-fed EPA or LA, only *fatp1* expression was downregulated by LA treatment (Article **V**) in concordance with the findings of Librán-Pérez et al (2013), where the transporters analyzed remained unchanged after fatty acid treatments suggesting that lipid internalization could finished at sampling time in this tissue.

Overall, fatty acids affected differently depending on cell origin and the approximation, *in vivo* or *in vitro*. In gilthead sea bream bone-derived MSCs, after the usage of an inhibitor of PPARγ and the consequent downregulated expression of the genes studied, it was suggested an inhibition of the adipogenic process, including lipid internalization, in part, due to the incapability of EPA to activate PPARγ and the companion transcription factors (Article **IV**). On the other hand, despite the presence of the same inhibitor, LA was able to maintain the upregulation of genes involved in lipid transport. Thus, fatty acids demonstrated to induce MSCs predestinated to osteoblastic linage to become adipocyte-like cells, and more concretely, fatty acids commonly found in FO appeared to stimulate adipogenesis via upregulation of *pparg* expression, whereas those commonly found in VO increased lipid internalization. Furthermore, fatty acids applied to rainbow trout preadipocytes in culture were demonstrated to induce adipocyte differentiation, but the presence of the fatty acids *per se* was suggested to inhibit *de novo* lipogenesis whereas lipid internalization was increased in these culture conditions (Article **V**). In the same species, when fatty acids were orally

administered, β-oxidation in adipose tissue was enhanced supposedly to provide energy for the increased *de novo* lipogenesis. Added to this, the stimulation of lipid storage appeared to be due to the activation of transporters by the increased presence of fatty acids. Therefore, although clear differences were not observed between the effects of EPA and LA in this trial, the results suggested that the administration of these fatty acids activated the characteristic metabolic turnover of the adipocyte.

## 5.5. Temperature effects on osteogenesis, adipogenesis and lipid homeostasis. Combined effects with substituted diets

Temperature is a major factor influencing fish larval development, thus rearing outside optimal temperatures can impair growth and lead to increased malformations, also compromising survival. Particularly, temperatures between 15 and 22ºC are considered within the adequate thermal range for gilthead sea bream (Mozes et al, 2011; Yúfera et al, 2011) and rearing at 22ºC was proposed during the larval period since enhanced growth and less malformations were observed (Tandler & Koven, 2011). In our studies, temperature effects at many levels from molecular, cellular, tissular to whole body were demonstrated not only through *in vitro* (Article **III**) but also *in vivo* (Article **III** and **VI**) approaches.

Bone-derived MSCs from gilthead sea bream incubated at high (28ºC) or at low (18ºC) temperatures showed noteworthy changes in gene expression of heat shock proteins 30 and 90b in comparison to control cells (23ºC) (Article **III**). This observation is in concordance with the link between stressful conditions and these genes as described in mammals (Currie, Moyes & Tufts, 2000; Matz et al, 1995; Wu, 1995). In general, expression of numerous osteogenic genes in the cells was decreased after being cultured either at 18 or at 28ºC (Article **III**), accordingly to the reduced expression of *col1a1* or *ocn* previously described in primary cultures of rat osteoblasts and Atlantic salmon muscle satellite cells

exposed to hypo or hyperthermia, respectively (Patel et al, 2012; Ytteborg et al, 2010d). Despite the generalized decrease observed in gene expression, *on* was upregulated in front of a temperature change probably as an initial cellular response to stress (Article **III**), since it has been described as a heat shock protein (Emerson et al, 2006; Martinek et al, 2007; Neri, Descalzi-Cancedda & Cancedda, 1992). Thus, temperature has an impact downregulating the expression of the principal genes involved in osteogenesis in gilthead sea bream while, *on* expression results proposed this gene as a possible indicator of stressful conditions in terms of temperature effects *in vitro*.

In the *in vivo* approach, gilthead sea bream was reared at 18ºC (LT) or at 22ºC (HT) and the expression of osteogenic genes was demonstrated to be affected differentially depending on the developmental stage. In LT fish, a downregulated expression in the osteogenic genes during embryo and larvae stages was observed when compared to those kept at 22ºC (HT). Despite the different gene expression levels, it was observed that the expression pattern of osteogenic genes during the different developmental stages was conserved in both groups, probably because the temperature regimes in this challenge were in the optimal temperature range for rearing this species (Yúfera et al, 2011). In our study, the most significant differences in expression were found in developmental stages where each gene was specifically described to be key (Article **III**). In LT compared to HT group, *bmp2* and *fib1a* involved in the first developmental steps were significantly downregulated in 1 and 10S stages respectively. In the same way, *col1a1* and *on* related to ECM production, were downregulated at hatching stage and finally *op* and *ocn,* involved in ECM mineralization, were decreased at early flexion (Article **III**). Thus, our results showed that embryogenesis is significantly the most sensitive developmental stage to temperature changes, and also this phase was demonstrated to be the stage in which imprinting mechanisms can impact on osteogenic genes in Atlantic cod (Bizuayehu et al, 2015). In this regard, thermal imprinting leading to long-term effects on bone turnover and gene expression was also demonstrated in gilthead sea bream exposed to a temperature regime during

early development (Mateus et al, 2017). In the same study, differences observed after a cold challenge where associated with the reduction of bone calcium content and the relative abundance of OCN among others in the ECM of bone. Altogether, the downregulation of key osteogenic factors in our study (i.e. *ocn*) (Article **III**) was suggested to be symptomatic of impaired responsiveness of bone, with the suppression of osteoblast differentiation thus, affecting homeostasis and bone remodelling as described in other fish species (Larsen et al, 2001; Suzuki & Hattori, 2002). In fact, these alterations provoked by temperature changes in developmental stages affect bone but also could produce persistent effects in adults in terms of muscle (García de la serrana et al, 2012; Johnston et al, 2009; López-Albors et al, 2003). In this sense, juvenile Atlantic salmon in a hyperthermic regime were found to present shorter and less mineralized vertebrae as well as higher rate of skeletal deformities as a consequence of the downregulated expression of genes involved in the ECM (Ytteborg et al, 2010a). In the second *in vivo* approach, gilthead sea bream were exposed during early development to four thermal regimes: LT, HT, low-to-high temperature (LHT) or high-to-low temperature (HLT), and then, 7-month old fish were exposed to a shift in water temperature (23 to 13°C). Results showed that, independently from the rearing temperature in juveniles 13 or 23ºC, expression of all genes related to bone remained similar in fish cultured at high temperatures on the early stages (HT and HLT), indicating that thermal imprinting during embryogenesis could contribute to beneficial effects in juvenile and adult skeletal tissue. On the contrary, fish reared during embryogenesis at low temperatures but in the juvenile stage either at low (LT) or high (LHT) temperature, gene expression was downregulated demonstrating a less beneficial thermal imprinting (Article **III**).

Overall, it was demonstrated that temperature influences the expression of genes involved in skeletal development in fish. This downregulation of key genes for bone formation, homeostasis and remodelling was also observed in gilthead sea bream affected by different skeletal malformations (Article **VII**).

Next, in Article **VI**, adult gilthead sea bream were fed diets with FO substitution by VO and reared at 21 or at 28ºC to mimic a future farming scenario, with diets low in FO content and elevated temperatures due to global climate change. In this challenge, fish fed a diet with PO substitution (P) and reared at 28ºC presented significant increased growth, while decreased HSI, VSI and MFI in comparison with fish fed with P diet at 21ºC. Increased growth at elevated temperatures was also demonstrated in Atlantic salmon (Handeland et al, 2000, 2003, 2008) and in a long-term experiment with gilthead sea bream (Guillaume et al, 2004). Furthermore, a high temperature is known to increase the digestibility of SFA presents in the diet (Ng et al, 2004; Olsen & Ringø, 1998). Thus, in our study, despite the high content of SFA present in diet P, due to its possible increased digestibility at elevated temperatures (i.e. consequently enhancing feed efficiency), was suggested to be the reason of the increased growth observed. Regarding effects in plasma metabolites from fish fed P diet, TG and NEFAs presented reduced levels in those fish reared at high temperature (Article **VII**), possibly supporting that high temperature improve lipid digestibility (and thus nutrient absorption) as suggested by Ng, Lim and Boey (2003), where in rainbow trout fed a PO diet at a low temperature an increased presence of undigested TGs in feces was observed. Although, these increased levels in plasma could also reflect the delay that stomach evacuation suffers at low temperatures as it was shown in salmon (Handeland et al, 2008).

Temperature also affected adipose and liver tissues of these fish fed P diet at a histological level, since fish reared at 28ºC significatively presented more smaller adipocytes as well as lipid droplets of reduced area in the liver, than those fish reared at 21ºC (Article **VII**). Our results indicated that at elevated temperatures there is an induction of adipogenesis, or an enhanced usage of TGs stored in adipose tissue and an increased fatty acid oxidation in the liver. On the contrary, the combination in our study of the fatty acids present in P, together with low temperature could be producing adipocytes of increased size through hypertrophy, as described in Medina-Gomez & Vidal-Puig (2005). According to this, fish fed P diet and maintained at 28ºC, showed decreased expression of

almost all genes involved in fatty acid transport either in liver or adipose tissue in comparison to fish fed P diet at 21ºC (Article **VI**). This effect could be related with an increased lipid transport with temperature, as it was previously described with PO usage in grass carp (Du et al, 2005). Thus, in Article **VI** it was suggested that the high temperature could be contributing to avoid hypertrophy of adipocytes and excessive lipid accumulation in the liver of fish fed P diet, possibly leading to the obtention of fish with a healthy adipose tissue by enhanced adipogenesis as shown in humans (Choe et al, 2016).

On the other hand, in fish reared at 28ºC differences in growth were not found among groups fed the three diets containing different VO: palm (P), rapeseed (R) and a combination of both (PR) (Article **VI**). Fish fed P diet presented slightly increased growth according to the findings of a recent study in gilthead sea bream, where fish fed a diet substituted with exclusively PO showed the highest growth when compared to nine different diets with VO substitutions (Sánchez-Moya et al, *under revision*). As mentioned above, digestibility of fatty acids present in the different substituted diets could increase with temperature depending also of the saturation, and in fact, dietary VO absorption is finally reflected in the fillet fatty acid composition (Francis et al, 2006; Izquierdo et al, 2005; Montero et al, 2005). In addition, different oil sources were demonstrated to affect in diverse ways, for example, in Atlantic salmon, a diet with RO induced more lipid accumulation in liver (Bell et al, 2001; Karalazos et al, 2007; Ruyter et al, 2006; Torstensen, Frøyland & Lie, 2004). Accordingly, in gilthead sea bream fed R diet, animals presented significantly increased lipid droplets in liver (Article **VI**), which could be possibly related to an increased lipolysis in adipose tissue, increased hepatic fatty acid synthesis, reduced fat oxidation and/or reduced fat export from liver (Postic & Girard, 2008). Our result contrast with the findings in the same species where fish fed a diet containing PO presented the highest amount of liver lipid content when compared to those fed SO or RO diets (Fountoulaki et al, 2009); thus, leaving a door open to the possibility that this opposite result could be due to different oil digestibility at this temperature.

Surprisingly, interesting results were obtained in fish fed PR diet (Article **VI**), obtaining a liver with significantly less area filled with lipid droplets and an adipose tissue with smaller adipocytes, that as discussed before could reflect an increased ability of the tissues for preadipocyte recruitment to store lipids to avoid suffering from hypertrophic adipocytes (Medina-Gomez & Vidal-Puig, 2005). As well as, the upregulation of *pparg*, *cd36* and *fatp1* in liver, and *pparb*, *pparg*, *hsl* and *fabp11* in adipose tissue of fish fed PR diet could be indicative of an activation of lipid metabolism in both tissues, with an increased fatty acid mobilization and use (Article **VI**). Thus, according to the findings in a recent study in sea bass (Torrecillas et al, 2017), the substitution of FO by a mixture of LO, RO and PO seems to assure a balanced amount of SFA and MUFA. In fact, no effects on growth, lipogenesis and lipid uptake was observed in sea bass fed the diet with this oil combination (60% FO substitution) when compared to a 100% FO diet (Richard et al, 2006). Even so, the combination of VO in the PR diet-fed fish presented equilibrated results in histology from adipose and liver tissues and a balanced lipid accumulation in this condition of water temperature (Article **VI**). With all this, it was demonstrated that temperature impacts on growth and on lipid digestibility and accumulation in fish; therefore, making clear the need of taking care of the combination of rearing temperature and lipids contained in feeds to assure proper metabolic function and animal health and welfare.

## 5.6. Skeletal malformations

The transcription expression pattern in bone is known to be susceptible to suffer modifications by genetic (Navarro et al, 2009; Negrín-Baez et al, 2015) and abiotic factors (Gisbert et al, 2008). Those factors could include nutrition and temperature, which have been demonstrated to impact by impairing bone responsiveness (Mateus et al, 2017) and have been associated to a higher number of vertebral deformities in fish (Ytteborg et al, 2010a). In fact, for the

proper development of bone, the need of the coordinated and sequential expression of specific osteogenic molecules has been previously described both, in mammals (Javed, Chen & Ghori, 2010) as well as in fish including gilthead sea bream (Article **III**).

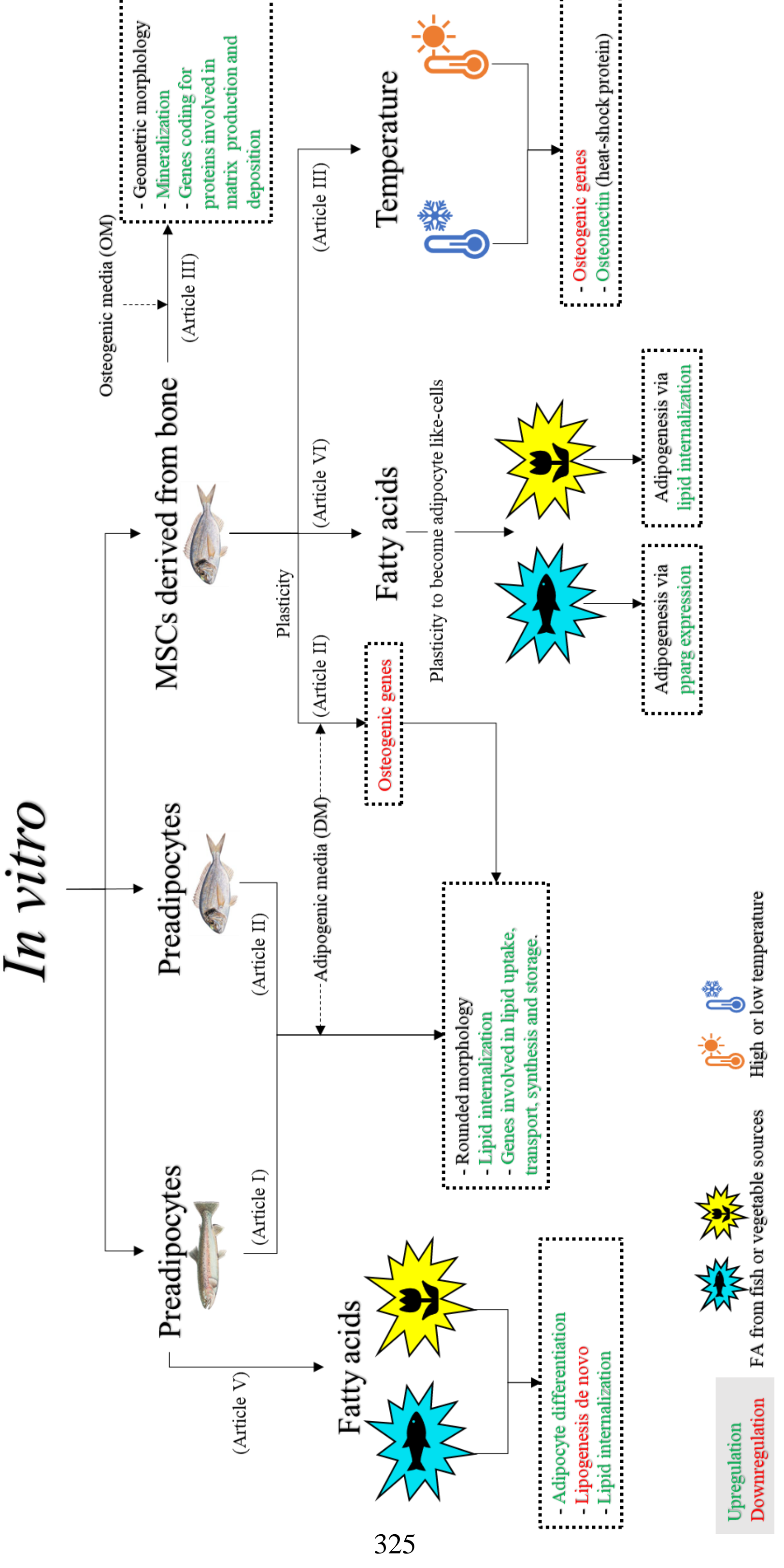
In this thesis, Article **VII** evaluated the gene expression of these molecules in juvenile gilthead sea bream affected by different malformations. Interestingly, cranial malformations did not reveal significant data, contrary to the results found in vertebral centra, where changes in gene expression seemed to be anomaly-related. In this sense, in gilthead sea bream showing LD or LSK malformations, genes involved in ECM mineralization and remodelling demonstrated to be most of them significantly downregulated, supporting the link between the altered expression and the occurrence of the anomaly. Furthermore, the gene expression pattern of the visually non-affected parts and the lordotic regions were similar, demonstrating that in these gilthead sea bream juveniles, the presence of a vertebral anomaly affects at transcriptional level the entire column (Article **VII**) contrarily to the results described in previous studies (Fjelldal et al, 2012; Kacem, Meunier & Bagliniere, 1998). Although Boursiaki et al (2019) found that despite scoliosis in this species is frequently found in the caudal region, both caudal and abdominal vertebrae were shorter than in normal fish, in support to our findings.

Proper skeletal development requires the recruitment of osteoblast cells (Article **III** and **IV**). In this sense, the upregulated expression of *runx2* in the column of malformed gilthead sea bream (Article **VII**) suggests a mismatch in the developmental stage and the bone status, with a higher amount of non-mature osteoblasts consequently leading to a more fragile bone (Liu and Lee, 2012; Ytteborg et al, 2010d). Regarding the ECM genes downregulated in malformed fish (Article **VII**), have been associated not only in fish but also in mammals with different genetic disorders; for example, the mutation in *col1a1* induces osteogenesis imperfecta, skeletal malformations and defects in bone growth (Fisher, Jagadeeswaran & Halpern, 2003; Steiner, Adsit & Basel, 2005). Therefore, a downregulated expression in malformed animals could suggest

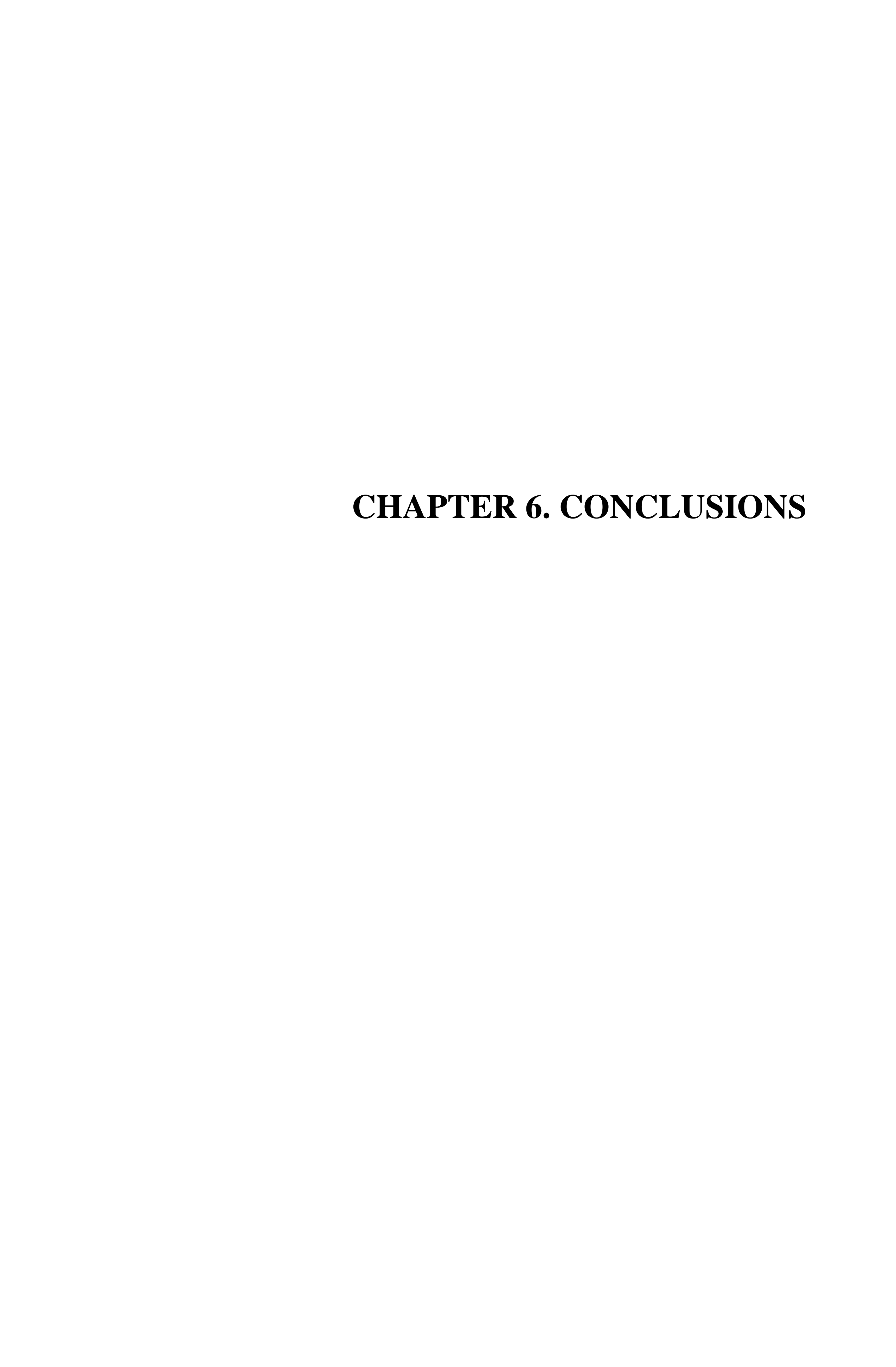
difficulties for bone to produce normal ECM. As well, the downregulated expression of non-collagenic ECM proteins as *on, op,* and *ocn* observed in our animals, agreed with Ytteborg et al (2010d) that suggested restrained differentiation in salmon cultured osteoblasts or a defect in late maturation as described in mammals (Boskey 1992; Termine et al, 1981). On the other hand, genes involved in remodelling (*ctsk, trap* and *mmp9*) are also important for controlling skeletal growth (Azuma et al, 2007; Rajaram et al, 2016), but these osteoclasts markers were not yet associated to malformations. Thus, increased expression of these genes in normal animals could suggest a balanced bone formation and resorption ratio (Article **VII**) that contrarily, as for example seen in mice, a reduced activity of osteoclasts due to a mutation in *ctsk* can lead to supernumerary teeth (Helfrich, 2005).

With all that, in Article **VII** was described the altered gene expression in deformed columns, although not enough information was obtained to discern if this expression pattern was the cause or the consequence of the skeletal deformity. Even so, these altered genes are suggested to be advantageous markers in identifying impaired bone formation leading to a malformed ossified tissue in gilthead sea bream.

*As a summary, schematic representations of the findings derived from the in vitro and in vivo approaches developed in the present thesis are included next.*


In vitro
Preadipocytes
Preadipocytes
MSCs derived from bone
(Article I)
(Article II)
(Article V)
Fatty acids
- Adipocyte differentiation
- Lipogenesis de novo
- Lipid internalization
Adipogenic media (DM)
- Rounded morphology
- Lipid internalization
- Genes involved in lipid uptake, transport, synthesis and storage.
Plasticity
(Article II)
Osteogenic genes
(Article VI)
Fatty acids
Plasticity to become adipocyte like-cells
Adipogenesis via pparg expression
Adipogenesis via lipid internalization
(Article III)
Temperature
- Osteogenic genes
- Osteonectin (heat-shock protein)
Osteogenic media (OM)
(Article III)
- Geometric morphology
- Mineralization
- Genes coding for proteins involved in matrix production and deposition
Upregulation
Downregulation
FA from fish or vegetable sources
High or low temperature

In vivo
(Article V)
Skeletal development
(Article III)
Characterization of osteogenic gene expression in 1 somite stage to early flexion.
(Article VI)
Fatty acids by gavage
FO substitution
Temperature
- De novo lipogenesis
- β-oxidation
- Lipid uptake
- Lipogenesis
- β-oxidation
P
R
PR
Lipid accumulation
Balanced lipid accumulation
(Article VI)
(Article III)
Increased growth
During development
- Adipogenesis
or
- Usage of stored TGs
- β-oxidation
- Osteogenic genes
- Embryogenesis more susceptible (imprinting)
+
- Reduced food intake
- Osteocalcin
- Impaired responsiveness of bone
(Article VII)
Skeletal deformities?
Alteration of the osteogenic genes' expression
Upregulation
Downregulation
Adipose tissue
Liver
VOs
FA from fish or vegetable sources
High or low temperature



# CHAPTER 6. CONCLUSIONS

1. Adipogenesis in rainbow trout and gilthead sea bream appears to require the transitory upregulation of various transcription factors (i.e. *cebpa* and *cebpb*) that in turn would modulate the expression of genes involved in lipid metabolism, regulating synthesis (*fas*), uptake (*fatp1* and *cd36*), storage (*plin2*) or mobilization (*hsl*) of lipids in the mature adipocyte.

2. *In vitro* osteogenesis in gilthead sea bream appears to involve at early stages the transient upregulation of *fib1a*, while later *bmp2, on* and *op* are upregulated, playing a role in the consolidation of osteoblast maturation and mineralization of the ECM. These cells mirrored accurately the data obtained with *in vivo* models, validating their use as a reliable model to investigate osteogenesis.

3. Cultured mesenchymal stem cells derived from gilthead sea bream bone have demonstrated their ability to differentiate into adipocyte-like cells after the addition of an adipogenic medium, maintaining osteogenic genes' expression low or unaltered and upregulating those involved in adipogenesis.

4. Fatty acids have demonstrated in bone-derived mesenchymal stem cells their potential to act as inductors of adipogenesis by different means, with fish oil-derived fatty acids (i.e. EPA) leading to healthier new cells formation through the upregulation of *pparg* gene expression, but vegetable oils-derived fatty acids (i.e. LA) inducing higher lipid accumulation (i.e. hypertrophic adipocytes) via stimulation of fatty acid transporters.

5. In preadipocyte cells from rainbow trout, fatty acids increase intracellular lipid accumulation, with ALA producing the highest effect. Activation of the adipogenic differentiation process and cell metabolic turnover is induced, with small differences considering the distinct oil sources-derived fatty acids.

6. Oral administration of EPA or LA to rainbow trout produces, according to transcriptional data, an activation of β-oxidation, lipogenesis and lipid transport in adipose tissue, contrarily to liver, but in all cases major differences were not observed between the two fatty acids.

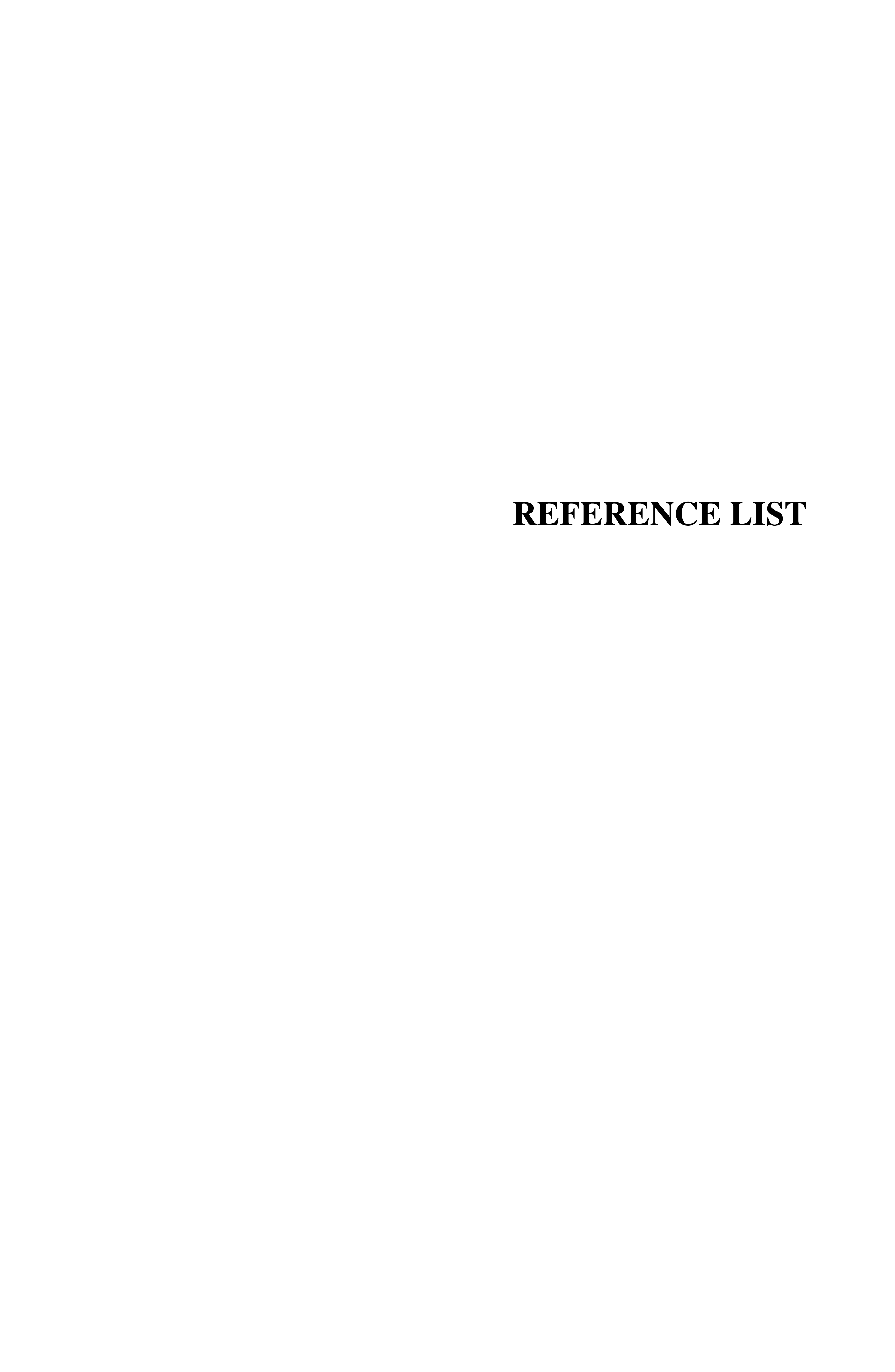
7. Temperature changes affect osteogenic genes' expression in gilthead sea bream both, in *in vitro* and *in vivo* models, pointing to *osteonectin* as a potential bone stress marker. Furthermore, temperature-challenged juvenile fish with different thermal histories during early development demonstrated the possible existence of embryonic thermal imprinting with long-term effects on bone.

8. Gilthead sea bream maintained at an elevated temperature present increased somatic growth and, changes at morphological and transcriptional levels suggesting an induction of adipogenesis or triglycerides utilization in adipose tissue, and increased fatty acid oxidation in liver thus, supporting the use of substituted diets by vegetable oils.

9. Concerning fish oil substitution, the combination of palm and rapeseed oils (PR diet) seems to give an equilibrated formulation to support gilthead sea bream to grow properly with a balanced lipid accumulation when reared at an elevated temperature.

10. The pattern of expression of genes related to bone development and matrix mineralization as well as those involved in resorption in gilthead sea bream showing vertebral column deformities, could be key to discriminate between potentially malformed or normal fish, whereas gene markers have not been identified in fish with operculum, dental or jaw deformities.

11. Overall, the present thesis has characterized the processes of adipogenesis and osteogenesis in rainbow trout and gilthead sea bream and demonstrated the plasticity of precursor cells. Moreover, it has shown that many factors, including nutrition and temperature, influence adipose tissue and bone development, besides whole-body energy metabolism, providing different models of study directed to improve a sustainable aquaculture in front of new challenges.



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# ANNEX

# ADDITIONAL PUBLICATIONS

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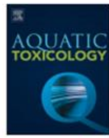

## Tributyltin and triphenyltin exposure promotes *in vitro* adipogenic differentiation but alters the adipocyte phenotype in rainbow trout

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## Ghrelin and Its Receptors in Gilthead Sea Bream: Nutritional Regulation

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## Temperature Affects Musculoskeletal Development and Muscle Lipid Metabolism of Gilthead Sea Bream (*Sparus aurata*)

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# Adipogenic Gene Expression in Gilthead Sea Bream Mesenchymal Stem Cells from Different Origin

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During the last decades, adipogenesis has become an emerging field of study in aquaculture due to the relevance of the adipose tissue in many physiological processes and its connection with the endocrine system. In this sense, recent studies have translated into the establishment of preadipocyte culture models from several fish species, sometimes lacking information on the mRNA levels of adipogenic genes. Thus, the aim of this study was to determine the gene expression profile of gilthead sea bream (*Sparus aurata*) primary cultured mesenchymal stem cells (MSCs) from different origin (adipose tissue and vertebra bone) during adipogenesis. Both cell types differentiated into adipocyte-like cells, accumulating lipids inside their cytoplasm. Adipocyte differentiation of MSCs from adipose tissue resulted in downregulation of several adipocyte-related genes (such as *lpl*, *hsl*, *ppar*α, *ppar*γ and *gapdh2*) at day 4, *gapdh1* at day 8, and *fas* and *ppar*β at day 12. In contrast, differences in *lxr*α mRNA expression were not observed, while *g6pdh* levels increased during adipocyte maturation. Gapdh and Pparγ protein levels were also detected in preadipocyte cultures; however, only the former increased its expression during adipogenesis. Moreover, differentiation of bone-derived cells into adipocytes also resulted in the downregulation of several adipocyte gene markers, such as *fas* and *g6pdh* at day 10 and *hsl*, *ppar*β, and *lxr*α at day 15. On the other hand, the osteogenic genes *fib1a*, *mgp*, and *op* remained stable, but an increase in *runx2* expression at day 20 was observed. In summary, the present study demonstrates that gilthead sea bream MSCs, from both adipose tissue and bone, differentiate into adipocyte-like cells, although revealed some kind of species- and cell lineage-specific regulation with regards to gene expression. Present data also provide novel insights into some of the potential key genes controlling adipogenesis in gilthead sea bream that can help to better understand the regulation of lipid storage in fish.

**Keywords: MSCs, adipogenesis, adipocyte, bone, *Sparus aurata***

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## INTRODUCTION

Traditionally adipose tissue was considered a mere energy store, synthesizing and accumulating triglycerides during caloric excess periods and releasing fatty acids and glycerol when needed, as under nutritional restriction. However, this changed with the discovery of the adipose tissue-produced hormone leptin in 1994 (1). Adipocytes and cells in the stromal vascular fraction of

adipose tissue produce many hormones, cytokines, and other molecules, with more than 50 described to date also in fish (2–8), which act on the central nervous system and peripheral organs regulating several processes, such as glucose and lipid metabolism (9, 10). Thus, it is now recognized that adipose tissue is an active contributor to the regulation of whole-body energy homeostasis.

Adipose tissue grows by increasing the size of existing adipocytes (hypertrophy), its number based on the formation of new adipocytes from precursor cells (hyperplasia), or both (11). These two adipocyte developmental types occur not only during the early life stages but also throughout life (12, 13). In addition to adipocytes, the adipose tissue also comprises a stromal vascular fraction formed by a heterogeneous population of cells, containing mesenchymal stem cells (MSCs) that include multipotent cells with the ability to differentiate into adipocytes, chondrocytes, and osteoblasts, among other cell lineages (14, 15). The process of adipocyte differentiation is divided in two steps and is influenced by hormones, growth factors, cytokines, and nutrients. First, the multipotent MSCs undergo a process known as determination (16, 17). This process results in cells that are morphologically similar to fibroblasts, which appear identical to MSCs, but are only able to differentiate into adipocytes. As a result, the cells in this post-determination state are called preadipocytes or adipoblasts. The second stage, the proper differentiation, consists in the formation of structurally mature adipocytes from preadipocytes and is commonly known as adipogenesis, where changes in cellular morphology, hormone sensitivity, and secretory capacity of the cells occur (18, 19). These changes are regulated through the coordinated expression of mainly transcription factors, which in turn act to activate transcription of genes that produce the adipocyte phenotype (20, 21). The peroxisome proliferator-activated receptor γ (Pparγ) is the central regulator of adipogenesis and is responsible for activating a number of genes involved in fatty acid binding, uptake, and storage, including lipoprotein lipase (*lpl*) or phosphoenolpyruvate carboxykinase, among others.

The interest in the adipogenic process and its regulation in fish has increased in the last years, because in aquaculture, the excessive fat accumulation experienced by some cultured species is generally perceived as an undesirable trait by the consumers and also has negative effects in terms of production, product lifetime, and fish health. Therefore, several primary cultures of preadipocytes have been established to better understand adipogenesis and its endocrine regulation in fish, including Atlantic salmon (*Salmo salar*) (8), red sea bream (*Pagrus major*) (22), rainbow trout (*Oncorhynchus mykiss*) (23), large yellow croaker (*Pseudosciaena crocea*) (24), grass carp (*Ctenopharyngodon idella*) (25), gilthead sea bream (*Sparus aurata*) (26), and common carp (*Cyprinus carpio*) (27). However, knowledge on the gene expression pattern during fish adipogenesis is usually scarce, especially in sparids, with only two microarray studies reported to date in salmonids, one in Atlantic salmon (7) and the other in rainbow trout (28).

Adipocytes and osteoblasts arise from a common precursor cell, which after the induction of certain transcription factors, differentiates into each one of these two cell types. As mentioned before, Pparγ is the master transcription factor for adipocyte differentiation, while runt-related transcription factor 2 (Runx2) is considered the one regulating osteogenic differentiation (20). The prevalence of skeletal malformations in hatchery-reared fish stimulated the establishment of cell culture models to study the mechanisms of bone formation and development in fish. The osteoblast models described to date have been two cell lines derived from vertebra and branchial arch of gilthead sea bream (29), one cell line derived from zebrafish (*Danio rerio*) calcified tissues (30), and three primary cultures; one derived from gilthead sea bream vertebra bone (31) and two of Atlantic salmon, one from white muscle precursor cells (32) and one from visceral fat precursor cells (33). Using the bone-derived cells from vertebra of gilthead sea bream, we have demonstrated that they are multipotent stem cells as they can be differentiated into either osteoblasts or adipocyte-like cells using an osteogenic or an adipogenic differentiation medium (DM), respectively (31). Nevertheless, the changes that occur in the transcriptional profile of these cells during such processes have not yet been investigated either.

Furthermore, another interest of the study of these cell culture models is that during the last decade in mammals, it has been speculated that the infiltration of bone marrow adipocytes during the development of osteoporosis in the elderly can be due to osteo-adipogenic transdifferentiation (34). In fish, the hematopoietic organ is the head kidney, and the bone marrow is from the beginning of its development filled with adipocytes, besides nerves, blood vessels, and connective tissue cells (35); therefore, we can hypothesize that such a process of adipocyte transdifferentiation from cells of the osteoblastic lineage may be also occurring in the adult, contributing to the whole fat content of the fish. As a first step toward exploring that possibility and using an *in vitro* approach, the aims of the current study were to (1) determine the transcriptional profile of gilthead sea bream preadipocytes during adipogenesis and (2) compare it with the gene expression pattern observed throughout the differentiation of bone-derived MSCs into adipocyte-like cells. To this end, we used primary cell cultures of gilthead sea bream precursor cells obtained from visceral adipose tissue and vertebra bone and analyzed well-characterized adipocyte differentiation markers and some osteogenic markers at different adipogenic stages.

## MATERIALS AND METHODS

### Animal Care

Animal care and experimental procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the Catalan government-established norms and procedures (permit numbers DAAM 7951; CEEA 169/14, and DAAM 6759; CEEA 243/12 for the preparation of primary cultures derived from adipose tissue or bone, respectively).

### Fish

Gilthead sea bream were obtained from a fish farm in Northern Spain and maintained in the animal facilities of the Faculty of Biology at the University of Barcelona. Fish were kept in 200 L

fiberglass tanks under 12-h light/12-h dark photoperiod at 21 ± 1°C, pH 7.5–8, 31–38% salinity and >80% oxygen saturation and fed *ad libitum* twice daily with a commercial diet (Skretting España SA, Burgos, Spain).

## Preadipocytes and Bone Cells Cultures

All plasticware for tissue culture was obtained from Nunc (Barcelona, Spain); and all the reagents were purchased from Sigma–Aldrich (Tres Cantos, Spain), unless stated otherwise. Cells were incubated at 23°C with 2.5% $CO_2$ during the whole duration of the experiments.

### Preadipocytes Cultures

Three or four juvenile gilthead sea bream of an average weight of 104 g were used for each culture. Preadipocytes were collected by mechanical disruption and enzymatic digestion of the visceral adipose tissue as described previously (26), plated at a density of $4.3 \times 10^4$ cells/cm$^2$ in gelatin-pretreated 6-well plates and maintained in growth media (GM) composed of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic solution (A/A), and supplemented with 60 mM NaCl. Cells were continuously cultured in GM until day 8, once confluence was reached, and then changed to a DM containing GM plus 10 µg/mL porcine insulin, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 0.25 µM dexamethasone, and 5 µL/mL lipid mixture (including cholesterol and fatty acids from cod liver oil) to induce adipocyte differentiation. After 3 days, the culture conditions were changed to an adipocyte medium (AM), consisting of GM plus lipid mixture (5 µL/mL), to keep the cells already differentiating until the end of the culture. Preadipocytes samples were collected at days 4 and 8 of culture (days −4 and 0, respectively) and samples of adipocytes at days 4, 8, and 12 after induction of differentiation. Prior to harvesting, cells were washed once with phosphate-buffered saline (PBS), recovered with TRI Reagent (Ambion, Alcobendas, Spain) using a cell scraper, then transferred to an RNase-free polypropylene tube, and kept at −80°C until RNA and protein extraction.

### Bone Cultures

A total of six juvenile gilthead sea bream of an average weight of 30 g were used for each culture. Bone-derived cells were isolated by mechanical disruption and enzymatic digestion of vertebra bone as described previously (31). Cells and small vertebra fragments were plated with GM supplemented with 19 mM NaCl and 1% fungizone (Invitrogen Life Technologies, Alcobendas, Spain), in a 10 cm culture dish. After 1 week, the fragments were removed and the attached cells collected with 0.25% trypsin–EDTA (Invitrogen Life Technologies, Alcobendas, Spain) and plated into new 10 cm plates with fresh GM. From here, the cells were routinely subcultured every time the cells reached about 70–80% confluence and used for a maximum of 10 passages. Differentiation into adipocyte-like cells was achieved as previously described (31). Briefly, 70–80% confluent cells were trypsinized from 10 cm culture dishes, seeded ($1 \times 10^4$ cells/cm$^2$), and cultured in 6-well plates with GM. The following day (i.e., day 0), media was changed first to DM, and 3 days later, changed to AM using the same experimental settings as explained in Section "Preadipocytes Cultures." Cell samples for RNA extraction were obtained at days 5, 10, 15, and 20 as described in Section "Preadipocytes Cultures."

## RNA and Protein Extraction

Simultaneous extraction of RNA and proteins from a single cell sample was performed using TRI Reagent (Ambion, Alcobendas, Spain) and following the manufacturer's recommendations.

### RNA Extraction and cDNA Synthesis

Total RNA was dissolved in DEPC-treated water (RNase-free) and stored at −80°C. RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain), and its integrity was analyzed by 1% (m/v) agarose gel electrophoresis. To eliminate any residual genomic DNA, total RNA (250 ng from preadipocytes and 1 µg from bone cells) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain), following the manufacturer's recommendations.

### Protein Extraction

Protein was extracted from the preadipocytes samples only and dissolved in RIPA buffer (Tris-HCl 50 mM, pH 7.4, NaCl 150 mM, EDTA 1 mM, NP-40 1%, Na-deoxycholate 0.25%, PMSF 1 mM, $Na_3VO_4$ 1 mM, NaF 1 mM, and protease inhibitor cocktail). Then, samples were homogenized using a "pellet pestle" for microtubes on ice, mixed in an orbital during 1 h at 4°C, and the supernatant was recovered after centrifugation during 30 min at maximum speed at 4°C. Total protein concentration was determined (595 nm) by Bradford assay using bovine serum albumin (BSA) as the standard protein (36).

## Quantitative PCR Analysis

In order to characterize the transcriptional profile occurring during adipocyte differentiation in gilthead sea bream, important genes implicated in adipogenesis and energy metabolism regulation were analyzed by quantitative PCR (qPCR). The genes comprise the following enzymes: fatty acid syntase (*fas*), *lpl*, glucose-6-phosphate dehydrogenase (*g6pdh*), hormone-sensitive lipase (*hsl*), and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) 1 and 2; and the following transcription factors: *ppar*α, *ppar*β, *ppar*γ, and liver X receptor alpha (*lxr*α). Moreover, the expression of four osteogenic genes, fibronectin 1a (*fib1a*), matrix Gla protein (*mgp*), osteopontin (*op*), and *runx2*, was determined in the cells derived from bone. In addition, elongation factor 1α (*ef1*α), ribosomal protein S18 (*rps18*), and 18S ribosomal RNA (*18s*) were tested as reference genes. qPCR was performed using a CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain) as previously described (37). Each qPCR reaction was performed in triplicate in a total volume of 5 µL, containing 2.5 µL of the iTaq Universal SYBR Green supermix (Bio-Rad, El Prat de Llobregat, Spain), 0.125–1.25 ng of cDNA template, 250–500 nM of each primer (**Table 1**), and milliQ water. Samples were amplified as follows: 95°C for 3 min, and then 40 cycles of 95°C for 10 s, followed by annealing 57–69°C for 30 s (primer-dependent, **Table 1**), followed by dissociation

**TABLE 1 | Primers used for real-time quantitative PCR.**

| Gene | Primer sequence (5′→3′) | Tm (°C) | Product size (bp) | *E* (%) | Acc. Num. |
|---|---|---|---|---|---|
| *lpl*_FW | GAGCACGCAGACAACCAGAA | 60 | 135 | 98.1 | AY495672 |
| *lpl*_RV | GGGGTAGATGTCGATGTCGC | | | | |
| *fas*_FW | TGGCAGCATACACACAGACC | 60 | 78 | 102.0 | AM952430 |
| *fas*_RV | CACACAGGGCTTCAGTTTCA | | | | |
| *g6pdh*_FW | CAGAATGAAAGATGGGATGGAGTC | 60 | 176 | 102.9 | AY754640 |
| *g6pdh*_RV | TTCAGGTAAATGGCTTCGTTCG | | | | |
| *hsl*_FW | GCTTTGCTTCAGTTTACCACCATTTC | 60 | 122 | 99.6 | EU254478 |
| *hsl*_RV | GATGTAGCGACCCTTCTGGATGATGTG | | | | |
| *gapdh1*_FW | CCAGCCAGAACATCATCC | 60 | 190 | 103.5 | DQ641630 |
| *gapdh1*_RV | GCAGCCTTGACGACCTTC | | | | |
| *gapdh2*_FW | CATGAAGCCAGCAGAGATCC | 57 | 196 | 105.5 | FM145063 |
| *gapdh2*_RV | GGTGGCCGGGTCATATTTC | | | | |
| *ppar*α_FW | TCTCTTCAGCCCACCATCCC | 62 | 116 | 104.2 | AY590299 |
| *ppar*α_RV | ATCCCAGCGTGTCGTCTCC | | | | |
| *ppar*β_FW | AGGCGAGGGAGAGTGAGGATGAGGAG | 69 | 188 | 108.3 | AY590301 |
| *ppar*β_RV | CTGTTCTGAAAGCGAGGGTGACGATGTTTG | | | | |
| pparγ_FW | CGCCGTGGACCTGTCAGAGC | 66 | 171 | 96.0 | AY590304 |
| pparγ_RV | GGAATGGATGGAGGAGGAGGAGATGG | | | | |
| lxrα_FW | GCACTTCGCCTCCAGGACAAG | 62 | 107 | 88.0 | FJ502320 |
| lxrα_RV | CAGTCTTCACACAGCCACATCAGG | | | | |
| *fib1a*_FW | CGGTAATAACTACAGAATCGGTGAG | 60 | 104 | 101.8 | FG262933 |
| *fib1a*_RV | CGCATTTGAACTCGCCCTTG | | | | |
| *mgp*_FW | TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC | 68 | 244 | 81.8 | AY065652 |
| *mgp*_RV | CGGGCGGATAGTGTGAAAAATGGTTAGTG | | | | |
| *op*_FW | AAAACCCAGGAGATAAACTCAAGACAACCCA | 68 | 153 | 85.0 | AY651247 |
| *op*_RV | AGAACCGTGGCAAAGAGCAGAACGAA | | | | |
| *runx2*_FW | ACCCGTCCTACCTGAGTCC | 60 | 122 | 96.1 | JX232063 |
| *runx2*_RV | AGAAGAACCTGGCAATCGTC | | | | |
| *ef1*α_FW | CTTCAACGCTCAGGTCATCAT | 60 | 263 | 85.0 | AF184170 |
| *ef1*α_RV | GCACAGCGAAACGACCAAGGGGA | | | | |
| *rps18*_FW | AGGGTGTTGGCAGACGTTAC | 60 | 164 | 98.3 | AM490061 |
| *rps18*_RV | CTTCTGCCTGTTGAGGAACC | | | | |
| *18s*_FW | TGACGGAAGGGCACCACCAG | 60 | 158 | 91.0 | AY550956 |
| *18s*_RV | AATCGCTCCACCAACTAAGAACGG | | | | |

*lpl, lipoprotein lipase; fas, fatty acid synthase; g6pdh, glucose-6-phosphate dehydrogenase; hsl, hormone-sensitive lipase; gapdh1, glyceraldehyde 3-phosphate dehydrogenase 1; gapdh2, glyceraldehyde 3-phosphate dehydrogenase 2; ppar*α*, peroxisome proliferator-activated receptor alpha; ppar*β*, peroxisome proliferator-activated receptor beta; ppar*γ*, peroxisome proliferator-activated receptor gamma; lxr*α*, liver X receptor alpha; fib1a, fibronectin 1a; mgp, matrix Gla protein; op, osteopontin; runx2, runt-related transcription factor 2; ef1*α*, elongation factor 1 alpha; rps18, ribosomal protein S18; 18s, 18S ribosomal RNA; FW, forward primer; RV, reverse primer; Tm, annealing temperature; bp, base pair; E, amplification efficiency; Acc. Num., accession number.*

step from 55 to 95°C with a 0.5°C increase every 5 s. A standard curve dilution series of a cDNA sample pool was constructed to determine the qPCR efficiency of each primer pair (**Table 1**), which was calculated using the CFX Manager Software (Bio-Rad). No template control (NTC), no reverse transcription control (RTC), and PCR control (PCR) were used to determine the overall performance of each qPCR assay. Relative expression levels of the target genes were determined by the Pfaffl method (38) using correction for primer efficiencies and normalizing the quantification cycle (Cq) value of each gene registered during the annealing step to that of *rps18* and *ef1*α, the most stable reference genes among the different culture stages ($P > 0.05$) using the CFX Manager Software (Bio-Rad). Data from preadipocytes and bone cells were obtained from 4–5 and 5–6 independent cultures, respectively.

## Western Blotting

For Western blot analyses, 8 μg of protein were loaded in each lane. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12%) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, El Prat de Llobregat, Spain) overnight at 4°C and 100 mA. Reversible Ponceau staining was used as a loading control (39). The PVDF membranes were blocked with 5% skimmed milk powder in Tris-Buffered Saline and Tween 20 (TBS-T) for 1 h 15 min and probed with rabbit polyclonal Pparγ (sc-7196) and goat polyclonal Gapdh (sc-20357) primary antibodies (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:200 overnight at 4°C on a tube rotator. Membranes were washed in TBS-T and probed with a horseradish peroxidase-conjugated anti-rabbit (sc-2004) or anti-goat (sc-2020) secondary antibody (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:10,000 for 1 h 15 min. Membranes were washed with TBS-T and chemiluminescent detection performed using an enhanced chemiluminescence kit (Pierce ECL Western blotting Substrate; Thermo Scientific, Alcobendas, Spain). Western blotting results were obtained from two independent cultures, and band intensities were quantified by scanning densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to Ponceau staining.

## Oil Red O Staining

To evaluate adipocyte differentiation in the bone-derived cells, intracellular neutral lipid accumulation was analyzed by Oil red O staining as explained in Ref. (31). Cells were grown as explained in Section "Bone Cultures" and samples obtained at days 5 and 15 of culture development. Quantification of cell lipid content was calculated as the absorbance measured at 490 nm divided by the read at 630 nm corresponding to cell protein content, which was obtained after Comassie blue staining for 1 h and dye extraction by incubation of the cells with 85% propylene glycol during 3 h at 60°C (31). Data are presented as fold change relative to day 5 of culture.

## Statistical Analyses

Data normality and homoscedasticity were assessed using Shapiro–Wilk and Levene's test, respectively. Independent samples' Student's *t*-test was used for comparison between two groups. For multiple mean comparisons of normal distributed data, one-way ANOVA was used followed by Tukey's or Dunnett's T3 *post hoc* tests in case of homogeneous or heterogeneous variance data, respectively. When data did not fit normal distribution, the non-parametric Kruskal–Wallis test, followed by Mann–Whitney test, were used. Statistical analyses were performed using SPSS Statistics version 20 (IBM, Armonk, NY, USA). Results were presented as mean ± SEM. $P < 0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

# RESULTS

## Gene and Protein Expression Profiles during Differentiation of Preadipocytes

Gilthead sea bream preadipocytes require the addition of a DM, a mixture of insulin, IBMX, dexamethasone, and lipid mixture, to differentiate into mature adipocytes (26). After differentiation induction, gilthead sea bream preadipocytes start to change its morphology from a fibroblast-like shape to an adipocyte-like form with an enlarged cytoplasm filled with lipids (26). The transcriptional profile during adipogenesis in gilthead sea bream preadipocytes was analyzed before (days −4 and 0) and after (days 4, 8, and 12) induction of differentiation. The mRNA levels of the early marker of adipocyte differentiation *lpl* decreased significantly from day −4 to day 4 and then gradually increased again (**Figure 1A**). *fas* and *g6pdh*, genes participating in the *via* of *de novo* lipogenesis from glucose and in the pentose phosphate pathway, respectively, showed opposite expression patterns, with *fas* gradually decreasing (**Figure 1B**), while *g6pdh* was significantly upregulated during most of the process (**Figure 1C**). The expression of the lipolysis-associated gene *hsl* was significantly higher in preadipocytes and late-differentiated adipocytes relative to cells at day 4 (**Figure 1D**). With regards to the adipocyte maturation marker *gapdh*, the expression of both isoforms (*gapdh1* and *gapdh2*) significantly diminished along with differentiation (**Figures 1E,F**).

Furthermore, regarding the expression of the transcription factors analyzed, *ppar*α and *ppar*β, nuclear receptors that regulate the beta-oxidation of fatty acids, were significantly downregulated from early and at late stages of differentiation, respectively (**Figures 2A,B**). The transcript levels of *ppar*γ, the nuclear receptor key in the process of adipocyte differentiation, decreased significantly after the addition of the DM (day 4), but then its expression was maintained along with adipocyte maturation (**Figure 2C**). Finally, the mRNA levels of *lxr*α, a nuclear receptor participating in the regulation of cholesterol homeostasis, was stable during the whole adipogenic process (**Figure 2D**).

Next, protein expression of Pparγ and Gapdh was also determined in lysates from undifferentiated (days −4 and 0) and differentiated (days 4 and 12) adipocytes. Results showed that Pparγ tended to increase up to day 4 and then diminished (**Figure 3A**), while Gapdh increased steadily during adipogenesis (**Figure 3B**).

## Lipid Accumulation during Adipogenesis of Bone-Derived Cells

Bone-derived MSCs of gilthead sea bream can become adipocyte-like cells using the same DM than in preadipocytes (31). Phenotypic changes from the cells with a fibroblast-like appearance to more rounded and lipid-filled cells with the morphological semblance of adipocytes were observed during adipogenesis (**Figure 4A**). Differentiation into adipocyte-like cells was tracked by Oil red O staining, which monitors lipid accumulation. Lipid content in the cells was gradually increasing during the process of adipocyte maturation, showing day 15 cells significant differences when compared to day 5 cells (**Figure 4B**).

## Gene Expression Profile during Adipogenesis of Bone-Derived Cells

The expression of all the genes implicated in lipid metabolism analyzed, such as *fas*, *g6pdh*, and *hsl* (**Figures 5B–D**), was significantly downregulated during adipogenesis of bone-derived cells. On the other hand, the expression of *lpl*, *gapdh1*, and *gapdh2* was unaffected (**Figures 5A,E,F**). Moreover, the gene expression of most of the transcription factors determined in the present study (*ppar*α, *ppar*β, and *lxr*α) was progressively downregulated (**Figures 6A,B,D**), with the exception of *ppar*γ that continued stable (**Figure 6C**).

With regards to the representative osteogenic genes analyzed, the expression of the three components of the extracellular matrix (*fib1a*, *mgp*, and *op*) remained unaltered during the whole process of adipocyte differentiation (**Figures 7A–C**). On the other hand, the key transcription factor controlling osteogenesis, *runx2*, showed increasing levels during differentiation (**Figure 7D**).

# DISCUSSION

Mesenchymal stem cells are multipotent cells that through a two-step process, lineage commitment of specific progenitors and maturation, can be induced to differentiate into cells of several tissue types, such as osteoblasts or adipocytes (20). Local, hormonal, and mechanical factors in MSCs, as well as intermediate precursors and differentiated cells, result in the activation

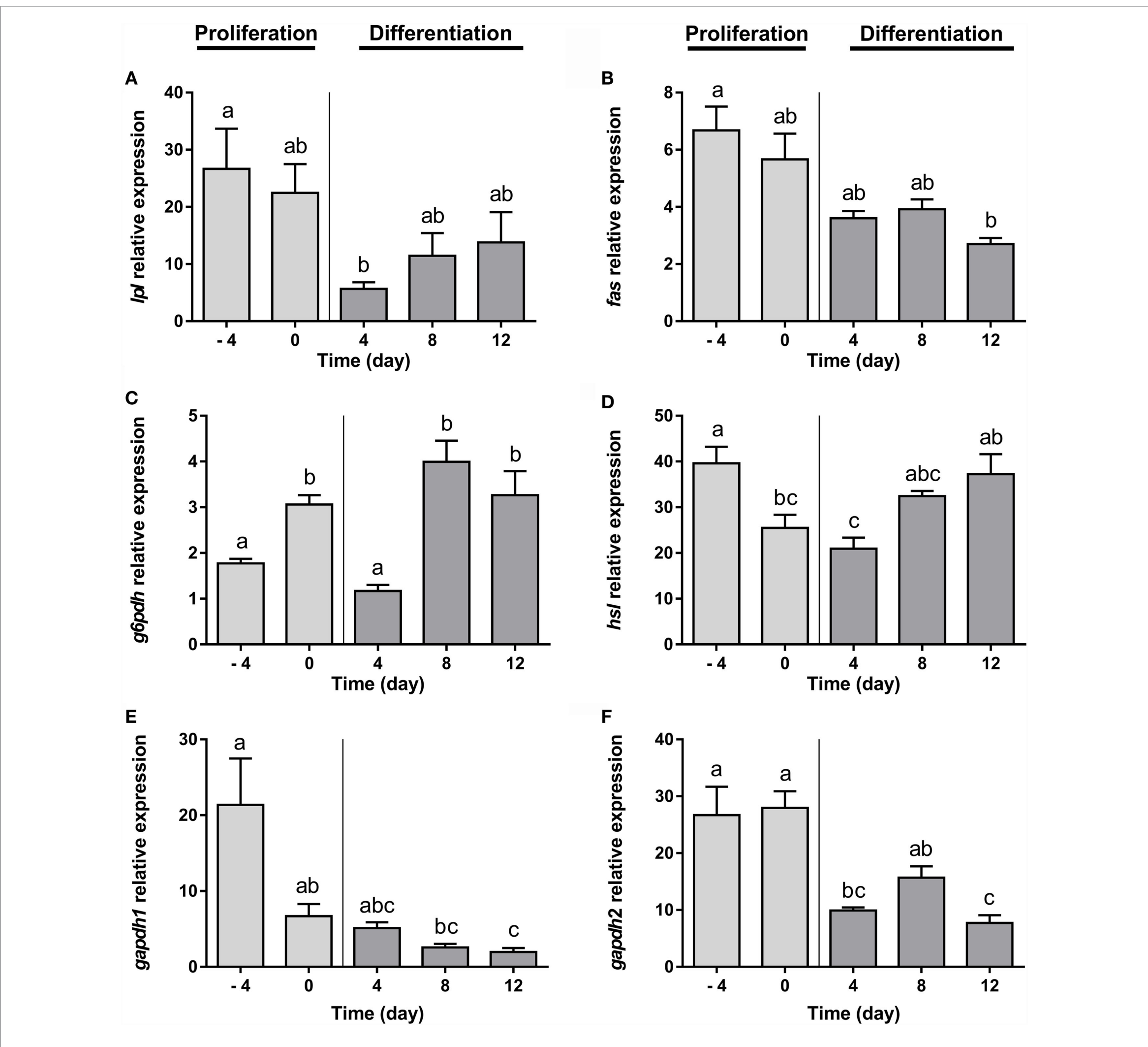


**FIGURE 1 | Gene expression profile of lipid metabolism-related genes in gilthead sea bream preadipocytes during adipogenesis**. The mRNA levels of *lpl* **(A)**, *fas* **(B)**, *g6pdh* **(C)**, *hsl* **(D)**, *gapdh1* **(E)**, and *gapdh2* **(F)** were measured by quantitative real-time PCR and normalized to *ef1*α and *rps18*. Samples were taken from preadipocytes (days −4 and 0 of culture) and differentiated adipocytes (days 4, 8, and 12 of culture). Values are means ± SEM, $n = 4–5$. Bars with different letters are significantly different ($P < 0.05$). *lpl*, lipoprotein lipase; *fas*, fatty acid synthase; *g6pdh*, glucose-6-phosphate dehydrogenase; *hsl*, hormone-sensitive lipase; *gapdh1* and *2*, glyceraldehyde 3-phosphate dehydrogenase 1 and 2, respectively; *ef1*α, elongation factor 1 alpha; *rps18*, ribosomal protein S18.

of a series of transcription factors and epigenetic mechanisms, which jointly control the balance of adipogenesis and osteoblastogenesis, including their transdifferentiation (40). Gilthead sea bream MSCs isolated from adipose tissue or vertebra bone can be differentiated into mature adipocytes using a medium supplemented with insulin, IBMX, dexamethasone, and lipid mixture (i.e., DM) (26, 31). Insulin, the most potent of these inducers in mammals, activates *ppar*γ expression (41, 42). IBMX and dexamethasone activate CCAAT enhancer-binding proteins *c/ebp*β and *c/ebp*δ expression, respectively (20), which in turn induce the expression of *c/ebp*α and *ppar*γ, the master regulators of adipogenesis. In preadipocyte primary cultures of some fish species, the addition of lipids in the differentiation cocktail is required to induce full maturation of adipocytes, most probably since polyunsaturated fatty acids, such as docosahexaenoic and eicosapentaenoic acids, are natural activators of Pparγ (43). A lipid mixture containing cholesterol and cod liver oil fatty acids (methyl esters), polyoxyethylenesorbitan monooleate, and D-α-tocopherol acetate has been used in Atlantic salmon (8), rainbow trout (23), large yellow croaker (24), and gilthead sea bream (26).

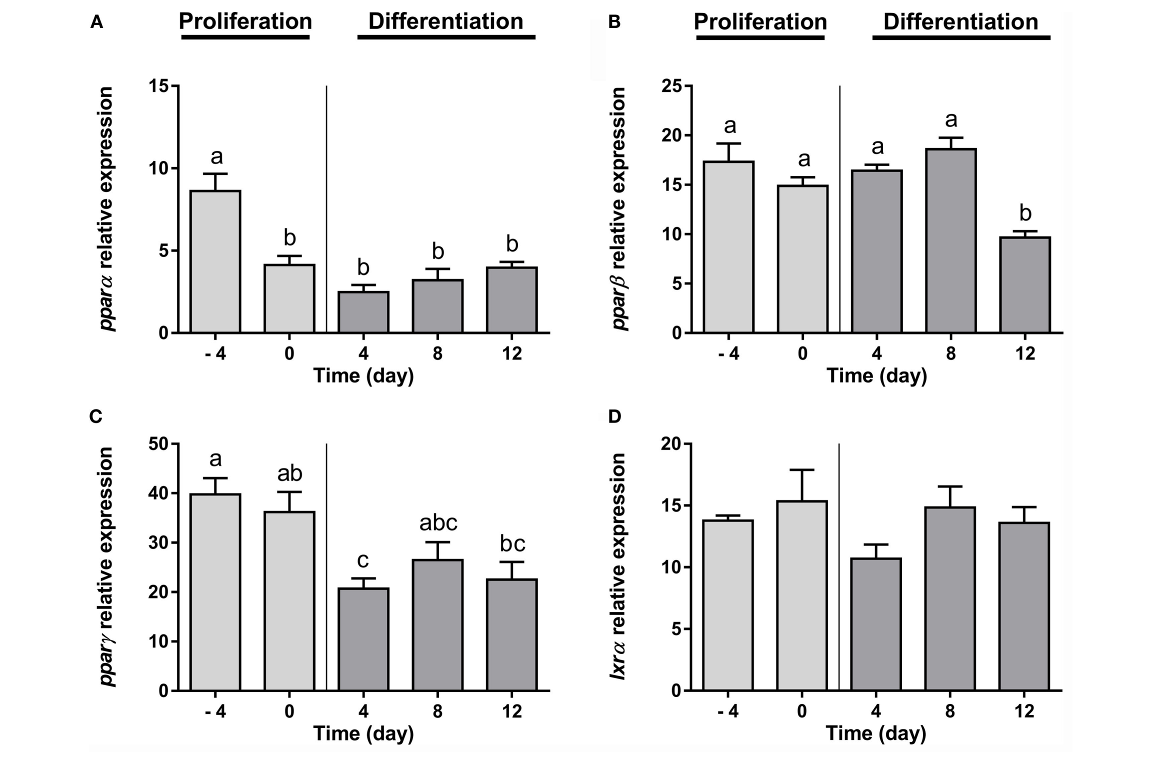


**FIGURE 2 | Gene expression profile of transcription factors in gilthead sea bream preadipocytes during adipogenesis**. The mRNA levels of *ppar*α **(A)**, *ppar*β **(B)**, *ppar*γ **(C)**, and *lxr*α **(D)** were measured by quantitative real-time PCR and normalized to *ef1*α and *rps18*. Samples were taken from preadipocytes (days −4 and 0 of culture) and differentiated adipocytes (days 4, 8, and 12 of culture). Values are means ± SEM, $n = 4–5$. Bars with different letters are significantly different ($P < 0.05$). *ppar*α, β, and γ, peroxisome proliferator-activated receptor alpha, beta, and gamma, respectively; *lxr*α, liver X receptor alpha; *ef1*α, elongation factor 1 alpha; *rps18*, ribosomal protein S18.

In common carp, linoleic and oleic acids have been employed in combination with T3 and troglitazone (27), whereas in red sea bream (22, 44) and grass carp (25), the use of a DME/F12 medium containing only linoleic acid was enough to induce adipocyte differentiation.

In the present study, the addition of the DM to the preadipocytes downregulated the expression of the early marker *lpl*, as it occurs during the early stages of differentiation in Atlantic salmon preadipocytes (7). However, *lpl* gene expression remained stable during the adipocyte differentiation of bone-derived cells; and in other fish species, such as large yellow croaker (24), rainbow trout (45), and red sea bream (22, 44), its expression was increased after induction of adipocyte differentiation. Thus, the regulation of *lpl* expression could be species-specific, or posttranscriptional mechanisms such as modulation at the activity level cannot be discarded. Fas is an enzyme that regulates the *de novo* biosynthesis of long-chain fatty acids catalyzing the formation of palmitate (46). In this study, *fas* mRNA expression decreased gradually during adipogenesis of both MSC types, contrary to that observed in Atlantic salmon (7) and red sea bream (44) preadipocytes, where its expression was higher and increased during adipocyte differentiation. In a recent study about *de novo* lipogenesis in Atlantic salmon preadipocytes, Bou and coworkers (47) showed that the use of palmitate decreases the expression of acetyl-CoA carboxylase (*acc*), the enzyme that catalyzes the formation of malonyl-CoA necessary for the fatty acid synthesis by Fas. Such data suggested that the synthesis of palmitate mediated by Fas may be blocking its own, and *acc* gene expression, through a negative feedback mechanism, similarly as reported in primary fetal rat calvarial cultured cells where palmitate reduced the expression of *fas* and *ppar*γ (48).

G6pdh, an enzyme of the pentose phosphate pathway that produces the NADPH necessary for the biosynthesis of fatty acids and cholesterol, was upregulated after the induction of differentiation in preadipocytes as it occurs in Atlantic salmon (7), although its expression decreased during adipogenesis of bone-derived cells. In 3T3-L1 cells, Parks and collaborators demonstrated that *g6pdh* overexpression upregulates most adipocyte marker genes (such as *fas* and *ppar*γ) and elevates the levels of cellular free fatty acids and triglycerides. Consistently, *g6pdh*

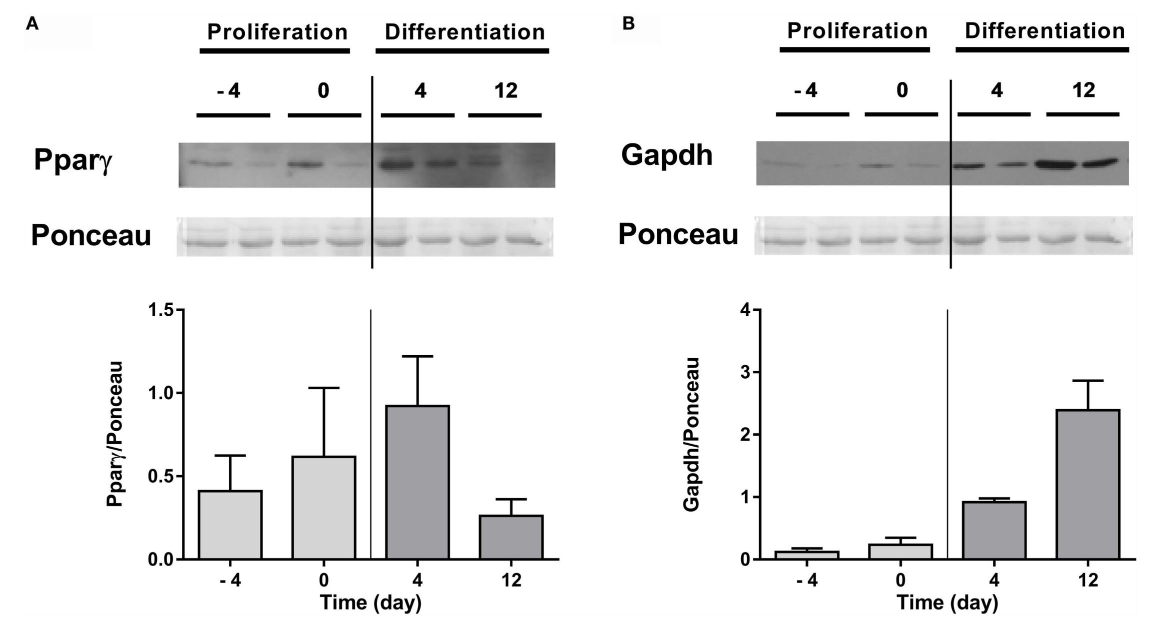


**FIGURE 3** | **Protein expression of Pparγ and Gapdh in gilthead sea bream preadipocytes during adipogenesis**. Representative Western blot, corresponding Ponceau, and quantification of Pparγ **(A)** and Gapdh **(B)** protein levels in preadipocytes (days −4 and 0 of culture) and differentiated adipocytes (days 4 and 12 of culture). Values are means ± SEM, $n = 2$. Pparγ, peroxisome proliferator-activated receptor gamma; Gapdh, glyceraldehyde 3-phosphate dehydrogenase.

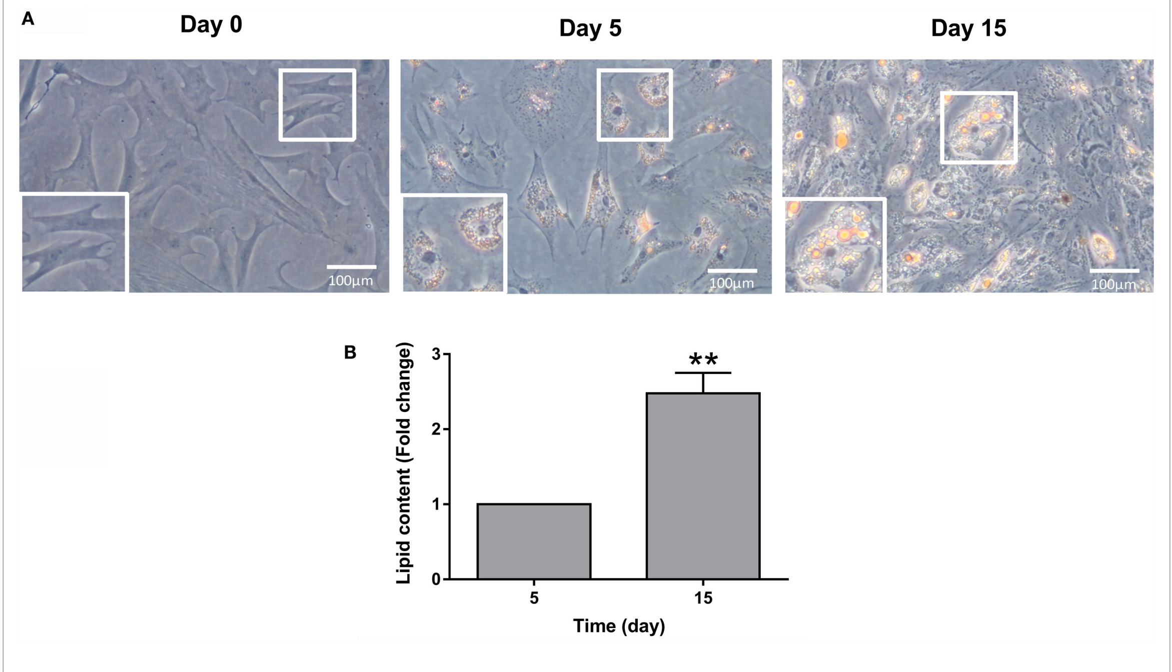


**FIGURE 4** | **Gilthead sea bream bone-derived cells during adipogenesis**. **(A)** Representative phase-contrast images of cells stained with Oil red O at days 0, 5, and 15 of culture. Magnification 20× and enlarged views. **(B)** Quantification of Oil red O staining at days 5 and 15 of culture. Values are mean ± SEM of five independent experiments with wells run in duplicate and presented as fold change relative to day 5 of culture (**$P < 0.01$).

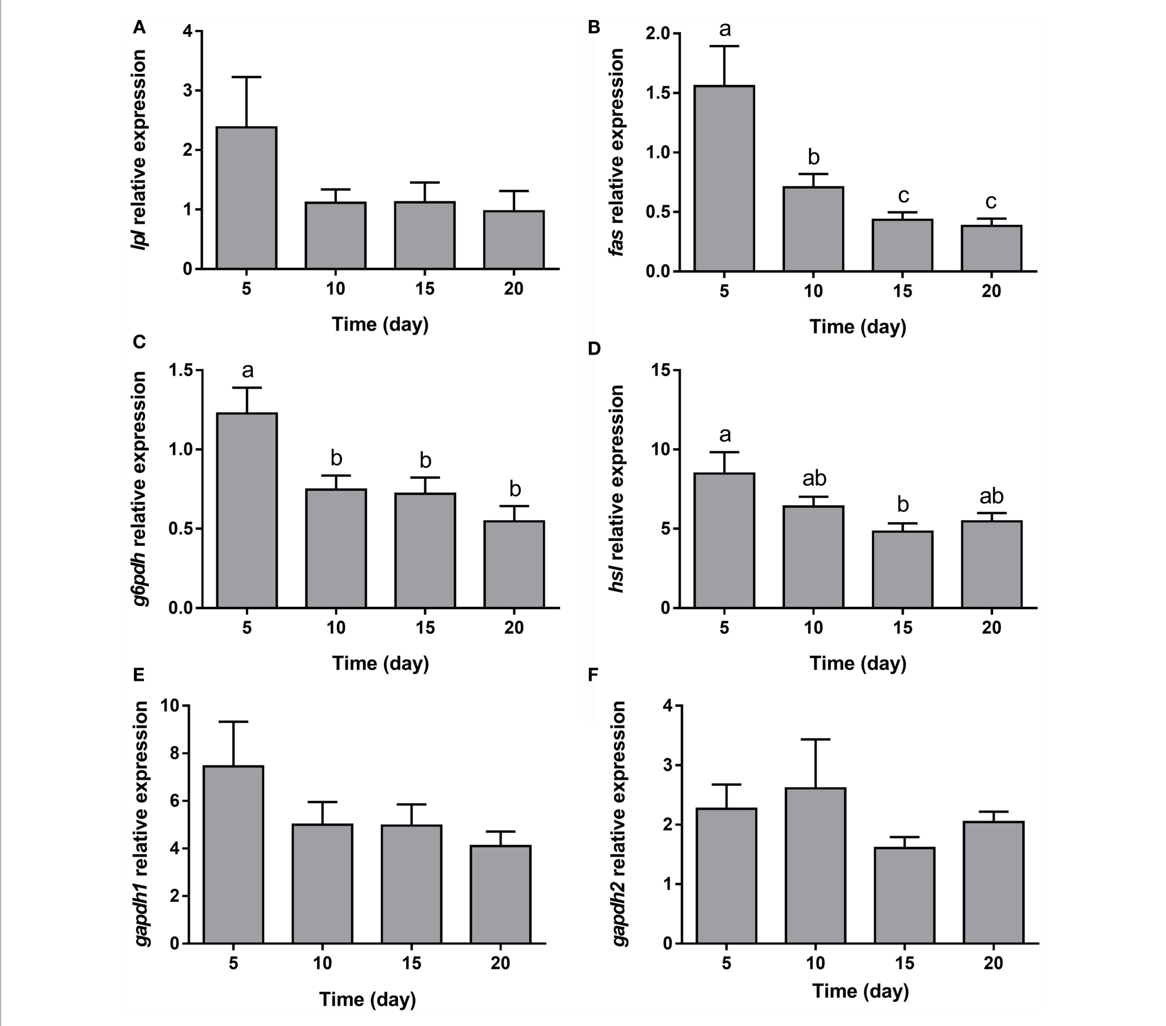


**FIGURE 5 | Gene expression profile of lipid metabolism-related genes in gilthead sea bream bone-derived cells during adipogenesis**. The mRNA levels of *lpl* **(A)**, *fas* **(B)**, *g6pdh* **(C)**, *hsl* **(D)**, *gapdh1* **(E)**, and *gapdh2* **(F)** were measured by quantitative real-time PCR and normalized to *ef1*α and *rps18*. Samples were taken from adipocyte-like cells at days 5, 10, 15, and 20 of culture. Values are means ± SEM, $n$ = 5–6. Bars with different letters are significantly different ($P < 0.05$). *lpl*, lipoprotein lipase; *fas*, fatty acid synthase; *g6pdh*, glucose-6-phosphate dehydrogenase; *hsl*, hormone-sensitive lipase; *gapdh1* and *2*, glyceraldehyde 3-phosphate dehydrogenase 1 and 2, respectively; *ef1*α, elongation factor 1 alpha; *rps18*, ribosomal protein S18.

knockdown *via* small interfering RNA attenuated adipocyte differentiation reducing lipid droplet accumulation, indicating that proper expression of *g6pdh* is required for adipogenesis as well as lipogenesis (49). Therefore, the different mRNA expression of *g6pdh*, observed during adipocyte differentiation between the two tissues, suggests that G6pdh may be playing different roles during adipogenesis (i.e., fatty acid synthesis and/or oxidation protection), and thus it can be used as a feature to identify adipocytes derived from each tissue type. Moreover, the lipolytic marker *hsl* decreased transiently its gene expression with differentiation to then increase again, in preadipocytes and to a lesser extent in bone-derived MSCs as well, in agreement with a previous study in human preadipocytes where *hsl* mRNA levels rose during adipocyte differentiation (50). This result suggests that the increase of lipid storage in the cells during adipogenesis may also promote lipolysis through Hsl in order to control its own intracellular levels of lipids. Furthermore, Gapdh produces the triglyceride glycerol required for the triglyceride synthesis being considered a late adipogenic marker. The gene expression of both isoforms of *gapdh* decreased along with adipocyte differentiation in preadipocytes but was unchanged in bone-derived MSCs, in contrast to that occurred at a protein level, where Gapdh

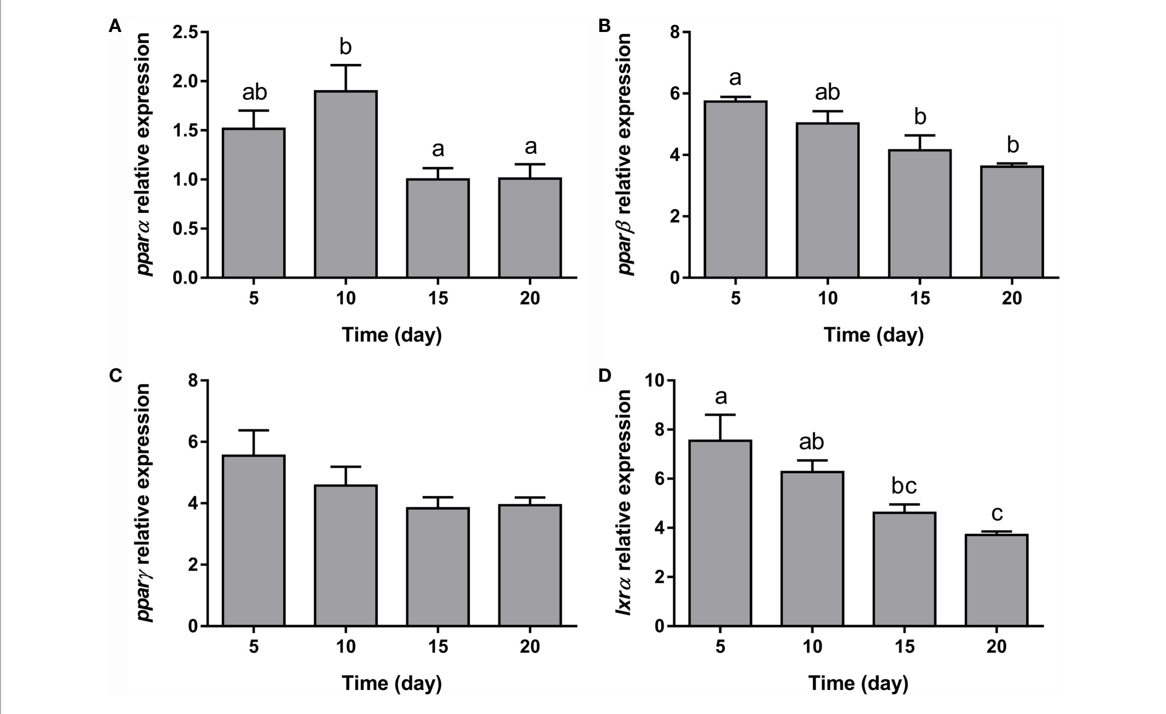


**FIGURE 6** | **Gene expression profile of transcription factors in gilthead sea bream bone-derived cells during adipogenesis**. The mRNA levels of *ppar*α **(A)**, *ppar*β **(B)**, *ppar*γ **(C)**, and *lxr*α **(D)** were measured by quantitative real-time PCR and normalized to *ef1*α and *rps18*. Samples were taken from adipocyte-like cells at days 5, 10, 15, and 20 of culture. Values are means ± SEM, $n = 5–6$. Bars with different letters are significantly different ($P < 0.05$). *ppar*α, β, and γ, peroxisome proliferator-activated receptor alpha, beta, and gamma, respectively; *lxr*α, liver X receptor alpha; *ef1*α, elongation factor 1 alpha; *rps18*, ribosomal protein S18.

appeared to increase from preadipocytes to differentiated adipocytes. In grass carp (25), Atlantic salmon (8), and rainbow trout (23), Gapdh gene expression was not determined, but its activity was also increased with adipocyte differentiation suggesting that perhaps some kind of posttranscriptional, translational, and/or protein regulation processes may be occurring (51).

Regarding the analyses of transcription factors, the expression of all *ppars* decreased early (*ppar*α), steadily (*ppar*γ), or late (*ppar*β) during preadipocyte differentiation and were similarly downregulated during the differentiation of bone-derived cells with the exception of *ppar*γ that remained stable. In previous works with Atlantic salmon, the gene expression of *ppar*α and the short form of *ppar*γ increased during differentiation, while *ppar*γ long was induced during the early phase and decreased at later stages of differentiation (52). Other studies in Atlantic salmon showed that the gene expression of *ppar*α and *ppar*β were unchanged during adipogenesis (53), whereas *ppar*γ was upregulated already in subconfluent cells (7), as observed in our study. In red sea bream, *ppar*α mRNA expression increased up to 7 days after induction and then decreased, while *ppar*β and *ppar*γ remained unaffected (44). In grass carp (25) and in large yellow croaker (24), *ppar*γ transcriptional expression increased gradually during cell differentiation. Here, the presence of the Pparγ protein increased from preadipocytes to early differentiated adipocytes, contrary to that occurred at a transcriptional level, suggesting that control at a posttranscriptional level exists. However, the present data about Pparγ protein expression during differentiation are in agreement with a previous study in rainbow trout preadipocytes where the protein level of Pparγ was higher in mature cells than in proliferating cells, suggesting that Pparγ participates as a transcription factor mostly during the early stages of the adipogenic process (23). Finally, gradual downregulation of *lxr*α mRNA levels was detected during adipocyte differentiation of bone-derived cells, while those were stable in differentiating preadipocytes in disagreement with other studies in 3T3-L1 cells (54) and rainbow trout preadipocytes (55), where its expression increased during differentiation. Lxr induction in the late wave of adipogenesis and its activation inhibits adipocyte conversion, increases glucose uptake, glycogen synthesis, cholesterol synthesis, and fatty acids efflux (56). To sum up, *lxr*α and *g6pdh* during maturation of cells from both tissue types showed a similar pattern of gene expression, suggesting that Lxrα can be involved in the expression of *g6pdh*, regulating the pentose phosphate pathway in gilthead sea bream.

The process of osteogenesis can be divided in three stages, commitment, extracellular matrix production, and mineralization.

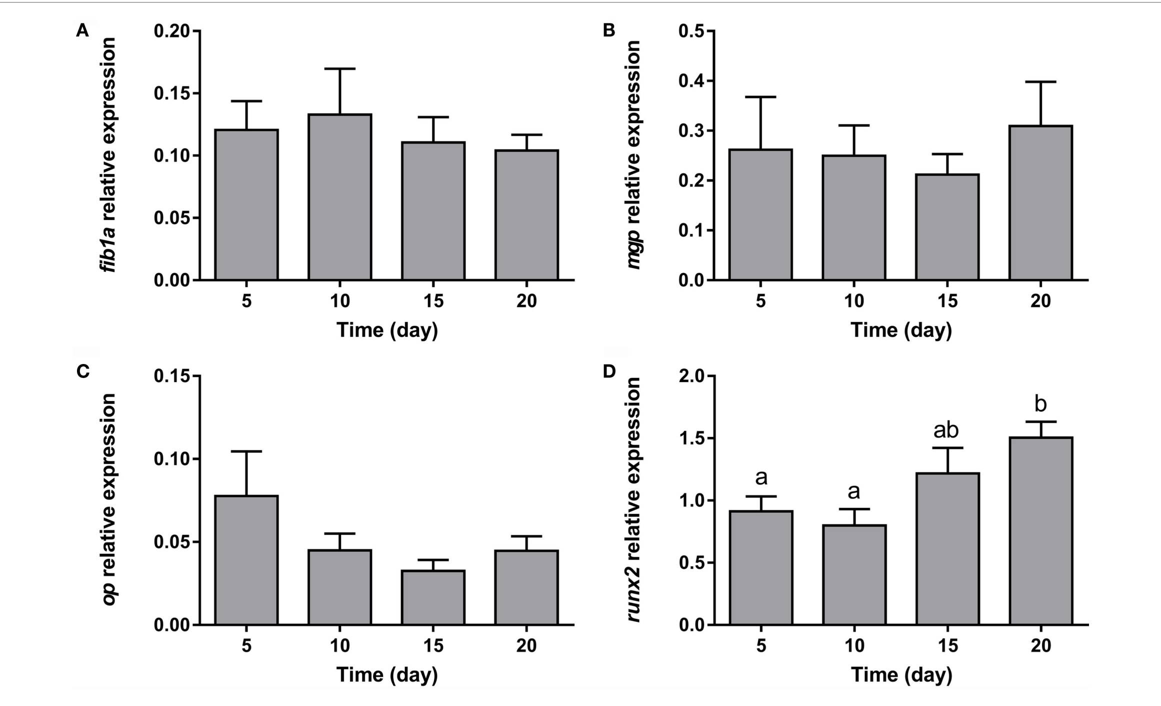


**FIGURE 7 | Gene expression profile of osteogenic markers in gilthead sea bream bone-derived cells during adipogenesis**. The mRNA levels of *fib1a* **(A)**, *mgp* **(B)**, *op* **(C)**, and *runx2* **(D)** were measured by quantitative real-time PCR and normalized to *ef1α* and *rps18*. Samples were taken from adipocyte-like cells at days 5, 10, 15, and 20 of culture. Values are means ± SEM, $n = 5–6$. Bars with different letters are significantly different ($P < 0.05$). *fib1a*, fibronectin 1a; *mgp*, matrix Gla protein; *op*, osteopontin; *runx2*, runt-related transcription factor 2; *ef1α*, elongation factor 1 alpha; *rps18*, ribosomal protein S18.

Fib1a, Op, and Mgp are components of the extracellular matrix. Fib1a is related to the initial state of osteoblast differentiation (57), and in previous studies with gilthead sea bream bone-derived cells, its gene expression increased at day 5 after addition of an osteogenic medium and decreased slowly during differentiation (58). On the other hand, Op and Mgp are key molecules during the phase of calcification of the extracellular matrix (59, 60), and in the gilthead sea bream osteoblast culture, its gene expression increased steadily when mineralization of the tissue started (58). These findings contrast with the results obtained here in the presence of an adipogenic media, where the expression of these osteogenic genes remained low and without variations, indicating a change in the transcriptomic profile of these cells when turning into adipocyte-like cells. Moreover, Runx2 and Osterix are considered key transcription factors required for osteogenic differentiation of MSCs (20). Runx2 promotes cell differentiation into immature osteoblasts inhibiting their commitment to the adipocyte lineage, while Osterix is required for the maturation phase (20). Nevertheless, during the *in vitro* osteogenic development of gilthead sea bream, bone MSCs' differences in *runx2* expression were not observed (58). Rat adipose-derived stem cells infected with a recombinant adenovirus carrying the *runx2* gene, decreased the gene expression of *lpl* and *pparγ* and reduced lipid droplet formation (61). In precursor cells isolated from visceral fat of Atlantic salmon differentiating into adipocytes or osteoblasts, Ytteborg and collaborators found that *pparγ* mRNA was absent in cultures given osteogenic medium, and *runx2* when adipogenic medium was added, suggesting that a similar corepressing mechanism also exists in fish (33). Nevertheless, in our study, the gene expression of *runx2* during adipogenesis was high and increased toward the end, explaining perhaps why the *lpl* and *pparγ* mRNA levels in bone-derived cells were not modified.

In summary, in the present study, we used primary cultures of MSCs derived from adipose tissue or vertebra bone of gilthead sea bream to investigate the expression profile of lipid metabolism-related genes and transcription factors during adipogenesis. Gilthead sea bream preadipocytes and bone-derived cells were able to differentiate into adipocyte-like cells after addition of a DM. However, the revealed gene expression profile during the process of adipogenesis contradicts some previous findings using similar experimental models in another vertebrate species. The differences between the present and other studies can be related to some kind of species or tissue-specific regulation, differences in the composition of the DM used to induce adipogenesis, or due to the existence of posttranscriptional, translational, and/or protein regulation processes. Future experiments will explore if this cell lineage determination provokes the complete activation

of adipocyte functions, and that the cells are not only able to accumulate lipids but also have other key characteristics, such as adipokines production or sensitivity to lipolytic or lipogenic stimuli, as it occurs in mature adipocytes.

## AUTHOR CONTRIBUTIONS

EC and IN conceived the idea and designed the experiments. CS and NR-H performed the cell cultures and analytical procedures and drafted the manuscript. All authors interpreted the data, revised the manuscript, and approved the final version.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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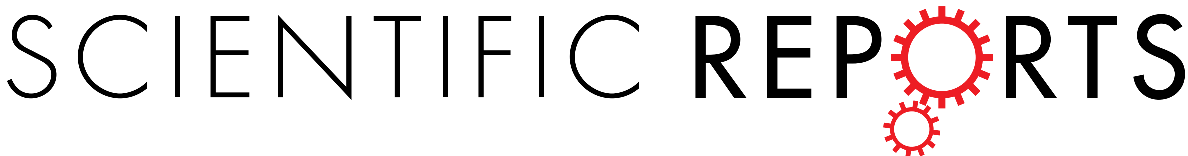

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# Temperature responsiveness of gilthead sea bream bone; an *in vitro* and *in vivo* approach

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**This study aimed to characterize the molecules involved in osteogenesis in seabream and establish using *in vitro/in vivo* approaches the responsiveness of selected key genes to temperature. The impact of a temperature drop from 23 to 13 °C was evaluated in juvenile fish thermally imprinted during embryogenesis. Both, *in vitro/in vivo*, *Fib1a*, appeared important in the first stages of bone formation, and *Col1A1*, *ON* and *OP*, in regulating matrix production and mineralization. *OCN* mRNA levels were up-regulated in the final larval stages when mineralization was more intense. Moreover, temperature-dependent differential gene expression was observed, with lower transcript levels in the larvae at 18 °C relative to those at 22 °C, suggesting bone formation was enhanced in the latter group. Results revealed that thermal imprinting affected the long-term regulation of osteogenesis. Specifically, juveniles under the low and low-to-high-temperature regimes had reduced levels of *OCN* when challenged, indicative of impaired bone development. In contrast, gene expression in fish from the high and high-to-low-temperature treatments was unchanged, suggesting imprinting may have a protective effect. Overall, the present study revealed that thermal imprinting modulates bone development in seabream larvae, and demonstrated the utility of the *in vitro* MSC culture as a reliable tool to investigate fish osteogenesis.**

Bone formation in fish does not occur during somitogenesis and the first skeletal structures appear after hatching. These structures consist of dermal bone (formed by intramembranous ossification) and cartilage replacement bone. The skeleton of the jaw and mouth only form after mouth opening with the onset of exogenous feeding. Subsequently, the first vertebrae appear in larvae at around 4 mm in body length, and only after early flexion (6 mm) does mineralization of these structures start to take place[1–4]. An almost fully mineralized skeleton is evident in larvae of 16 mm body length[1] or at about 30 days post-hatching[4]. Concerning the molecules involved in the formation and turnover of bone, transcriptomes from gilthead sea bream (*Sparus aurata*) gill arch and vertebra have recently been generated and revealed homologs of many mammalian skeleton-related transcripts[5]. Many of the identified genes corresponded to transcription factors that control osteoblast differentiation (i.e. runt-related transcription factor 2 (Runx2/Cbfa1), osterix/Sp7), or components of the extracellular matrix (ECM), including structural proteins, collagens, proteoglycans, as well as non-collagenous proteins that regulate mineral deposition such as osteonectin/SPARC (ON), osteopontin/Spp1 (OP) and osteocalcin/BGP (OCN). The study by Vieira *et al.*[5], unveiled the conservation of transcripts present in bone and cartilage of gilthead sea bream compared to mammals, although the tissue and cell specific localization of these transcripts remains to be established. However, with the increasing number of *in vitro* fish cell models developed in recent years[6,7], including the gilthead sea bream primary culture of mesenchymal stem cells (MSCs) derived from vertebrae, established by our group[8], it is now possible to characterize at a cellular level the role of identified candidate markers during osteoblastogenesis.

Gilthead sea bream is one of the most important farmed species in Spain, which is ranked fourth in total production volume in the Mediterranean area and third in the European Union[9]. Although the production of gilthead sea bream in aquaculture systems is well consolidated, the high incidence of skeletal deformities is still an important bottleneck for the sustainability of this industry. Moreover, since this species is commercialized as

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whole fish, skeletal anomalies lead to a downgrade in the quality of the product and a reduction in its value. The most common deformities found in gilthead sea bream are those that affect the opercular complex, vertebral column and haemal or caudal body regions[10–12], which may result in ca. to 50% of lost production at the end of the hatchery phase[13]. These deformities may also reduce growth rate, increase mortality and negatively impact animal welfare[14].

The aetiology of skeletal deformities is uncertain, but temperature is one of the most important abiotic factors linked to this problem and fish grown at elevated temperatures exhibit increased levels of vertebral anomalies[13–15]. In fact, fluctuations in water temperature are associated with abnormal muscle growth and heterochrony of muscle formation, whereas temperature effects on bone growth have been associated with deformation of the vertebral bodies in fish larvae, which leads to spinal deformities[16,17]. In addition, body shape and meristic characters (i.e. dorsal spines) are also significantly affected by environmental temperature during the early life stages of the European sea bass, *Dicentrarchus labrax*[18]. In Atlantic salmon (*Salmo salar*), faster growth induced by hyperthermia significantly modified gene transcription in osteoblasts and chondrocytes, which was associated with an increased number of deformities and modified bone tissue structure and composition[19].

In the Mediterranean Sea, gilthead sea bream is frequently exposed to severe temperature changes during winter, which is one of the causal factors of winter syndrome in reared populations[20]. This pathology causes chronic mortality during the coldest months of the year and acute death episodes when the water temperature rises again[21,22]. Mortality rates are usually around 7–10% of the fish stock, although in some very acute cases, they may be as high as 80%[23]. Winter syndrome is a multifactorial condition that triggers a stress response[24,25], depresses the immune system[21,26], affects several tissues including the skeletal muscle, exocrine pancreas, liver and digestive tract[20,25,27,28], and may cause lesions in the brain and kidney[23]. The effect of thermal imprinting in embryos and larvae and on the response of the skeleton to a temperature drop (to simulate winter conditions) was recently studied in adult gilthead sea bream[29]. Overall, the study reported that although thermal imprinting failed to modify bone homeostasis in optimal ambient water temperatures, it did change the bones responsiveness during a cold challenge.

Following on from our previous work, in the present study, we addressed the hypothesis that, modifications in the skeleton due to water temperature are linked to its effect on the gene expression of key osteogenic molecules during development. To this end, we analysed *in vivo* how thermal regimes during development influenced the expression of genes involved in osteogenesis and used an *in vitro* primary culture of MSCs from juvenile gilthead sea bream vertebrae to investigate how temperature modified bone tissue specific gene expression. We then assessed *in vivo* how thermal regimes experienced during early development influenced the expression of bone tissue specific genes in adults exposed to a cold challenge.

## Results

**Bone cells development and temperature effects *in vitro*.** Orthologs of mammalian osteogenic genes have recently been identified in gilthead sea bream by transcriptome analysis, however their specific expression in bone cells during the process of osteoblastogenesis remains elusive. The transcriptional profile of gilthead sea bream bone-derived MSCs differentiating into osteoblasts under mineralizing conditions induced by incubation with an osteogenic medium (OM) was compared to that of cells growing in growth medium (GM) for 20 days and is presented in Fig. 1. A significant interaction between days in culture and media was observed for fibronectin 1a (*Fib1a*), Bone Morphogenetic Protein 2 (*BMP2*) and *OP* mRNA levels. Nevertheless, significant differences were not observed with regard to transcript abundance through time in cells growing in GM for any of the genes studied (Fig. 1). Moreover, in cells incubated in OM, the gene expression of *Runx2*, a key transcription factor driving osteogenesis of MSCs, and of collagen type 1 alpha-1 (*Col1A1*), and tissue non-specific alkaline phosphatase (*TNAP*), did not vary significantly as differentiation progressed (Fig. 1A,D,H). Matrix Gla protein (*MGP*) showed similar results, but probably as an effect of the culture media ($P = 0.029$), an increase in its expression was observed at day 20 in cells growing in OM (Fig. 1G). *Fib1a* expression decreased gradually during MSCs differentiation, and significantly higher mRNA levels were found in the OM group compared to the GM group at days 5 and 10 (Fig. 1B). In contrast, *BMP2*, *ON* and *OP* expression increased during culture under mineralizing conditions and significantly higher mRNA levels were detected at day 20 relative to the previous days (Fig. 1C,E,F). A significant increase in transcript abundance in the OM group compared to the GM group was noted for *OP* at days 15 and 20 (Fig. 1F), and for *BMP2* and *ON* at day 20 (Fig. 1C,E).

The expression of osteogenic gene transcripts from cells incubated in OM at 18 and 28 °C in comparison with the control temperature of 23 °C is represented as a heat map in Fig. 2. The relative expression data obtained at each of the three temperatures can be found in Table S1. For most genes, *MGP*, *TNAP*, *BMP2*, *Fib1a* and *OP*, expression was down-regulated in response to an increase or reduction in temperature. However, this is probably not a generalized non-specific response to change in temperature, since the other osteogenic gene analysed (*ON*) showed a contrary pattern. Similarly, heat shock protein 90b (*HSP90b*) gene expression increased in the first 6 h after temperature challenge and then subsequently decreased. In contrast, mRNA levels of *HSP30* and *ON* responded differently to temperatures. At 18 °C the *HSP30* mRNA levels increased over time, while at 28 °C they fell; the gene expression of *ON* was opposite to *HSP30*, since at 18 °C mRNA levels decreased over time and at 28 °C they increased (Fig. 2).

**Bone development and temperature effects *in vivo*.** Since cell differentiation and proliferation is not always proportional to bone/cartilage formation by osteoblasts/chondrocytes or resorption by osteoclasts, the most suitable predictors of bone mineralization available are: (i) the abundance of ECM proteins, (ii) the activity of cell-type specific enzymes, or (iii) the expression levels of genes encoding these proteins. The expression of key osteogenic genes during embryonic and larval development of gilthead sea bream exposed to two different rearing temperatures was studied and is presented in Fig. 3.

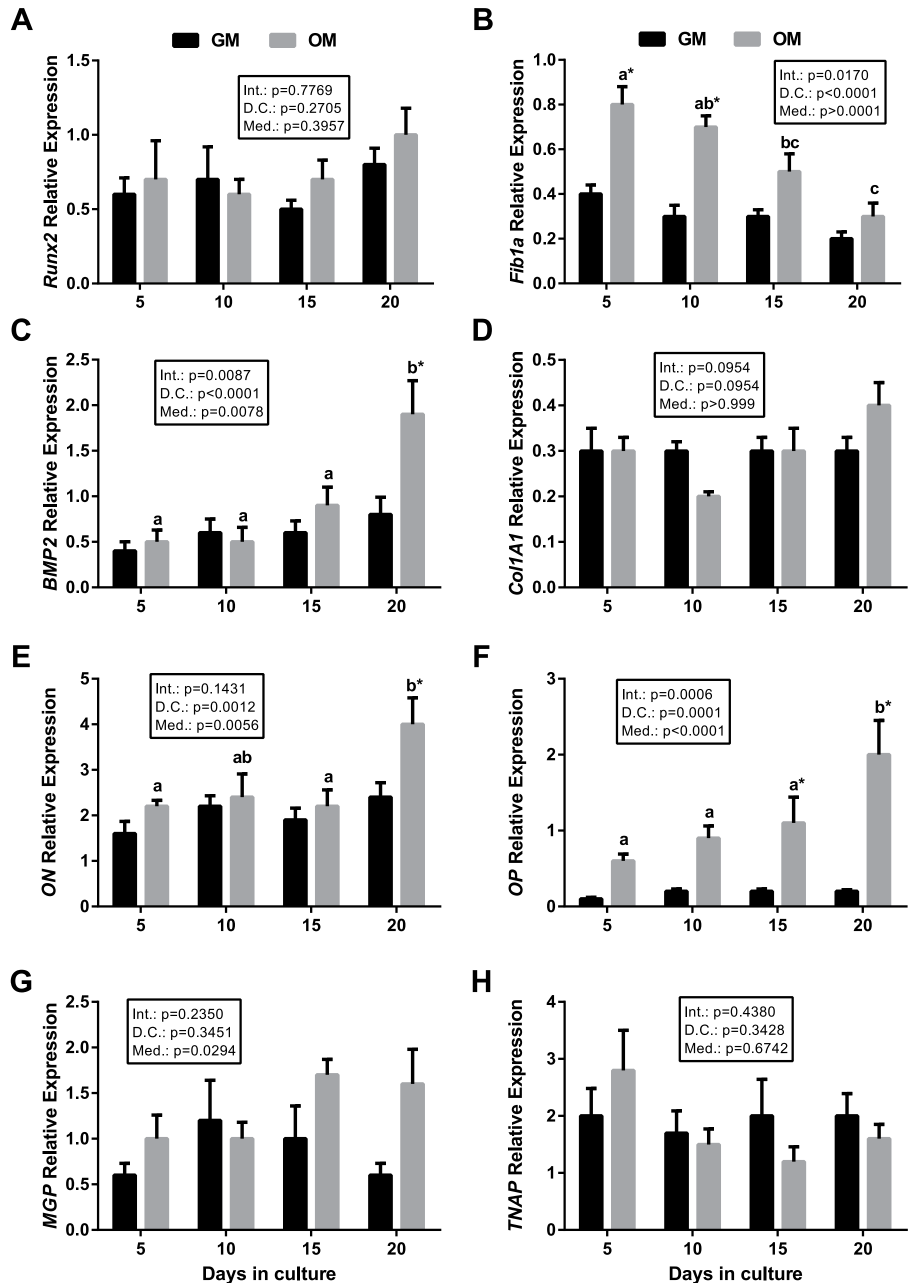


**Figure 1.** Expression of osteogenic genes in gilthead sea bream differentiating bone-derived primary cell cultures *in vitro*. Quantitative gene expression relative to the geometric mean of *RPS18* and *EF1α* for (**A**) *Runx2*, (**B**) *Fib1a*, (**C**) *BMP2*, (**D**) *Col1A1*, (**E**) *ON*, (**F**) *OP*, (**G**) *MGP* and (**H**) *TNAP* in cells cultured in growth (GM) or osteogenic (OM) media at days 5, 10, 15 and 20. The results are shown as the mean ± s.e.m. (n = 5–8). Different letters indicate significant differences throughout time within groups (upper case for GM and lower case for OM) and asterisks indicate significant differences between groups at each culture day ($p < 0.05$). Int.: Interaction, D.C.: Days in culture, Med.: Media.

A significant interaction between developmental stage and rearing temperature was observed for all genes studied except *TNAP*. *Fib1a* and *BMP2* had their highest expression during somitogenesis at both temperatures, although expression was significantly higher in the high temperature group (HT, 22 °C) compared to the low

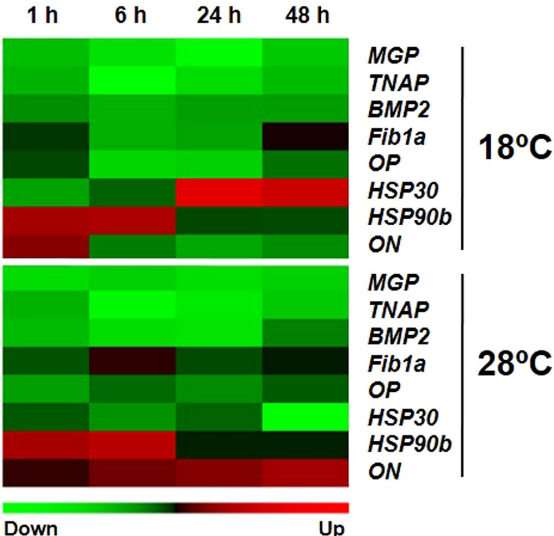


**Figure 2.** Heat map representing changes in osteogenic genes expression in gilthead sea bream bone-derived primary cell cultures incubated at different temperatures. Quantitative gene expression was relative to the geometric mean of *RPL27* and *Ub* for *MGP*, *TNAP*, *BMP2*, *Fib1a*, *OP*, *HSP30*, *HSP90b* and *ON*. Changes in gene expression were determined in cells after 13 days growing in osteogenic medium (OM) and incubated at two different temperatures (18 and 28 °C) and normalized in relation to the control temperature of 23 °C at 1, 6, 24 and 48 h (n = 5–8). Rows (mRNA transcripts) in the heat map were standardized following a standard score normalization. Red and green shading, respectively, indicate the highest and lowest expression levels, as specified in the scale bar at the bottom of the figure. Each block represents the average standard-score normalization for the 5–8 cultures sampled at each time-point.

temperature group (LT, 18 °C), at 1 somite stage for *BMP2* and at 10 somites stage for *Fib1a* (Fig. 3A,B). The transcription of *Fib1a* and *BMP2* at both temperatures decreased progressively until hatching, after which they remained stable. In contrast, *Col1A1* and *ON* transcript abundance was low during somitogenesis and increased from hatching up to the early flexion stage in both HT and LT groups (Fig. 3C,D). Furthermore, although in the latter stages the mRNA levels of these genes were always higher in the HT group, statistically significant differences were only detected at hatch. The *OP*, *MGP* and *OCN* mRNA levels in the LT and HT groups were very low up until hatch but at mouth opening (*MGP*) and early flexion (*OP* and *OCN*) a significant increase in transcript abundance existed between the two groups, although it should be noted that only 2 samples (pools of larvae) were available for analysis for the latter developmental stage (Fig. 3E–G). Finally, *TNAP* transcripts were very abundant up until hatch and then were significantly down-regulated after mouth opening in both LT and HT groups, and were not significantly different between the temperature groups (Fig. 3H).

The analysis of gene expression in bone from 7-month old gilthead sea bream exposed to the four thermal regimes (LT, HT, low-to-high temperature LHT or high-to-low temperature HLT) was not significantly different between the treatment groups (Fig. 4). However, when fish were exposed to a shift in water temperature (23 to 13 °C) a significant reduction in expression was observed for *OCN* in the LT and LHT groups (Fig. 4F).

## Discussion

In the present study we aimed to characterize the molecules involved in osteogenesis in gilthead sea bream and to determine how temperature modified their expression using both *in vitro* and *in vivo* approaches. Moreover, we also investigated the temperature-induced gene responses in bone tissue by challenging juvenile fish that had undergone thermal imprinting during their embryonic and larval stages.

Differentiation of gilthead sea bream bone-derived MSCs into osteoblasts *in vitro* requires the addition of an OM. Shortly after induction, the cells started to change their morphology from a fibroblast-like form to a geometrical shape, and mineralizing nodules became progressively more evident[8], indicating the characteristic stages of lineage commitment, ECM production and ECM mineralization. These results are similar to those reported by Fernández *et al.*[30], using another fish bone-derived cell line. These processes are illustrated in the present study by cell morphology and at a transcriptional level (Fig. 5A). Lineage determination and differentiation of osteoblasts from MSCs involves multiple regulatory actors, specific transcription factors, environmental conditions and mineral availability. Among the transcription factors, Runx2 is crucial and it is well-known that it is required for osteoblast differentiation in mammals[31] and coordinates the expression of genes such as *OP*, *OCN* and alkaline phosphatase[32,33]. In fish, Runx2 is also an important early indicator of the osteogenic capacity of cells[15] and several authors have shown in fish and mammals that *Runx2* expression increases at very early stages[34–37]. However, in the present study we did not observe significant changes in *Runx2* expression in the cell cultures, which may be

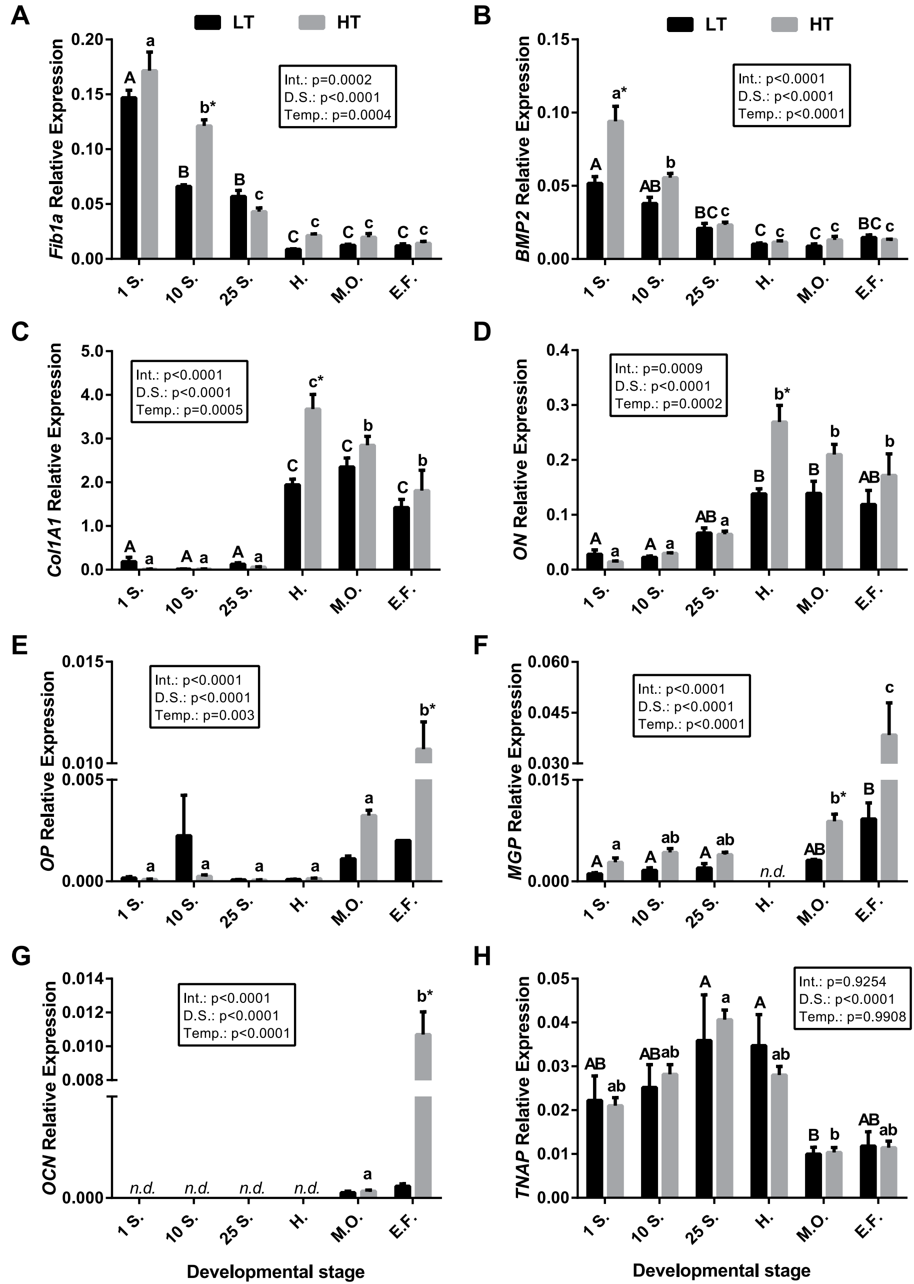


**Figure 3.** Expression of osteogenic genes in gilthead sea bream embryos and larvae reared at two different temperatures. Quantitative expression relative to *RPS18* of (**A**) *Fib1a*, (**B**) *BMP2*, (**C**) *Col1A1*, (**D**) *ON*, (**E**) *OP*, (**F**) *MGP*, (**G**) *OCN* and (**H**) *TNAP* at different developmental stages (1 somite [1S.], n = 6; 10 somites [10 S.], n = 6; 25 somites [25S.], n = 6; hatch [H.], n = 6; mouth opening [M.O.], n = 6; early flexion [E.F.], n = 2) in fish reared at two temperatures (18 [LT] and 22 °C [HT]). Results are shown as the mean ± s.e.m. Different letters indicate significant differences through time within groups (upper case for LT and lower case for HT) and asterisks indicate significant differences between groups at each developmental stage ($p < 0.05$). Although it should be taken into account that only 2 samples (each corresponding to a pool of larvae) were available for the developmental stage of early flexion. n.d.: Non-detected, Int.: Interaction, D.S.: Developmental stage, Temp.: Temperature.

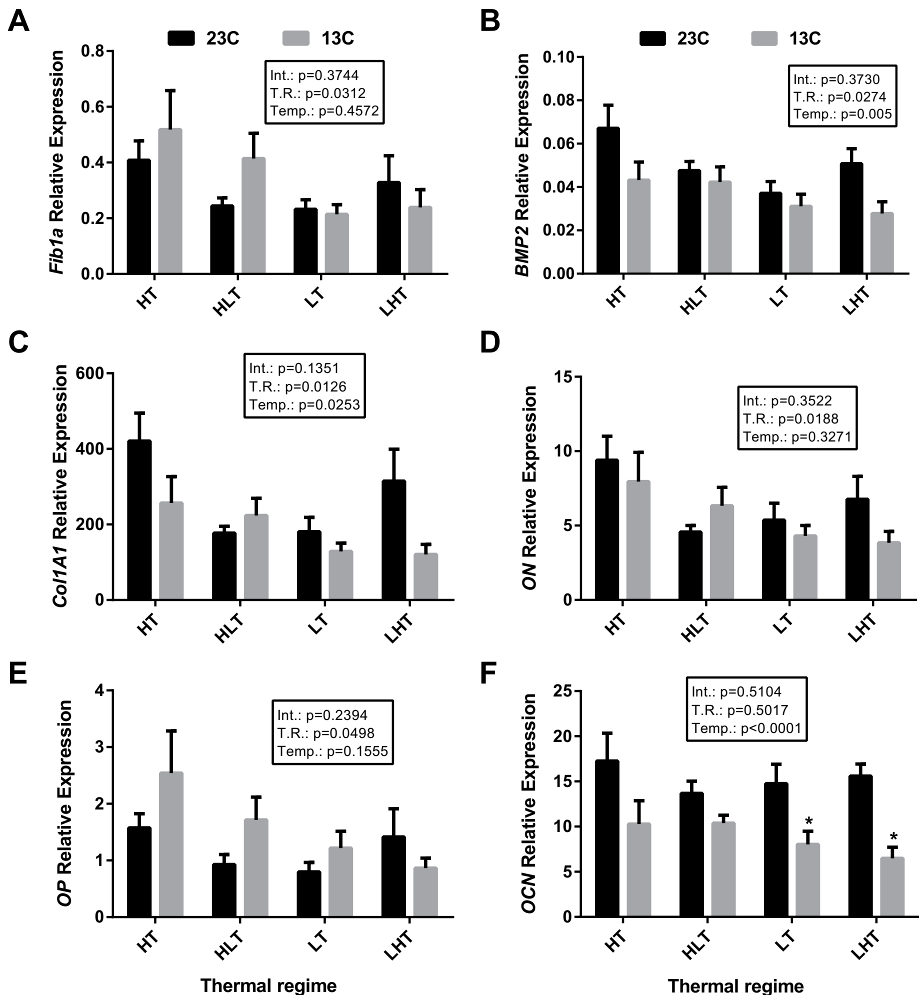


**Figure 4.** Expression of osteogenic genes in gilthead sea bream juveniles after a temperature challenge *in vivo*. Quantitative expression relative to *RPL27* of (**A**) *Fib1a*, (**B**) *BMP2*, (**C**) *Col1A1*, (**D**) *ON*, (**E**) *OP* and (**F**) *OCN*. Quantitative PCR was performed with samples from gilthead sea bream reared at different temperature regimes during embryogenesis (LT [18–18 °C]; LHT [18–22 °C]; HT [22–22 °C]; HLT [22–18 °C]) and then maintained at 23 °C (LT n = 9, LHT n = 10, HT n = 9, HLT n = 9) or challenged with a drop in water temperature from 23 °C to 13 °C (LT n = 7, LHT n = 9, HT n = 9, HLT n = 9) at 7 months' post-hatch. Results are shown as the mean ± s.e.m. No significant differences were observed among thermal regimes within each temperature treatment (23 or 13 °C), but asterisks indicate significant differences between temperature treatments for each thermal regime ($p < 0.05$). Int.: Interaction, T.R.: Thermal regime, Temp.: Temperature. Variation in sample number for different experimental groups was due to the low yield and quality of RNA extracted from some samples.

because sampling only occurred at day 5. In fact, regulation of *Runx2* levels in gilthead sea bream MSCs undergoing differentiation into osteoblasts was recently detected 6 h after induction (Riera-Heredia, unpublished data). The secreted factor BMP2 is involved in bone commitment and is a widely used growth factor for *in vitro* bone induction[38,39], although lineage determinant effects of BMPs on MSCs are highly dependent on receptor type and dose[40]. Contrary to expectations, increased *BMP2* expression was not observed in early bone cell cultures. Nonetheless, BMP2 has also been associated later in the process of ECM mineralization and bone nodule formation during MSCs differentiation in mammals[41,42], which is coherent with the *BMP2* mRNA expression profile detected in the gilthead sea bream MSC cultures described herein. Furthermore, a similar expression pattern was reported in a gilthead sea bream chondrocyte-like VSa13 cell line, since *BMP2* gene expression was also strongly induced during mineralization, but did not occur in the osteoblast-like VSa16 cell line[43].

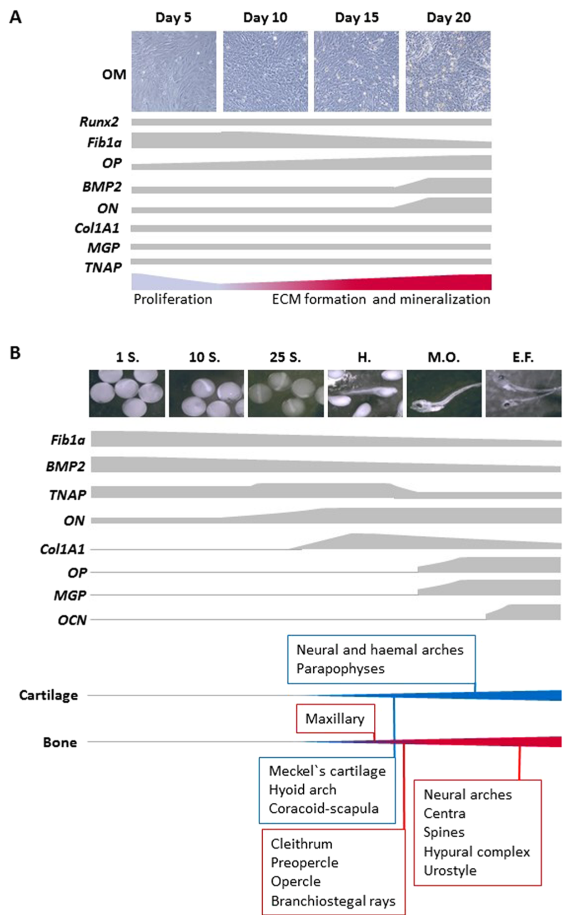


**Figure 5.** Schematic representation summarizing the expression of osteogenic genes in gilthead sea bream *in vitro* and *in vivo* assays. Representative images of (**A**) bone-derived primary cell cultures growing in osteogenic medium (OM) at days 5, 10, 15 and 20, and (**B**) gilthead sea bream at different developmental stages during embryogenesis (1 somite [1S.]; 10 somites [10S.]; 25 somites [25S.]; hatch [H.]; mouth opening [M.O.]; early flexion [E.F.]). In (**A**), the processes of proliferation and extracellular matrix (ECM) mineralization according to Capilla *et al.*[8] are schematically indicated with the thick to narrow transition or vice versa representing the approximate rate during culture development. In (**B**), the developmental osteology of gilthead sea bream indicating the main skeletal structures that are formed is shown[1–3]. Cartilage is represented by the blue line and endochondral and dermal bone formation is represented in red; the blue - red transition represents the onset of endochondral (cartilage replacement bone) formation. The narrow to thick transition of the line for cartilage or bone formation represent the increasing number of structures that develop during development. In sea bream, skeletogenesis begins soon after hatching with the development of cartilaginous and bony structures necessary for exogenous feeding, vision, opening and closing the mouth, expanding and narrowing the oral and branchial areas and propulsion. The comparison between the *in vitro* and *in vivo* results shows that of the genes analysed *Fib1a* is most important during the early development of bone, which is in agreement with its more structural role, while *ON*, *OP*, *MGP* and *OCN* are more abundant in later stages when the deposition of mineral needs to be tightly regulated for the proper formation of bone. In contrast, *BMP2*, *Col1A1* and *TNAP* had divergent expression patterns between the *in vitro* and *in vivo* situation probably due to their diverse roles in tissues during development.

Concerning ECM structural elements, Fib1a is produced by osteoblasts and accumulates in the ECM; its expression is higher during the early stages of osteoblast differentiation and declines during cell maturation[44–47]. In our MSC cultures, *Fib1a* expression was highest at day 5 and progressively decreased thereafter, confirming a likely role in the first stages of osteogenesis and cell adhesion[46–48]. Col1A is a component that comprises 90% of the bone ECM, it is expressed during osteoblast differentiation together with some non-collagenous proteins (i.e. ON and OP) and serves as a useful marker of early mineralization. ON acts as a modulator of cell–matrix interactions and has been recognized as a mediator of the early phase of ECM production[49] and OP is involved in

ECM mineralization by regulating calcium phosphate deposition and in bone remodelling by mediating osteoclast attachment to the mineralized ECM during resorption[50–53]. Notwithstanding this timely role, the expression of *ON* and *OP* usually increases during maturation[15,54], as occurred in our cells, which presented a significant up-regulation by day 20, confirming that these molecules seem to be required for bone mineralization in fish. The results for *OP* agreed with those reported in control *versus* mineralized VSa13 and VSa16 fish cell lines[55], although under the same conditions *ON* was also shown to be down-regulated by the same authors. During this initial phase of ECM production, an increase in *MGP* and *TNAP* concomitant with *Col1A1*, was expected based on the homology between fish and mammalian systems[56,57]; nevertheless, higher *MGP* mRNA levels were only found at day 20 in OM cells relative to cells growing in GM in our study, but no changes in *TNAP* expression were found. This may indicate that cells during this period were more prone to mineralize than to differentiate as suggested by the *MGP* and *TNAP* mRNA levels; only at later stages of differentiation, has mineralization been found to be negatively regulated by increased *MGP* expression[6].

Summarizing the *in vitro* characterization analysis, Fib1a appears to be involved in the regulation of the very early stages of the bone cells in culture, possibly in the establishment of the osteoid, while at later stages BMP2, ON, OP and probably MGP, appear to play a role in the consolidation of osteoblast maturation and mineralization of the ECM. Interestingly, the gene expression profiles reported in the present study during embryonic and larval development (Fig. 5B) are concordant with those obtained in our *in vitro* culture model, with the exception of BMP2, demonstrating the relevance and robustness of this *in vitro* system for studies of osteogenesis in this fish species. Furthermore, the gene expression profiles reported herein for *BMP2*, *ON*, *OP* and *OCN* during embryogenesis also agree with those previously presented for this fish species[43,55,58–60].

*Fib1a* had the highest levels of expression during the somite stages, whereas *Col1A1* and *ON* transcript levels were increased at hatch, confirming their important role in structuring the bone ECM[46–48] and establishing ECM production[49,54], respectively. Such up-regulation at hatch could be related *in vivo* with the early formation of the cranial skeleton (neurocranium and spachnocranium) and the caudal fin elements that are of fundamental importance for the proper development of sensory systems, exogenous feeding and survival[61], since at this stage the vertebral column and fins are not yet differentiated[1,3]. Then, the onset of bone mineralization at mouth opening was marked by increased transcript levels of *MGP* and *OP*, supporting their central role in osteoblast maturation and ECM mineral deposition, mainly in the already formed viscerocranial structures. Finally, *OCN* was significantly up-regulated at early notochord flexion *in vivo* in agreement with its major osteogenic role, although due to the low sample number (n = 2) available in the present study for this stage, further confirmatory analysis is required. The up-regulation of *OCN* could be associated with the appearance at this developmental stage of skeletal structures formed by intramembranous ossification (i.e. vertebral centra, neural and haemal spines) and the mineralization of cartilage replacement bones (i.e. hypurals, neural and haemal arches) since at this time the notochord is still not segmented[1–3]. In fact, although we used whole larvae for gene expression analysis and OCN is not exclusively present in skeletal tissues[31], it is the most abundant non-collagenous protein in the bone ECM and is considered a late marker of osteoblast differentiation, essential for appropriate maturation of hydroxyapatite crystals and bone mineralization[62–64]. Probably this is why this molecule was undetected in our *in vitro* cultures, because the level of mineralization/maturation of the cells was too low to induce its expression.

Temperature is a primary factor affecting fish larval development, and rearing fish at temperatures outside their optimal range, which in the case of temperate Sparids is below 15 °C and above 22 °C, leads to increased skeletal anomalies[65]. Specifically, for gilthead sea bream, although the usual hatchery temperatures are between 16 and 18 °C[66], rearing at 22 °C for the entire larval period has been proposed since larvae have less malformations and also have enhanced growth[67]. Overall, while the mechanisms controlling osteogenesis in fish are not fully clarified, it is generally accepted that temperature changes transcription in bone-forming and cartilage-forming cells.

When the responsiveness of gilthead sea bream bone-derived MSCs to a change in temperature was evaluated, an increase in *HSP30* and *HSP90b* transcripts was observed, which may be associated with exposure to stressful conditions as previously reported[68–70]; thus confirming the potentially compromised status of these cells. Most of the osteogenic genes studied in the bone-derived MSCs were down-regulated in response to either an increase or decrease in temperature. A reduction in the osteogenic genes *Col1A1*, *ON* and *OCN* has previously been reported in rat osteoblast primary cultures exposed to hypothermia[71], and also when Atlantic salmon primary muscle satellite cells that were induced to differentiate into osteoblasts *in vitro* were exposed to a sudden rise in temperature[7]. Thus, although a change in temperature (both an increase and decrease) caused an apparent generalized reduction in gene transcription in our cells, the response was gene specific, as has previously been reported[7,71]. In this sense, our results showed that *ON* was primarily up-regulated in response to temperature change. ON is an ECM glycoprotein that has also been described as a heat shock protein having chaperone-like properties[72,73]. In this context, we cannot eliminate the possibility that the rapid response of *ON* to temperature may be an initial cellular response to stress that is linked with its role in the prevention of collagen denaturation, as has previously been suggested[74]. Overall, the *in vitro* results suggest *ON* gene expression in fish bone may be a candidate indicator of stressful conditions, while the decrease in expression of the other genes may be indicative of a temperature induced check in the osteogenic process.

To evaluate the effects of temperature *in vivo*, fish were reared under two different thermal regimes, and lower expression of all osteogenic genes studied in the embryo and larvae incubated at 18 °C (LT) compared to fish kept at 22 °C (HT) was encountered. Interestingly, the gene expression patterns throughout development were not modified due to incubation temperature, probably since both thermal regimes were within the optimal temperature range for gilthead sea bream rearing[65]. In this sense, although bone development might be accelerated in the HT fish, the consequences with regard to the incidence of malformations is not known. Moreover, in all cases the differences in transcript levels were most significant at the developmental time at which the corresponding gene had its peak of expression: at the stages of 1 and 10 somites for *BMP2* and *Fib1a*, respectively, at hatching for

*Col1A1* and *ON* and at early flexion for *OP* and *OCN*. In the latter case substantial differences in gene transcript expression were detected, but since only 2 samples (each corresponding to a pool of larvae) were available for this developmental stage, further confirmation is required. Since the potential temperature effects on fish from different experimental treatments were corrected by sampling using developmental stage as a reference, these data indicate that the most temperature sensitive developmental period is embryogenesis, when imprinting mechanisms can influence the expression of osteogenic genes (i.e. epigenetic modification of chromatin and histones, DNA methylation)[75]. Furthermore, the results of our study indicate that, in common with skeletal muscle and other physiological systems, the skeleton of gilthead sea bream is affected by a short-term change in temperature during embryogenesis. Moreover, in various teleost species the embryonic rearing temperatures produced persistent effects by affecting gene expression in adults even when grown at a common temperature from hatch onwards[76–78].

Mateus *et al.*[29], recently demonstrated in gilthead sea bream that the temperature regime applied during early development caused thermal imprinting with long-term effects on bone turnover and gene expression. The present study reinforces the previous observations and identifies, using gene markers as a proxy and an *in vitro* culture model, the molecular and cellular effects. In fact, juvenile fish imprinted with the LT and LHT thermal regimes had reduced transcription of some genes in the bone when the water temperature fell from 23 to 13 °C, suggesting bone formation and mineralization was checked and this may be another negative consequence of winter syndrome. The significant differences observed for *OCN* agree with that reported previously[29], where the cold challenge in these fish caused a reduction in the bone calcium content and the relative abundance of *OCN* and osteoglycin, a small leucine-rich proteoglycan found in the ECM of bone. In the same study[29], the LHT and LT groups also had the most notable down-regulation of the endocrine factors controlling bone growth (i.e. insulin-like growth factor 1 and glucocorticoid and thyroid receptors). The reduced expression of some key regulatory and osteogenic factors was proposed to be indicative of impaired responsiveness of bone, suppressing osteoblast differentiation and affecting bone homeostasis and remodelling[79,80]. In support of this notion, hyperthermic Atlantic salmon showed down-regulation of ECM genes (i.e. *Col1A1*, *ON* and *OCN*) and at the juvenile stage had shorter and less mineralized vertebral bodies and a higher rate of skeletal malformations than fish reared under a normal temperature regime[19]. On the other hand, mRNA levels of all the genes under the HT and HLT treatments remained similar irrespective of the rearing temperature of the juveniles. This may show the possible beneficial effects for the skeletal tissue in juveniles and adults, of thermal imprinting during embryogenesis. We hypothesize that under the present experimental conditions, epigenetic mechanisms such as modifications in chromatin and histones or DNA methylation were in place to maintain the expression of the osteogenic genes that had a higher transcript abundance in the HT and HLT groups during their early development, whereas the changes caused by temperature imprinting in the LT and LHT groups were less beneficial. In view of the variable response of the gene transcript expression to a drop in temperature in the different thermally imprinted groups, we conclude that the response is specific and not generalized to either, i) reduced water temperature or ii) the reduced food intake in the 13 °C group (1% *versus* 3% of the controls at 23 °C). The specificity of the response of the thermally imprinted fish to a temperature drop was further supported by the similar level of abundance of the reference genes (also in the *in vitro* study), in the control and challenged fish, and this counters the idea that the results obtained arose from a global repression in transcription due to temperature/nutrient reduction.

Overall, the present study provides novel and detailed data characterizing the gene networks regulating the process of osteogenesis in developing gilthead sea bream exposed to thermal imprinting. We further demonstrated the potential of the gilthead seabream MSCs *in vitro* system as a reliable model to investigate osteogenesis and revealed that it mirrors accurately the data obtained with *in vivo* models. Thus, this study provides, not only better knowledge with regards to bone development and temperature induced effects, but also presents a promising tool for future studies aimed at unravelling the mechanisms underlying the high incidence of skeletal deformities in aquaculture.

## Methods

**_In vitro_ studies.** *Primary cultures of bone-derived cells from gilthead sea bream.* Gilthead sea bream were obtained from a fish farm in the North of Spain (Tinamenor S.L., Pesués) and maintained in the animal facilities of the Faculty of Biology at the University of Barcelona. Fish were kept in 200 L fiberglass tanks under a 12 h light/12 h dark photoperiod and fed *ad libitum* twice daily with a commercial diet (OptiBream™, Skretting, Burgos, Spain). All animal handling procedures were carried out in accordance with the guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of the University of Barcelona (permit numbers CEEA 243/12 and DAAM 6759). Primary cultures of gilthead sea bream bone-derived MSCs were performed as previously described[8]. Briefly, six fish (average weight 23 g) per culture were used, they were sacrificed by a blow to the head, and the vertebral column was removed and cleaned. The vertebrae were diced-up into smaller fragments using a scalpel and then, two subsequent digestions of 30 and 90 min, respectively, were performed with 0.125% Type II collagenase. Next, the tissue fragments obtained were washed with Dulbecco's Modified Eagle Medium (DMEM) supplemented with a 1% antibiotic/antimycotic solution (A/A) and plated with complete GM composed of DMEM with 10% fetal bovine serum, 1% Fungizone and 1% A/A and incubated at 23 °C and 2.5% $CO_2$. After 1 week, the vertebrae fragments were removed, and the cells collected with 0.25% trypsin-EDTA and routinely sub-cultured for a maximum of 10 passages. All plastic ware was obtained from Nunc (Barcelona, Spain). The trypsin–EDTA and Fungizone solutions were from Invitrogen (El Prat de Llobregat, Spain), and all the other reagents were purchased from Sigma-Aldrich (Tres Cantos, Spain).

*Characterization of osteogenesis in vitro.* In order to characterize the stages of development of the cell cultures, trypsinised cells in suspension were counted and plated in 6-well plates with GM at a density of $10^5$ cells per well (n = 8 independent experiments). On the following day (day 0), the media was changed to grow the cells under

| Gene | Primer sequence (5′-3′) | Tm (°C) | Acc. Num. | Efficiency (%) |
|---|---|---|---|---|
| *BMP2* | F: GGAGAAGCAGCGTGGATTAAACACGAAT<br>R: GGCCTGCGCCTCAGTCCAAACATATT | 68 | AY500244 | 103.9 |
| *Col1A1* | F: GAGATGGCGGTGATGTGGCGGAGTC<br>R: GCCTGGTTTGGCTGGATGAAGAGGG | 68 | DQ324363 | 91.7 |
| *EF1α* | F: CTTCAACGCTCAGGTCATCAT<br>R: GCACAGCGAAACGACCAAGGGGA | 60 | AF184170 | 94.2 |
| *Fib1a* | F: CGGTAATAACTACAGAATCGGTGAG<br>R: CGCATTTGAACTCGCCCTTG | 60 | FG262933 | 101.8 |
| *HSP30* | F: GGTGACTGACGGGAAAGAGA<br>R: CTGAGGAGGAGGTGCTGTTC | 60 | GU060312 | 93.1 |
| *HSP90b* | F: TTCACGCATGGAAGAAGTTG<br>R: GGTCCACCACACAACATGAA | 56 | DQ012949 | 86.0 |
| *MGP* | F: TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC<br>R: CGGGCGGATAGTGTGAAAAATGGTTAGTG | 68 | AY065652 | 89.2 |
| *OCN* | F: TCCGCAGTGGTGAGACAGAAG<br>R: CGGTCCGTAGTAGGCCGTGTAG | 60 | AF048703 | 90.7 |
| *ON* | F: AGGAGGAGGTCATCGTGGAAGAGCC<br>R: GTGGTGGTTCAGGCAGGGATTCTCA | 68 | AY239014 | 97.1 |
| *OP* | F: AAAACCCAGGAGATAAACTCAAGACAACCCA<br>R: AGAACCGTGGCAAAGAGCAGAACGAA | 68 | AY651247 | 95.3 |
| *RPL27* | F: AAGAGGAACACAACTCACTGCCCCAC<br>R: GCTTGCCTTTGCCCAGAACTTTGTAG | 68 | AY188520 | 97.4 |
| *RPS18* | F: AGGGTGTTGGCAGACGTTAC<br>R: CTTCTGCCTGTTGAGGAACC | 60 | AM490061 | 100.3 |
| *Runx2* | F: ACCCGTCCTACCTGAGTCC<br>R: AGAAGAACCTGGCAATCGTC | 60 | JX232063 | 96.1 |
| *TNAP* | F: CATCGCAACCCTTTTCACAGTCACCCG<br>R: AACAGTGCCCAAACAGTGGTCCCATTAGC | 68 | AY266359 | 103.2 |
| *Ub* | F: CGGAAGTAAGAGGAACCAACAC<br>R: AAGCAGTCAGAATGCAAAGTCA | 56 | AM955423 | 78.4 |

**Table 1.** Primers used for real-time quantitative PCR. F, forward primer; R, reverse primer; Tm, annealing temperature; Acc. Num., accession number.

control (GM) or mineralizing conditions using an OM, which consisted of GM supplemented with 50 µg/ml L-ascorbic acid, 10 mM β-glycerophosphate and 4 mM $CaCl_2$. These cell cultures (GM and OM) were then sampled at different time points (days 5, 10, 15 and 20) with pools of 2 wells collected into 1 ml of Tri Reagent® solution (Life Technologies, Alcobendas, Spain) and stored at −80 °C until gene expression analyses were performed.

*Differentiating bone cells at three temperatures.* Cultivated bone-derived cells were grown in OM until day 13 to have differentiated cells in a partly mineralized ECM (n = 7 independent experiments). Then, the cell plates were divided into three different temperature groups (time 0). One group was kept at 23 °C (control), a second group was transferred to an incubator at 18 °C and a third group was moved to 28 °C. Cell samples from the three temperature groups were thereafter harvested for gene expression analyses at the following time points: 1, 6, 24 and 48 h after the temperature challenge. Results from cells incubated at the two experimental temperatures (18 and 28 °C) were analysed relative to cells maintained at the control temperature of 23 °C and standardized (i.e. standard score normalization). A heat map with these values was then generated using PermutMatrix version 1.9.3[81].

**In vivo studies.** *Animals and ethics statement.* Rearing of gilthead sea bream embryos, larvae and juveniles was performed at the Institute for Aquaculture and Food Technology Research, (IRTA, Sant Carles de la Ràpita, Spain), in a temperature-controlled seawater recirculation system (IRTAmar™). Animal handling procedures were carried out in accordance with the guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of IRTA (4998-T9900002).

*Embryogenesis characterization at two temperatures.* The effect of temperature on embryonic and larval development was determined by rearing fish under a constant temperature of 18 °C (LT) or 22 °C (HT). Fertilized eggs (110 mL/tank) of gilthead sea bream (fertilization rate of 92%) were maintained in duplicate 30 L tanks from embryogenesis up until the larval-juvenile transition. A detailed description of the experiment is provided in García de la serrana *et al.*[75]. Samples of each stage, 6 per tank, were removed by quickly netting the larvae, sacrificed with an overdose of anaesthetic (150 mg/L of MS-222; Sigma, Tres Cantos, Spain) and snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde. Embryos and larvae were sampled at: 1 (n = 6), 10 (n = 6) and 25

somites (S., n = 6), hatch (H., n = 6), mouth opening (M.O., n = 6) and early notochord flexion (E.F., n = 2), and to compensate for the potential effects of temperature on the developmental progression, the samples of fish from the two different experimental groups were collected using developmental stage as the reference.

*Temperature drop challenge.* For this experiment, fish reared under four different thermal regimes during embryonic and larval development were studied. A schematic representation of the experimental trial and sampling details can be found in Mateus *et al.*[29], where biometric and bone homeostasis parameters are reported. After hatching, the temperature was either maintained (LT, 18 °C or HT, 22 °C groups, respectively) or changed from 22 to 18 °C (HLT) or 18 to 22 °C (LHT) up until the larval-juvenile transition. Juvenile fish were then transferred to duplicate 2000 L tanks per treatment connected to a recirculating sea water system (5–10% water renewal per day, IRTAmar™) at 22–23 °C and were fed 3% body mass (w/w) with a commercial diet (OptiBream™, Skretting, Spain).

For the cold challenge experiment, fish were age matched (7 months' post-hatch), although significant differences ($P < 0.001$) in weight and length existed between the fish reared under the different thermal regimes during embryogenesis as previously reported[29]. Duplicate tanks of fish from the four different thermal regimes, were either, (a) maintained at the same temperature of 23 ± 1 °C or (b) exposed to a temperature challenge of 13 ± 1 °C for 15 days. The circuit consisted of 200 L fibreglass tanks in a semi-closed sea water system at pH 7.5–8.0, 35–36‰ salinity and >80% oxygen saturation and maintained under a 12 h light/12 h dark photoperiod. Fish from the control group were fed at the rate of 3% body weight daily using a commercial diet (OptiBream™), while fish from the cold challenge were fed at a rate of only 1%, and uneaten food was siphoned daily from the bottom of the experimental tanks. These feeding ratios were adjusted to the differences observed in intake due to rearing temperatures during acclimation to keep the tanks clean and with good water quality conditions. Ten fish from each condition were sacrificed with an overdose of phenoxyethanol (450 ppm) and samples of vertebrae were collected. No mortality occurred during the experimental trial and the gilthead sea bream exhibited no signs of distress during the experiment.

**Gene expression analyses.** *RNA extraction and cDNA synthesis.* Total RNA was isolated using Tri Reagent® following the manufacturer's instructions and quantified using a NanoDrop2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain), and the quality analysed by 1% (w/v) agarose gel electrophoresis. One µg of total RNA per sample was DNase treated (Life Technologies, Alcobendas, Spain) and used to synthesise first-strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Vallès, Spain), following the manufacturers' instructions.

*Quantitative real-time PCR (qPCR).* The qPCR assays were conducted according to the requirements of the MIQE guidelines[82] using a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). Prior to the analyses, the specificity of the reaction, absence of primer-dimers, as well as the most appropriate cDNA working dilution for each assay was determined by running a dilution curve with a pool of samples. Reactions were performed in triplicate (methodological replicates) and contained cDNA, iQ SYBR Green Supermix (Bio-Rad) and 250 nM (final concentration) of sense and antisense primers (Table 1). The protocol consisted of 1 cycle of 3 min at 95 °C and 40 cycles of 10 s at 95 °C and 30 s at 55–68 °C (primer dependent, see Table 1), followed by an amplicon dissociation analysis from 55 to 95 °C with a 0.5 °C increase every 30 s. A single peak was observed for each of the qPCR reactions and this confirmed reaction specificity. SYBR Green fluorescence was recorded during the annealing-extending phase of cycling. Target transcript abundance was analysed using the delta-delta Ct method[83] with the CFX Manager Software (Bio-Rad). In the *in vitro* studies, gene expression results were normalized using the geometric mean of ribosomal protein S18 (*RPS18*) and elongation factor 1 alpha (*EF1a*) for the culture development experiment and ribosomal protein L27 (*RPL27*) and ubiquitin (*Ub*) for the temperature experiment. *RPS18* and *RPL27* were used to standardise the gene expression during the *in vivo* experiments of embryogenesis and the adult temperature challenge, respectively. In each case, the reference gene(s) selected were those for which stability was confirmed by running the GeNorm algorithm implemented in the CFX Manager Software (Bio Rad).

**Statistical analyses.** Statistical analyses of all parameters were performed in SPSS Statistics version 20 (IBM, Armonk, NY, USA). Normality was analysed using the Shapiro-Wilk test and homogeneity of variance using a Levene's test. Statistical significance was assessed by two-way analysis of variance (two-way ANOVA) followed by Tukey *post-hoc* test. Significant differences were taken at $p < 0.05$ for all statistical tests performed. Data are presented as mean ± standard error of the mean (s.e.m.). Significant differences through time within groups (i.e. days in culture, developmental stages or thermal regimes) are shown with different letters and significant differences between treatments (i.e. culture media or rearing temperature) are shown with an asterisk.

**Data availability.** All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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## Author Contributions
D.M.P. and E.C. conceptualised the study; N.R.-H. performed all the *in vitro* cultures and corresponding laboratory analyses; E.G. run the *in vivo* trials; R.M., A.P.M. and R.A.C. performed the laboratory analyses corresponding to the *in vivo* experiments; N.R.-H., D.M.P. and E.C. analysed and interpreted the data; I.N., J.G., D.M.P. and E.C. acquired funding; N.R.-H., E.G., J.G., D.M.P. and E.C. drafted and critically reviewed the manuscript. All authors read and approved the final paper.

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RESEARCH ARTICLE

# Fatty acids from fish or vegetable oils promote the adipogenic fate of mesenchymal stem cells derived from gilthead sea bream bone potentially through different pathways

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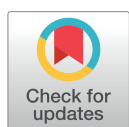

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## Abstract

Fish are rich in n-3 long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, thus they have a great nutritional value for human health. In this study, the adipogenic potential of fatty acids commonly found in fish oil (EPA and DHA) and vegetable oils (linoleic (LA) and alpha-linolenic (ALA) acids), was evaluated in bone-derived mesenchymal stem cells (MSCs) from gilthead sea bream. At a morphological level, cells adopted a round shape upon all treatments, losing their fibroblastic form and increasing lipid accumulation, especially in the presence of the n-6 PUFA, LA. The mRNA levels of the key transcription factor of osteogenesis, *runx2* significantly diminished and those of relevant osteogenic genes remained stable after incubation with all fatty acids, suggesting that the osteogenic process might be compromised. On the other hand, transcript levels of the main adipogenesis-inducer factor, *pparg* increased in response to EPA. Nevertheless, the specific PPARγ antagonist T0070907 appeared to suppress the effects being caused by EPA over adipogenesis. Moreover, LA, ALA and their combinations, significantly up-regulated the fatty acid transporter and binding protein, *fatp1* and *fabp11*, supporting the elevated lipid content found in the cells treated with those fatty acids. Overall, this study has demonstrated that fatty acids favor lipid storage in gilthead sea bream bone-derived MSCs inducing their fate into the adipogenic *versus* the osteogenic lineage. This process seems to be promoted via different pathways depending on the fatty acid source, being vegetable oils-derived fatty acids more prone to induce unhealthier metabolic phenotypes.

## Introduction

In the last decades, both the world population and the consumption of fish and seafood per capita have increased and will continue to rise. Fish products are rich in n-3 long chain

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polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids [1], which are crucial nutrients for overall health [2]. For these reasons, scientific research is indispensable to improve aquaculture production under sustainable conditions, which implies among others, a reduction in the use of fish oil in aquafeeds formulation [3]. The alternatives are vegetable oils, which in contrast to fish oil, are richer in n-6 or n-9 PUFA such as linoleic (LA, 18:2n-6), oleic (18:1n-9) or alpha-linolenic (ALA, 18:3n-3) acids [4]. Moreover, fish (especially marine) may have limited ability to convert $C_{18}$ PUFA to $C_{20/22}$ [4], [5] so, it should be considered that feeding fish with highly substituted diets can result in tissues with lower n-3 LC-PUFA content [6], [7]. Apart from changes in the fatty acid composition of the fish filet [8], [9], [10], dietary vegetable oils in excess can cause adipose tissue and hepatic metabolic alterations [11], [12] or affect the immune system [13], [14]. Besides, low concentrations of dietary EPA and DHA during development, have been related to increased incidence of skeletal malformations [15], [16]. Overall, these can lead to unhealthier or low-quality fish having consequences in aquaculture production.

Fish bone consists, as in other vertebrates, of several cell types including progenitor cells or mesenchymal stem cells (MSCs) that differentiate into osteoblasts after appropriate induction [17], [18]. There are many regulators involved in the process of osteoblastogenesis, but runt-related transcription factor 2 (Runx2), is the main transcription factor controlling lineage determination and osteogenic genes expression [19]. Once differentiated, osteoblasts produce the bone extracellular matrix (ECM) or osteoid, where key components such as osteonectin (ON), osteopontin (OP) and osteocalcin subsequently regulate mineral deposition [20], [21], [22]

Interestingly, mammalian adipocytes can arise from the same MSCs as osteoblasts and a high degree of plasticity has been observed between the two cell lineages, even in very advanced maturation stages [23]. During the onset of the adipogenic process, transcription factors such as CCAAT/enhancer binding protein β and δ (C/EBPβ and C/EBPδ) are activated, which in turn, induce the expression of *c/ebpa* and peroxisome proliferator-activated receptor γ (*pparg*) [24]. These factors successively promote the transcription of specific genes mainly related with lipid metabolism like fatty acid synthase (*fas*) or the hormone sensitive lipase (*hsl*) [25]. Adipose tissue can grow not only by elevating the cellular number from resident precursors (hyperplasia) but also by increasing the size of existing adipocytes (hypertrophy), by accumulating lipids into their cytoplasm [26]. For that matter, fatty acid transporter proteins like FATP1 or the FAT translocase/CD36, together with lipoprotein lipase (LPL), are relevant actors that facilitate the fat uptake [27]. However, despite being the adipose tissue the largest body energy reserve, considered vital for the maintenance of energy homeostasis [28], its growth by hypertrophy has been associated with less responsive adipocytes to hormones and metabolites (i.e. insulin). This situation in humans derives in hypertrophic obesity and is closely linked to major health issues such as diabetes, hyperlipidemia or cardiovascular diseases [29], [30].

As indicated, the decision of MSCs fate can be affected by cell surrounding microenvironment and might be modulated by endocrine and dietary conditions. Moreover, the signals that induce adipogenesis, at the same time act as inhibitors of osteoblastogenesis, and *vice versa* [23]. Therefore, depending on the stimulus they receive, MSCs differentiate into one or another lineage. In mammals, low dietary n-3/n-6 ratios reduce bone formation and cause greater bone resorption [31], [32], [33], [34]. In the same way, changes in dietary fatty acids could modify the bone health and whole fat content due to this cellular interconversion, but although this is clear in mammals [33] it has not been proved in fish yet. Recently, culture models of MSCs have been established in fish from various adult tissues including fat and

bone, and those MSCs have been demonstrated to hold the plasticity to differentiate into lineages different from the original tissue [18], [35], [36], [37] [38], [39].

In this context, the aim of the present work was to study the effects of the fatty acids EPA and DHA, present mainly in fish oil, and those of LA and ALA, common in vegetable oils (such as soybean, rapeseed and linseed oils), on fat deposition and the expression of both adipogenic- and osteogenic-related genes, in MSCs derived from gilthead sea bream vertebrae. The study hypothesis was that these fatty acids, supplemented in the media, could induce the differentiation of bone-derived MSCs toward the adipogenic *versus* the osteogenic lineage, potentially producing phenotypically different adipocytes depending on the fatty acid source.

## Materials and methods

### Animals and ethics statement

Gilthead sea bream (*Sparus aurata*) were obtained from the Tinamenor fish farm (Cantabria, Spain) and maintained in the animal facilities of the Faculty of Biology at the University of Barcelona. Sexually immature juveniles of an average weight of 30g were kept in 200 L fiberglass tanks under a 12 h light/12 h dark photoperiod and fed *ad libitum* twice daily with a commercial diet (Optibream, Skretting, Burgos, Spain). All animal handling procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by the Ethics and Animal Care Committee of the University of Barcelona, (permit numbers CEEA 210/14 and DAAM 6759).

### Primary cultures of bone-derived MSCs and experimental design

Primary cultures of gilthead sea bream bone-derived MSCs were performed as previously described [35]. Briefly, three juvenile gilthead sea bream were used for each culture. The fish were sacrificed by a blow to the head, and bone-derived MSCs were isolated from a piece of vertebra by mechanical disruption and enzymatic (i.e. collagenase) digestion. After several washes, cells and small vertebra fragments were plated with growth medium (GM) consisting on Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution and supplemented with 19mM NaCl and 1% fungizone (Invitrogen Life Technologies, Alcobendas, Spain), in 10cm culture dishes. After 1week, the fragments were removed, and the attached cells collected with 0.25% trypsin–EDTA (Invitrogen Life Technologies) and plated into new 10cm plates with fresh GM. From here, the cells were routinely subcultured every time they reached about 70–80% confluence and used for a maximum of 10 passages.

To perform the experiments the cells were seeded at a density of $1\times10^4$ cells/cm$^2$ in 24 well plates for the viability test and the lipid quantification assay and in 6 well plates for the gene expression analyses. The fatty acids were applied 3–4 days after plating and the duration of the treatments were of 6 h for gene expression analyses, 24 h to determine viability and 6, 24, 48 and 72 h to quantify lipid accumulation. In all cases, two wells were used for each experimental condition. The fatty acids selected were the following: EPA and DHA since are the most abundant n-3 fatty acids in fish oil sources, and LA and ALA because are essential fatty acids that are present at a high percentage in vegetable oils commonly used in fish feeds (i.e. soya and rapeseed oils). The fatty acids obtained from Cayman Chemical Company (Michigan, USA), were first dissolved in ethanol and used at a final concentration of 200 μM both, individually and in all tested combinations unless stated otherwise. Final concentration of ethanol was very low (below 1%) and did not cause any negative effects in cell viability as confirmed in preliminary assays (ethanol concentrations up to 10% were tested for 24 h, S1 Fig). For the PPARγ antagonists experiment, two commonly used covalent PPARγ ligands T0070907 (2-Chloro-

5-nitro-N-4-pyridinyl-benzamide) and GW9662 (2-Chloro-5-nitro-N-phenylbenzamide) were used. These two compounds are referred to as antagonists because they physically block ligand binding by covalently modifying the Cys285 located in an orthosteric pocket embedded in the ligand-binding domain, although they do not have comparable effects with regards to transcription [40], [41]. Both were obtained from Sigma-Aldrich (Tres Cantos, Spain), diluted with dimethyl sulfoxide (DMSO) and applied together with the fatty acids at a final concentration of 10 μM according to previous literature [42].

## MTT cell assay

The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to evaluate cell viability as previously described elsewhere [35]. Briefly, cells from 3–4 independent cultures were incubated the last 3 h of the total 24 h treatment with a final concentration of 0.5 mg/mL of MTT. Then, cells were washed with PBS, resuspended in 250 μL of DMSO per well and absorbance was read immediately using a microplate reader (Infinite 200, Tecan). Cell viability values were obtained from the absorbance measured at 570 nm, with 680 nm as the reference wavelength.

## Oil Red O staining

Intracellular neutral lipid accumulation was analyzed by Oil red O (ORO) staining as explained in [35]. Briefly, cells were fixed with 10% formalin for 1 h, subsequently rinsed with PBS, stained with 0.3% ORO prepared in 36% tri-ethyl phosphate for 2 h, and then rinsed with distilled water. Quantification of cell lipid content was calculated as the absorbance measured at 490 nm divided by the read at 630 nm (Infinite 200, Tecan) corresponding to the protein content. The latter was obtained after Comassie blue staining for 1 h and dye extraction by incubation of the cells with 85% propylene glycol during 3 h at 60˚C [35]. Data are presented as fold change relative to the control (n = 3). The staining effectiveness was evaluated with a Zeiss Axiovert 40C (Carl Zeiss Inc., Germany) inverted research grade microscope equipped with a Canon EOS 1000D digital camera (magnification 20x).

## RNA extraction and cDNA synthesis

The cells were lysed with a cell scraper and TRI Reagent (Applied Biosystems, Alcobendas, Spain) in a total volume of 1 mL per each two wells. Total RNA was extracted according to the manufacturer's recommendations, dissolved in DEPC-treated water (RNase-free), quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Alcobendas, Spain) and stored at −80˚C. To eliminate any residual genomic DNA, total RNA (1 μg) was treated with DNase I (Invitrogen, Alcobendas, Spain) and converted into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain), following the manufacturer's instructions.

## Quantitative PCR analyses

To characterize the transcriptional profile occurring during the differentiation of bone-derived MSCs into adipocyte-like cells, key genes implicated in osteogenesis, adipogenesis and energy metabolism regulation were analyzed by real-time quantitative PCR (qPCR). The genes evaluated comprise the following: the transcription factor *runx2*, and ECM components: fibronectin 1a (*fib1a*), matrix Gla protein (*mgp*), *op* and *on* for the osteogenic genes. The transcription factors or nuclear receptors: *pparg*, retinoid X receptor (*rxr*) and *cebpb*; the enzymes: fatty acid synthase (*fas*), lipoprotein lipase (*lpl*) and hormone-sensitive lipase (*hsl*); fatty acid

transporters: *cd36*, fatty acid transport protein 1 (*fatp1*) and fatty acid binding protein 11 (*fabp11*) for the adipogenic genes. In addition, elongation factor 1 alfa (*ef1a*), ribosomal protein S18 (*rps18*), and beta-actin (*b-actin*) were tested as reference genes.

qPCR was performed using a CFX384 thermocycler (Bio-Rad, El Prat de Llobregat, Spain) as previously described [38]. Each qPCR reaction was performed in triplicate in a total volume of 5 μL, containing 2.5 μL of the iTaq Universal SYBR Green supermix (Bio-Rad, El Prat de Llobregat, Spain), 2 μL of diluted cDNA, 0.125 μL of each primer (250 nM) (Table 1), and milliQ water. Samples were amplified as follows: 95˚C for 3 min, and then 40 cycles of 95˚C for 10 s, followed by annealing 60–68˚C for 30 s (primer-dependent, Table 1), followed by a dissociation step from 55 to 95˚C with a 0.5˚C increase every 5 s. A standard curve with a dilution series of a cDNA sample pool was constructed to determine the qPCR efficiency of each primer pair (Table 1), which was calculated using the CFX Manager Software (Bio-Rad). To determine the overall performance of each qPCR assay three control samples were used: no template control (NTC), no reverse transcriptase control (RTC), and PCR control (PCR). Relative expression levels of the target genes were determined by the Pfaffl method [43], using correction for primer efficiencies and normalizing the quantification cycle (Cq) value of each gene, registered during the annealing step to that of *b-actin* and *rps18*, the most stable reference genes among the different conditions ($P > 0.05$) determined using the CFX Manager Software (Bio-Rad). Data were obtained from 4–6 independent cultures.

### Statistical analyses

Data normality and homoscedasticity were assessed using Shapiro–Wilk and Levene's test, respectively. Independent samples' Student's t-test was used for comparison between two groups (each experimental treatment *versus* the control). For multiple mean comparisons (among fatty acid treatments) of normal distributed data, one-way ANOVA was used followed by Tukey's or Dunnett's T3 *post hoc* tests in case of homogeneous or heterogeneous variance data, respectively. When data did not fit normal distribution, the non-parametric Kruskal–Wallis test, followed by Mann–Whitney test, were used. Statistical analyses were performed using SPSS Statistics version 20 (IBM, Armonk, NY, USA). Results are presented as mean ± SEM. $P < 0.05$ was considered to indicate a statistically significant difference. Graphs were generated using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com).

## Results

### Fatty acids effects in cell viability and differentiation

Preliminary analyses demonstrated that cell viability was unaffected by the addition of the different fatty acids up to the 200 μM concentration tested (S1 Fig). On the other hand, a dose response was observed with regards to lipid accumulation upon a 48 h treatment with each one of the four fatty acids (EPA, DHA, LA and ALA), showing at the 100 μM concentration significantly higher intracellular lipid content compared to lower doses and the control condition without fatty acids (Fig 1A). Moreover, the images obtained after ORO staining of the cells upon all treatments (EPA and LA shown in Fig 1B as a representation) confirmed this observation, being the 200 μM concentration the one causing higher lipid accumulation and therefore, the one selected for the following experiments. In addition, we could observe in these images the change of cell morphology in response to the treatments, becoming the cells more rounded with an enlarged cytoplasm while losing the fibroblastic shape of MSCs.

To further determine the effects through time of selected fatty acids on lipid accumulation, individual treatments with EPA and LA as representatives from the highly present fatty acids

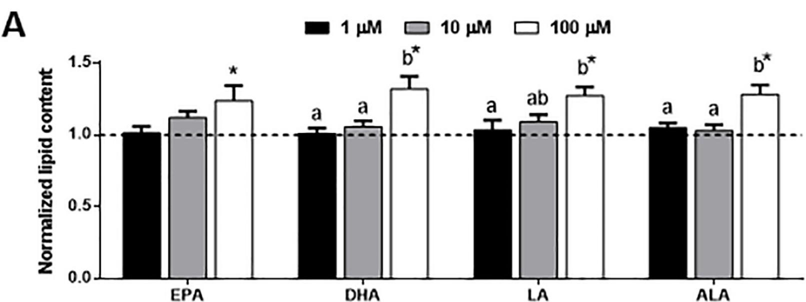
**Table 1. Primers sequences.**

| Gene | Primer sequence (5'→3') | Tm (˚C) | Efficiency (%) | Acc. Num. |
|---|---|---|---|---|
| *runx2* | **F:** ACCCGTCCTACCTGAGTCC | 60 | 104.1 | JX232063 |
| | **R:** AGAAGAACCTGGCAATCGTC | | | |
| *pparg* | **F:** CGCCGTGGACCTGTCAGAGC | 66 | 94.1 | AY590304 |
| | **R:** GGAATGGATGGAGGAGGAGGAGATGG | | | |
| *rxr* | **F:** CCCGGATGCAAAAGGTCTCT | 60 | 99.7 | - |
| | **R:** ATGCTCCAGACACTTGAGGC | | | |
| *cebpb* | **F:** ATGCGCAACTTGGAGACTCA | 60 | 95.5 | - |
| | **R:** GATTAGACAAGCGGCCCAGT | | | |
| *fib1a* | **F:** CGGTAATAACTACAGAATCGGTGAG | 60 | 96.7 | FG262933 |
| | **R:** CGCATTTGAACTCGCCCTTG | | | |
| *mgp* | **F:** TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC | 68 | 101.1 | AY065652 |
| | **R:** CGGGCGGATAGTGTGAAAAATGGTTAGTG | | | |
| *on* | **F:** GTGGTGGTTCAGGCAGGGATTCTCA | 68 | 94.3 | AY239014 |
| | **R:** AGGAGGAGGTCATCGTGGAAGAGCC | | | |
| *op* | **F:** AAAACCCAGGAGATAAACTCAAGACAACCCA | 68 | 91.9 | AY651247 |
| | **R:** AGAACCGTGGCAAAGAGCAGAACGAA | | | |
| *fas* | **F:** TGGCAGCATACACACAGACC | 60 | 95.7 | AM952430 |
| | **R:** CACACAGGGCTTCAGTTTCA | | | |
| *lpl* | **F:** GAGCACGCAGACAACCAGAA | 60 | 108.3 | AY495672 |
| | **R:** GGGGTAGATGTCGATGTCGC | | | |
| *hsl* | **F:** GCTTTGCTTCAGTTTACCACCATTTC | 60 | 92.0 | EU254478 |
| | **R:** GATGTAGCGACCCTTCTGGATGATGTG | | | |
| *cd36* | **F:** GTCGTGGCTCAAGTCTTCCA | 60 | 96.8 | - |
| | **R:** TTTCCCGTGGCCTGTATTCC | | | |
| *fatp1* | **F:** CAACAGAGGTGGAGGGCATT | 60 | 102.7 | - |
| | **R:** GGGGAGATACGCAGGAACAC | | | |
| *fabp11* | **F:** CATTTGAGGAGACCACCGCT | 60 | 107.5 | - |
| | **R:** ACTTGAGTTTGGTGGTACGCT | | | |
| *b-actin* | **F:** TCCTGCGGAATCCATGAGA | 60 | 106.9 | X89920 |
| | **R:** GACGTCGCACTTCATGATGCT | | | |
| *ef1a* | **F:** CTTCAACGCTCAGGTCATCAT | 60 | 97.4 | AF184170 |
| | **R:** GCACAGCGAAACGACCAAGGGGA | | | |
| *rps18* | **F:** AGGGTGTTGGCAGACGTTAC | 60 | 107.3 | AM490061 |
| | **R:** CTTCTGCCTGTTGAGGAACC | | | |

Primers used for real-time quantitative PCR. F, forward primer; R, reverse primer; Tm, annealing temperature; Acc. Num., GenBank accession number.

https://doi.org/10.1371/journal.pone.0215926.t001

in fish and vegetable oils respectively, plus the mixtures EPA+DHA as the fish oil combination, LA+ALA as the vegetable oils one and EPA+LA as the combination containing one fatty acid of each source, were tested at 6, 24, 48 and 72 h. All treatments caused a significant increase in cell lipid content compared to the control condition. Moreover, a time-dependent response up to 48 h (remaining high at 72 h) was also observed by treatments including LA alone or in combination (Fig 2A). The effects of these fatty acids inducing cell lipid accumulation and differentiation (i.e. rounding up) compared to the control condition were confirmed by the microscopic visual evaluation of the culture (Fig 2B). According to these results, the 6 h treatment was selected to evaluate gene expression in subsequent experiments, in order to observe



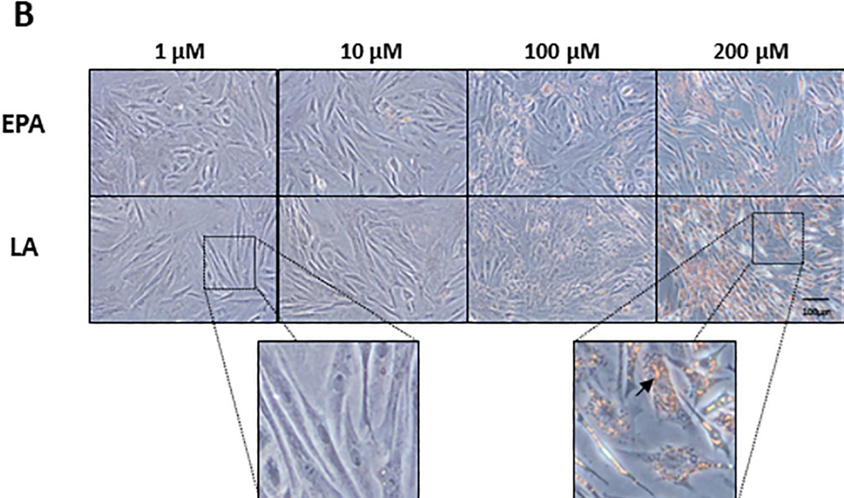




**Fig 1. Dose response of fatty acids on lipid accumulation.** (A) Quantification of lipid content normalized by protein and (B) representative phase-contrast images of gilthead sea bream bone-derived cells after staining with Oil red O. Cells were treated at day 4 with different concentrations of individual fatty acids, or were left untreated as control (dashed line in A) for 48 h. In (A) data are shown as mean + SEM (n = 3–4). Significant differences ($p<0.05$) among concentrations are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. In (B) magnification 20x and enlarged views, arrow indicates lipid droplets. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

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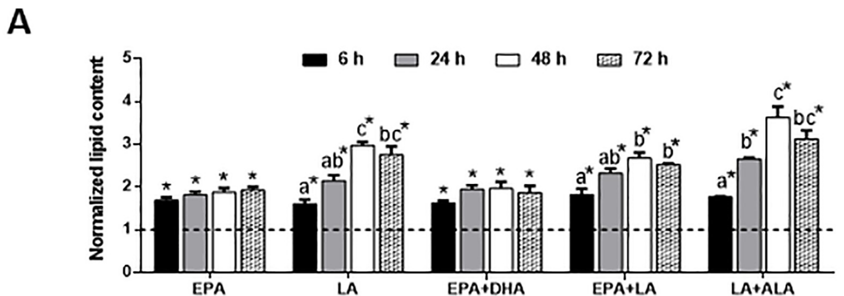
the immediate effects of these fatty acids at a transcriptional level inducing changes on cell metabolism.

## Fatty acids effects in gene expression

The gene expression of *runx2*, the key transcription factor of the osteogenic process, was significantly down-regulated by all fatty acids, either applied individually or combined, compared to the control condition, although differences were not observed among treatments (Fig 3A and 3B). Contrarily, the principal genes involved in the first steps of adipogenesis, were up-



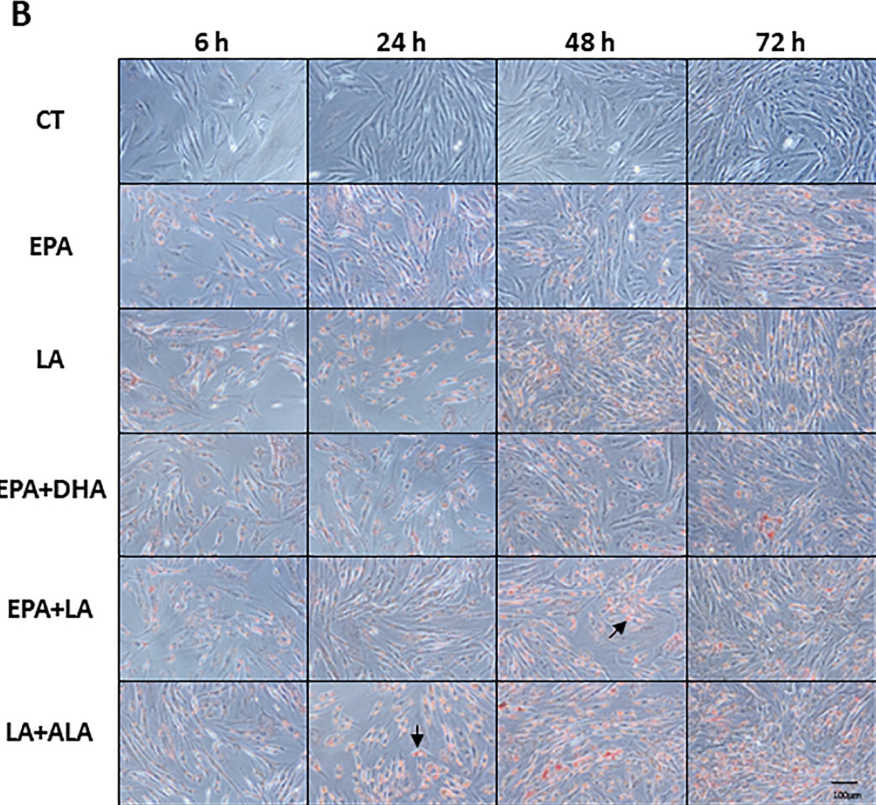




**Fig 2. Time course of lipid accumulation by fatty acids.** (A) Quantification of lipid content normalized by protein and (B) representative phase-contrast images of gilthead sea bream bone-derived cells stained with Oil red O. Cells were treated at day 4 with selected individual or combined fatty acids (200 μM), or were left untreated as control (CT, dashed line in A) for 6, 24, 48 and 72 h. In (A) data are shown as mean + SEM (n = 3–4). Significant differences ($p<0.05$) among time-points are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. In (B) magnification 20x, arrows indicate lipid droplets. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

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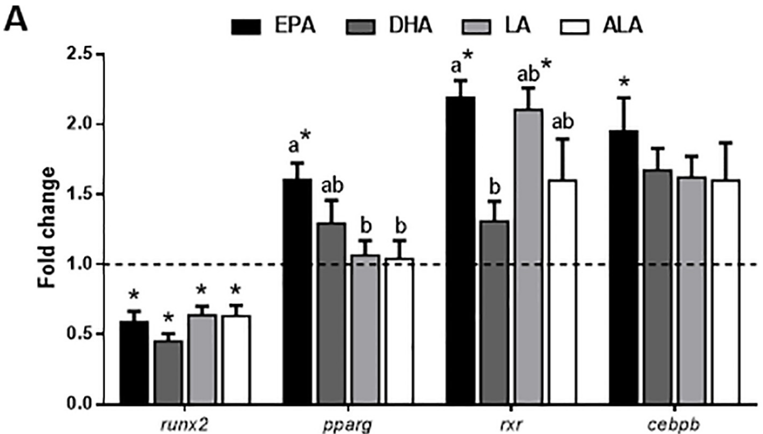

**Fig 3. Fatty acids effects on transcription factors gene expression.** Relative expression of genes related to the processes of osteogenesis (*runx2*) and adipogenesis (*pparg*, *rxr* and *cebpb*) normalized to *b-actin* and *rps18* in gilthead sea bream bone-derived cells. Cells at day 4 were treated with different (A) individual or (B) combined fatty acids or were left untreated as control (dashed lines) for 6 h. Data are shown as mean + SEM (n = 3–4). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.


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regulated after EPA treatment, significantly for *pparg*, *cebpb* and *rxr*, and the latter also by LA treatment, when compared to the control. In addition, EPA-treated cells showed significant differences with respect to those treated with LA or ALA for *pparg* gene expression and with DHA as well in the case of *rxr* (Fig 3A). Furthermore, the different combinations caused patterns of expression for these genes according to the fatty acids included in the mixture (Fig 3B). Namely, the combinations containing one (i.e. DHA) or specially the two fatty acids present in fish oils, significantly up-regulated the transcript levels of the adipogenic genes *pparg* and *cebpb*, compared to the control condition and the combination of LA+ALA. In addition,

the mRNA levels of *rxr* and *cebpb* were significantly lower in response to the combinations containing LA (especially in the one of LA+ALA), respect to the EPA+DHA mixture (Fig 3B).

The expression analysis of osteogenic genes involved in ECM formation and/or mineralization showed how these remained unaltered in cells in the presence of fatty acids either applied alone or in combination. Nevertheless, the treatments with two fatty acids combined caused *on* and *op* to have a lower, but not significant expression, compared to the control (Fig 4A and 4B). Moreover, genes encoding lipid metabolism-related enzymes and fatty acid transporters were studied to unravel whether a possible regulation of pro-adipogenic genes was related to the process of differentiation of MSCs into adipocyte-like cells. EPA treatment caused an increase in *hsl* mRNA levels, although only significant when compared to DHA-treated cells, while *fas* and *lpl* remained stable (Fig 5A). Concerning the fatty acid transporters, EPA, LA and ALA significantly up-regulated the mRNA levels of *fabp11* compared to the control (Fig 5B). Even applying combinations of the different fatty acids to the cells, the gene expression of *fas*, *lpl*, *hsl* and *cd36* remained unaffected (Fig 5C and 5D). Nevertheless, the combination of the two fatty acids more common in vegetable oils (LA+ALA), significantly up-regulated the transcript expression of *fabp11* in comparison to the combination with the fatty acids EPA +DHA and the control condition. On the other hand, *fatp1* levels were significantly higher in response to LA+ALA when compared to the combinations containing EPA and either one of these two fatty acids from vegetable oils, but not with the control (Fig 5D).

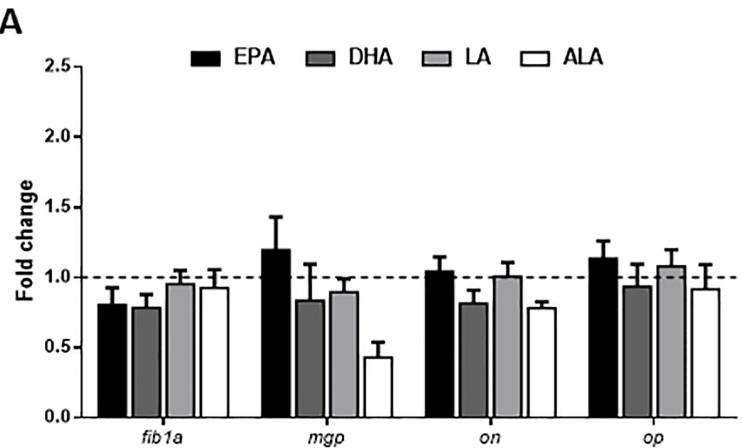
### Effects of PPARγ antagonists in gene expression

Two different antagonists of PPARγ were applied to cells treated with either EPA or LA, to elucidate the potential different mechanism of action of these fatty acids inducing the differentiation of the bone-derived MSCs into adipocyte-like cells. First, viability assays were performed to assure non-toxicity of the products (S1 Fig). Next, taking into account that the transcriptional effects of each fatty acid alone in comparison to the control were already reported, the condition of each fatty acid in the absence of antagonists was used as the corresponding control in this set of experiments. Cells treated with EPA and T0070907 showed an overall decrease in expression for the genes studied, which was significant in comparison to the treatment with the other antagonist, GW9662 for *rxr* and *cebpb* and, to the control condition for the fatty acids transporters *cd36*, *fatp1* and *fabp11* (Fig 6A). Contrarily, the cells treated with LA and either one of the two antagonists, did not show any significant changes in gene expression (Fig 6B).

## Discussion

This study has focused on the characterization of the likely differential effects of fatty acids typical from fish oil (EPA and DHA) and those most commonly found in vegetable oils (LA and ALA) on cellular plasticity and metabolism. To this end, we used as a model an *in vitro* culture of MSCs derived from vertebra bone of gilthead sea bream (*S. aurata*), one of the most cultivated species in Mediterranean aquaculture. The main objective was to evaluate the lineage-induction potential over the MSCs of these fatty acids, not only for its possible relevance in fish nutrition and welfare, but also to validate the cell system to further study the multipotentiality of piscine MSCs and their regulation.

The first step was to check that the incubation with the fatty acids was not causing any deleterious effect on the cells. Thus, an MTT assay was run showing that none of the fatty acids significantly affected bone-derived MSCs viability at concentrations up to 200 μM, although a slight rise in viability could be seen with the 100 μM concentrations, maybe related to the increased metabolic activity of the cells along the induced process of differentiation. These



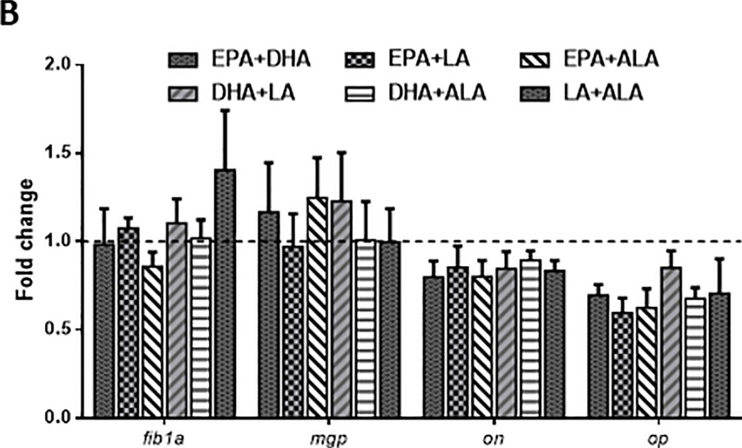




**Fig 4. Fatty acids effects on osteogenic genes expression.** Relative expression of genes related to the process of osteogenesis (*fib1a*, *mgp*, *on* and *op*) normalized to *b-actin* and *rps18* in gilthead sea bream bone-derived cells. Cells at day 4 were treated with different (A) individual or (B) combined fatty acids or were left untreated as control (dashed lines) for 6 h. Data are shown as mean + SEM (n = 3–4). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

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results coincide with those in another study, where fatty acid treatments were performed on human MSCs grown in osteogenic media, to see their possible effect on osteoblastogenesis [44]. Also, cell viability was verified after applying different fatty acids (e.g. EPA, DHA and arachidonic) at a 100 μM concentration on the skeletal VSa16 cell line of gilthead sea bream, demonstrating that such treatments can stimulate proliferation without signs of toxic effects [45].

Bone marrow MSCs in mammals retain a high degree of plasticity and their fate is affected by cell culture medium composition [23]. In the present study, accumulation of lipid content induced by fatty acids added to the media was dose-dependent as confirmed through

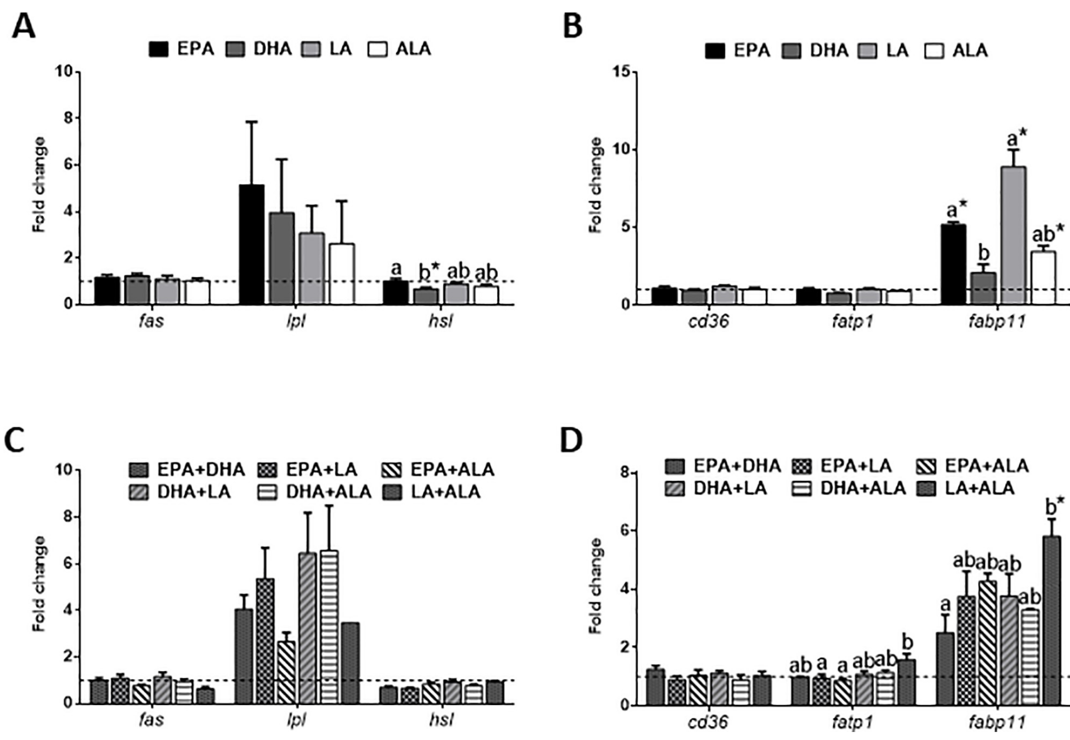


**Fig 5. Fatty acids effects on adipogenic genes expression.** Relative expression of genes related to lipid metabolism, including (A, C) enzymes (*fas*, *lpl* and *hsl*) and (B, D) fatty acid transporters (*cd36*, *fatp1* and *fabp11*) normalized to *b-actin* and *rps18* in gilthead sea bream bone-derived cells. Cells at day 4 were incubated with different (A, B) individual or (C, D) combined fatty acids, or were left untreated as control (dashed lines) for 6 h. Data are shown as mean + SEM (n = 3–4). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; ALA: α-linolenic acid.

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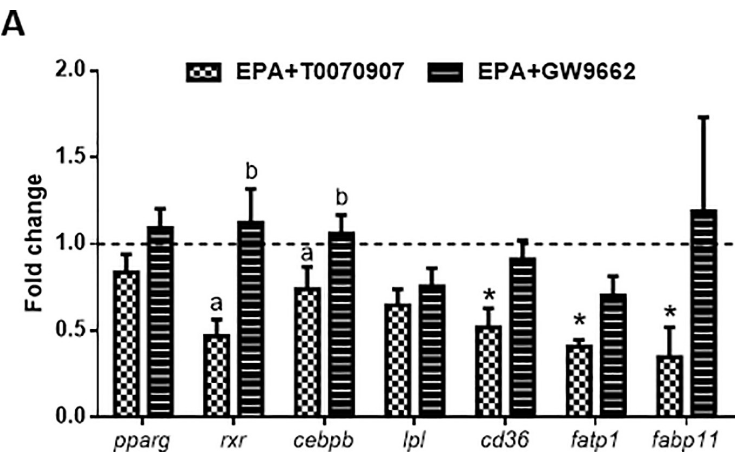
quantification and microscopy observation of the morphological changes, and the intensification of the red aura occupied in the cells by ORO staining. Previously, the ability of MSCs derived from bone to differentiate, in addition to osteoblasts, into adipocyte-like cells was demonstrated, by applying adipogenic media containing two different concentrations of lipid mixture; thus, confirming their multipotentiality [35]. The presence of fatty acids in such specific media seems to be critical to shoot the adipogenic process in undetermined fish cells [37], [46], [47], [48], and this has also been observed in avian adipocyte precursor cells [49]. Accordingly, in the present study, the cells began to accumulate lipids potentially inducing adipocyte-like differentiation in response to the fatty acid treatments. However, LA and the combinations containing this fatty acid were those that produced a greater effect, whereas the combination of the two fatty acids mainly present in fish oil (EPA+DHA) caused lower lipid deposition. Similarly, in mature salmon adipocytes it was observed that fatty acids from vegetable oils (i.e. oleic acid) were able to induce more triacylglycerol accumulation than fatty acids characteristic of fish oils [50]. Other studies have also shown this lower capacity of fatty acids from fish oils to be stored in adipose cells, such as those performed on 3T3-L1 pre-adipocytes, where DHA reduced dose-dependently fat deposition likely by suppressing lipid filling [51]. Overall, the data suggest that the n-6 PUFA LA may stimulate the uptake and depot of extracellular fats in these adipocyte-like cells, more than the other treatments tested.



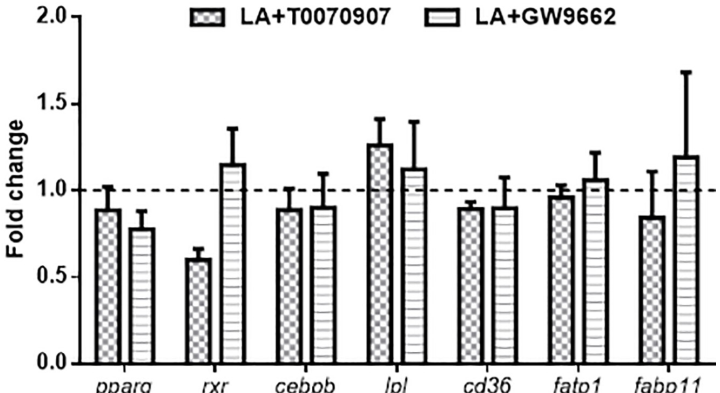




**Fig 6. PPARγ antagonists effects in adipogenic genes expression.** Relative expression of genes related to adipogenesis and lipid metabolism (*pparg*, *rxr*, *cebpb*, *lpl*, *cd36*, *fatp1* and *fabp11*) normalized to *b-actin* and *rps18* in gilthead sea bream bone-derived cells. Cells at day 4 were incubated with the fatty acids (A) EPA or (B) LA in the absence (dashed line, used as control) or presence of a PPARγ antagonist (T0070907 or GW9662) for 6 h. Data are shown as mean + SEM (n = 3–4). Significant differences ($p<0.05$) among treatments are indicated by different letters. Asterisks show significant differences ($p<0.05$) with the control. EPA: eicosapentaenoic acid; LA: linoleic acid.

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Next, to further evaluate the effects of fatty acids on MSCs lineage determination, expression of relevant driving genes was analyzed. *runx2* codifies for a key transcriptional activator that promotes osteoblastogenesis thus, inhibiting the determination and subsequent differentiation of MSCs into other cell lineages [24]. On the other hand, PPARγ, a member of the hormone nuclear receptors family, after interaction with specific ligands such as LC-PUFA, activates the transcription of genes involved in adipogenesis and lipid metabolism determining

the adipocyte phenotype of MSCs [25]. Moreover, induction of *pparg* expression can result in the inhibition of differentiation toward osteoblasts, as it has been described in some mammalian studies by acting as a suppressor of *runx2* [23], [52], [53]. Besides, overexpression of *runx2* in rat MSCs derived from adipose tissue produces a decrease in the expression of *pparg* [54]; consequently, these two transcription factors seem to act by negatively regulating each other. In Atlantic salmon, *pparg* is also silenced when the culture medium used is osteogenic, while *runx2* has its expression inhibited in the presence of an adipogenic medium [39]. In agreement with these observations, in our study, all fatty acid treatments, either alone or in combination, down-regulated *runx2*, although only EPA was able to significantly increase *pparg* gene expression. In fact, when treating the gilthead sea bream osteoblast-like VSa16 cell line with EPA, a decrease in the mRNA levels of *runx2* was also reported [45]. Accordingly, an increase in the transcript levels of *pparg* was also caused in human MSCs due to EPA treatment [44]. Despite significant differences were not observed with DHA alone, up-regulation of this key adipogenic gene was found with all combinations containing this fatty acid. Interestingly, both EPA and DHA had been considered for years, natural ligands of *pparg*, and to have greater potency on activating this transcription factor, compared to the n-6 PUFA (i.e. LA) [55]; so, these findings propose a direct effect of these n-3 LC-PUFA stimulating adipogenesis, as previously described in mammalian models [25]. Furthermore, *cebpb* expression was also up-regulated in response to EPA and by the combination EPA+DHA, supporting that the initiation of the adipogenic process is taking place upon those treatments. In fact, at least in mammals, *cebpb* contributes to stimulate *pparg* expression during early adipogenesis [56], [57]. Moreover, similar results were found when the gene expression of *rxr* was analyzed, since EPA, but also LA, could significantly up-regulate it. The nuclear receptor RXR forms a heterodimer among others with PPARγ, to regulate the transcription of genes related to lipid metabolism, thus also driving adipocyte differentiation [58]. Nevertheless, knowledge on the PPARγ-RXR heterodimers, as well as their response to fatty acids in fish is very limited.

To further evaluate if the bone-derived MSCs are deviated from the osteogenic process when the fatty acid treatments are applied, the expression of various genes related to both early osteogenesis and late mineralization of bone ECM was determined [36]. The mRNA levels of *fib1a*, *mgp*, *on*, and *op* remained constant in response to all the treatments, similarly as in [38], in the same cell model after addition of a standard adipogenic medium. With these results, we could suggest that the osteogenic process, to which these cells were previously predestined in their tissue microenvironment, has stopped. This deregulation of MSCs determination and/or trans-differentiation has been related not only in mammals, but also in fish, with developmental disorders or disease states, such as distraction osteogenesis in rats [59], bone loss in osteoporotic human patients [60] and reduced or malformed vertebrae in Atlantic salmon [61], [62], [63], [64], in which, diet specifically, has been shown as a causative factor [65].

Regarding expression of adipocyte markers, specifically genes that codify for key enzymes such as *fas*, involved in the synthesis of fatty acids, remained stable, maybe due to a direct inhibition of *de novo* synthesis caused by the addition of fatty acids into the culture medium. Similarly, differences could not be found in *lpl* or *hsl* gene expression during differentiation into adipocytes of rainbow trout cultured pre-adipocytes when the whole transcriptional profile of this process was analyzed [66]. Nevertheless, increased gene expression in the late phases of adipocyte differentiation has been reported for *lpl* in red sea bream [47] and Atlantic salmon [50].

Concerning the genes involved in the uptake and transport of fatty acids, *cd36* is, among others, a target gene of PPARγ [25]. In this context, PPARγ activation was found to induce *cd36* expression and adipocyte differentiation of the arterial rat VSMCs line [67]. In our study, the increased *pparg* mRNA levels at 6 h incubation in response to EPA, but not in *cd36*, suggest

a possible delayed up-regulation of the latter. Furthermore, in Atlantic salmon and rainbow trout, the mRNA levels of *fatp1* increased during the differentiation of pre-adipocytes, at earlier stages than *fabp11*, indicating that the former has an important role in the induction of adipogenesis and in the uptake of fatty acids from the environment [27], [68]. In our study, the combination of LA+ALA or LA alone caused an increase in *fatp1* and *fabp11* mRNA levels compared to other combinations. Altogether, these results indicated a more direct stimulation of fat transporters expression by fatty acids of vegetal origin, especially LA, suggesting a possible mechanism of action to induce adipogenesis through enhancing fatty acid uptake. These observations together with the elevated capacity of lipid accumulation when cells were treated with LA are in agreement with a previous study in gilthead sea bream, in which diets with high content in vegetable oils induced adipocyte hypertrophy [12]. On the other hand, DHA and more remarkably EPA, showed a higher potential to stimulate adipogenesis via up-regulation of *pparg* gene expression, thus inducing the adipocyte-like phenotype but with lower lipid content. Hence, since smaller adipocytes have the metabolic advantage of retaining insulin sensitivity and protect other tissues from lipotoxicity according to works in mammals [69], further studies would be of great interest to demonstrate if fish oil-derived fatty acids would lead to healthier cells as well in fish.

To corroborate the differential action of EPA and LA driving adipocyte-like development of bone-derived MSCs through PPARγ activation, their effects in combination with two PPARγ antagonists were evaluated. According to the results, LA action was unaffected, demonstrating the direct effect of this fatty acid on the transport of lipids due to *fabp11* and *fatp1* up-regulated gene expression. With regards to EPA treatment, the antagonist GW9662 did not cause any change, but the use of the specific antagonist T0070907, triggered a remarkable down-regulation on transcript levels of the three fatty acid transporters and the factors *rxr* and *cebpb*, although not of *pparg*. Accordingly, in mammals GW9662 shows negligible effects on transcription compared to T0070907, which displays properties of an inverse agonist, showing effects on transcription opposite to well-known PPARγ agonists such as [40], [41]. Similarly, the antiobesogenic effect of these antagonists has been shown in zebrafish larvae *in vivo*, although without performing transcriptomic analyses [42], [70]. Overall, the current data would suggest an inhibition of the adipogenic process, including lipid internalization, mediated at least in part by the incapability of EPA to activate PPARγ and the companion transcription factors. In agreement with this hypothesis, other authors using this same antagonist showed a decrease in *cd36* mRNA levels not depending on the increased gene expression of *pparg*, but elevated PPARγ activity [71]. Thus, blockage of EPA action by T00709007 indicates that via the action of this transcription factor, EPA may also up-regulate fat transporters to ultimately stimulate adipocyte differentiation of MSCs. These results agree with the capacity of n-3 LC-PUFA to promote the formation of healthy new adipocytes [72]. An example of a similar scenario may be the antidiabetic treatment with the full PPARγ agonists, the thiazolidinediones (i.e. troglitazone or pioglitazone), which have been shown, at least in rodent models, to favor remodeling of the adipose tissue by promoting pre-adipocyte recruitment for hyperplastic growth [73], [74].

## Conclusions

Gilthead sea bream bone-derived MSCs treated with one or two combined fatty acids undergo morphological and transcriptional changes, increasing lipid accumulation as well as the expression of adipogenic genes while decreasing or maintaining stable those related to the osteogenic process. This confirms the plasticity of these cells and supports their use as a model to study MSCs fate modulation. Besides, these findings should be also considered when

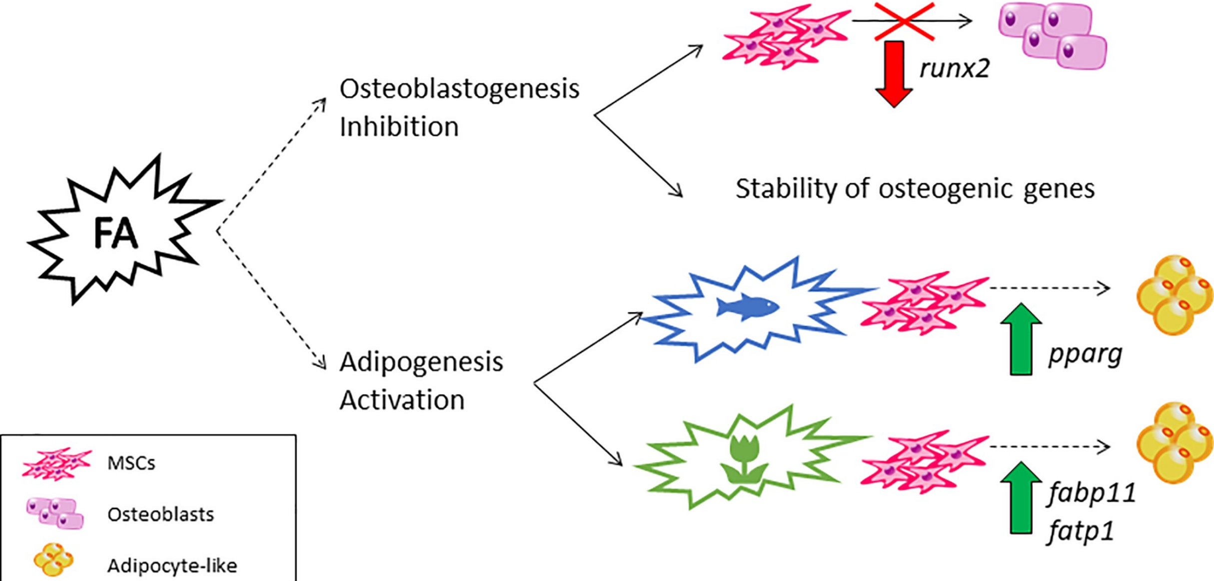


**Fig 7. Summary of fatty acids effects on gilthead sea bream bone-derived cells.** Schematic representation summarizing the effects of fatty acid (FA) treatments over bone-derived mesenchymal stem cells (MSCs) of gilthead sea bream. The fatty acids produce an inhibition of the osteogenic process through causing a down-regulation of *runx2* and a stabilization of the osteogenic genes relative expression. On the other hand, fatty acids induce adipogenesis, with those fatty acids characteristic from fish oils apparently via up-regulating *pparg* mRNA levels and in contrast, those typical from vegetable oils increasing the relative gene expression of fatty acid transporters (*fabp11* and *fatp1*), thus potentially enhancing cell lipid accumulation.

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studying fish bone structure and function, since at least in humans, there is a correlation between the appearance of bone marrow fat and the reduced bone forming capacity observed during diabetes and aging [75], [76]. Our data also suggest that fatty acids might be inducing adipogenesis potentially through different pathways, with fish oil-derived fatty acids such as EPA causing mainly formation of new adipocytes through activation of PPARγ, whereas vegetable fatty acids like LA appear to rather induce a process of fat accumulation in committed pre-adipocytes (Fig 7). These results advise that fatty acids from plant origin should be wisely used in aquafeeds, as they could induce the formation of less sensitive and functional hypertrophic adipocytes as previously suggested [12]. While we should be cautious because most of our data is based on a transcriptional level, and further studies are required to validate these observations; overall, this needs to be considered in feeds formulation to carefully find a balance according to the nature of the oil sources to ensure a healthy and high-quality fish.

## Supporting information

**S1 Fig. Effects of ethanol, fatty acids and antagonists treatments on cell viability.** Viability of gilthead sea bream bone-derived cells at day 4 determined by means of the MTT assay. Cells were treated (**A**) for 24 h with different concentrations of ethanol; or for 6 h (**B**) with different concentrations of selected fatty acids (EPA and LA) or were left untreated as control (dashed line), and (**C**) with the fatty acids EPA or LA in the absence (dashed line) or the presence of a PPARγ antagonist (T0070907 or GW9662). Data are shown as mean + SEM (n = 3). Significant differences ($p<0.05$) among concentrations are indicated by different letters. Asterisks indicate significant differences ($p<0.05$) with the corresponding control. EPA: eicosapentaenoic

acid; LA: linoleic acid.
(TIF)

**S1 File. Submit R1.zip contains the files with the complete raw data.**
(ZIP)

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ORIGINAL ARTICLE

# Gene expression analyses in malformed skeletal structures of gilthead sea bream (*Sparus aurata*)

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## Abstract

The incidence of skeletal anomalies in reared fish has been translated for years in important economic losses for the aquaculture industry. In the present study, we have analysed the gene expression of extracellular matrix components and transcription factors involved in bone development in gilthead sea bream presenting different skeletal anomalies: lordosis (LD), lordosis–scoliosis–kyphosis (LSK) or opercular, dental or jaw malformations in comparison with control (CT) specimens. Results showed a possible link between the presence of LD and LSK and the significant downregulation of genes involved in osteoblasts' maturation and matrix mineralization (collagen type 1-alpha, osteopontin, osteocalcin, matrix Gla protein and tissue non-specific alkaline phosphatase), as well as in bone resorption (cathepsin K and matrix metalloproteinase 9) compared to CT animals. Contrarily, the key osteogenic transcription factor *runx2* was upregulated in the malformed vertebra suggesting impaired determination of mesenchymal stem cells towards the osteoblastic lineage. Despite the gene expression patterns of the other malformed structures were not affected in comparison with CT fish, the results of the present study may contribute in the long term to identify potential candidate gene profiles associated with column deformities that may help reducing the incidence of appearance of skeletal anomalies in this important aquaculture species.

KEYWORDS
lack of operculum, lordosis–scoliosis–kyphosis, osteogenesis, skeletal anomalies

## 1 | INTRODUCTION

Aquaculture research is focused on obtaining the most sustainable and profitable production, minimizing the cost, but assuring a good high-quality product. Notwithstanding, one of the major problems being faced in the last decades is the elevated presence of skeletal anomalies, leading to important economic losses. Those fish that are severely affected either die, have to be early discarded during the selection process or downgraded because they are not suitable for the market. The incidence varies greatly among farms, species and batches, and even depending on the accuracy of the different detection methods used (for a review see Boglione et al., 2013). Nevertheless, in Spanish aquaculture, up to 30% of marine fish larvae have, on average, at least one type of malformation (Afonso et al., 2000; Boglione, Gagliardi, Scardi, & Cataudella, 2001). Gilthead sea bream (*Sparus aurata*) is a valuable Mediterranean species, for which a 27% incidence of skeletal anomalies, with a survival rate of only 5% of these malformed specimens, was reported three decades ago (Andrades, Becerra, & Fernández-Llebrez, 1996). Nonetheless, 15%–50% of *Sparidae* juveniles with severe anomalies are nowadays culled out of the productive cycle at the end of the hatchery phase (Boglione & Costa, 2011), indicating this is still an important bottleneck.

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Deformities can appear elsewhere, causing serious problems to the individuals (e.g., affecting feeding and growth), compromising their welfare and survival, as reported for European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (Divanach et al., 1996; Koumoundouros, Maingot, Divanach, & Kentouri, 2002; Matsusato, 1986). Some of the most common affectations described are in the cranial region, affecting the operculum (OPC), jaw (JW) or dentary bone (DNT), as well as in the vertebral centra, where lordosis (LD), scoliosis, kyphosis or a combination of these three anomalies leading to a more severe malformation referred to as LSK could be observed. Among the skull anomalies, the gill cover has been recorded to affect up to 80% of gilthead sea bream individuals (Koumoundouros, 2010) and pugheadness to be the more frequent among the DNT and JW anomalies also in this species. Even so, in Mediterranean marine species including gilthead sea bream, LD is one of the best-studied malformations. In that species, haemal LD, for example, can be found in about 13% of the specimens (Georgakopoulou, Katharios, Divanach, & Koumoundouros, 2010), whereas kyphosis or scoliosis is rarely observed. Among the non-genetic factors discussed as causative of LD (along the entire vertebral centra), there are the non-inflation of the swim bladder, inappropriate tank hydrodynamism or feeding behaviour, although discrepancies exist in the literature about their relative importance (reviewed in Boglione et al., 2013). In Atlantic salmon (*Salmo salar*), temperature seems to be the most potent causative factor of spinal anomalies (Wargelius et al., 2010); however, genetic factors have also been postulated as responsible in non-salmonid fish. In gilthead sea bream, development of the LSK syndrome has been associated with family structure (Afonso et al., 2000), and consanguinity has been shown to increase the incidence of LD and lack of an OPC, having both characters high heritability (Afonso et al., 2009; Navarro et al., 2009). More recently, García-Celdrán et al. (2015) and Negrín-Báez et al. (2015) also reported a significant relationship between the prevalence of LD, LSK and lack of OPC and the mating of broodstock of different origins suffering from the same deformity, thus confirming the importance of inheritance in the presence of these malformations in gilthead sea bream.

Despite both, simple Mendelian genetics and polygenic determinants could explain at least in part the incidence of skeletal anomalies, studies evaluating alterations in specific genes are limited in fish. In this sense, zebrafish (*Danio rerio*) carrying a mutation in the collagen type 1 alpha-1 (*col1a1*) has been identified as a model of osteogenesis imperfecta, a type of skeletal dysplasia (Fisher, Jagadeeswaran, & Halpern, 2003). Moreover, several mutants for genes involved in arch development have also been reported in the same species presenting craniofacial abnormalities (Piotrowski et al., 1996; Schilling, Walker, & Kimmel, 1996). In gilthead sea bream, transcripts characteristic of bone cells have been recently identified and their expression associated with the different processes that occur during osteogenesis (Riera-Heredia et al., 2018; Vieira et al., 2013), although studies potentially relating dysregulation of these genes with the emergence of malformations are, as far as we know, absent in this species. The principal transcription factor involved in the control of osteoblast differentiation is Runt-related transcription factor 2 (Runx2) (Vieira et al., 2013). Then, COL1A1 plays an important role in structuring and stablishing the production of the extracellular matrix (ECM), while the tissue non-specific alkaline phosphatase (TNAP) and the non-collagenous proteins, osteonectin (ON), osteopontin (OP) and osteocalcin (OCN), are required for osteoblast maturation and ECM mineralization during bone development (Riera-Heredia et al., 2018). Besides, considering abnormalities are more frequent in larval and juvenile fish as they exhibit increased bone growth and turnover rates, factors controlling bone resorption should be also considered. Characteristic markers of osteoclasts involved in bone matrix degradation, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK) and matrix metalloproteinase 9 (MMP9), have been also identified in gilthead sea bream (Vieira et al., 2013).

The main objective of this study was to increase knowledge about the involvement of gene factors in the onset of skeletal anomalies in gilthead sea bream. Special attention is paid to the relationship between the expression of potential candidate genes and the occurrence of a specific deformity. The ultimate goal is to further use this information to optimize the aquaculture production of high-quality fish with reduced incidence of bone malformations.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals and ethics statement

Gilthead sea bream (*S. aurata*) were obtained from the hatchery Tinamenor S.L. (Pesués). Fish were reared on standard hatchery conditions at 20–22°C, 90%–110% $O_2$ saturation and 15:9 daily photoperiod and were fed throughout the day on a diet containing 56% protein and 18% lipid (Perla, Skretting). Animal handling procedures complied with the guidelines of the European Union Council (86/609/EU), and Spanish and Catalan Governments legislation and were approved by the Ethics and Animal Care Committee of the University of Barcelona. Forty-seven animals from the same batch of production with or without skeletal anomalies and an average weight of 2.7 ± 0.1 g were preselected by the hatchery personnel trained to do the usual discarding of malformed animals and transferred to the University of Barcelona. Transportation was done by road with the fish in sealed cubitainers with saturated oxygen at very low density to minimize stress. The fish were sampled at arrival, and the identification of anomalies was done according to visual inspection by all the researchers taking part in the sampling. Figure 1 shows representative images of the different malformed animals, and the sampling was performed as schematically illustrated in Figure 2. Samples ($n$ = 6–10 per group) were taken from control (CT) normal specimens or animals with LD, lordosis–scoliosis–kyphosis (LSK), or malformations in the OPC, DNT or JW. For the column malformations, once the cleaning of the vertebrae was performed, the phenotype was confirmed as the one identified during the external visualization. Samples of vertebrae from the LD specimens were taken both from affected (LDA) and normal non-affected (LDN) portions of similar size, although the location along the column for each sample may have varied from one fish to another depending on the specific

LD LSK OPC DNT JW

1 cm

**FIGURE 1** Representative images of the specimens sampled with lordosis (LD), lordosis–scoliosis–kyphosis (LSK), operculum (OPC), dental (DNT) or jaw (JW) malformations. Scale bar 1 cm

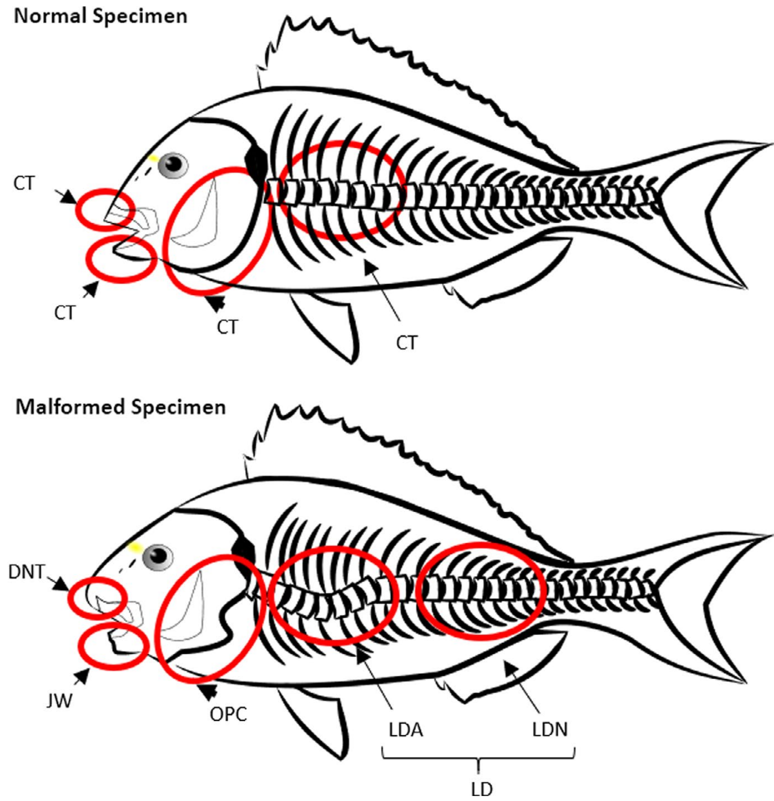



**FIGURE 2** Schematic representation of the bone samples extracted from a normal and a malformed specimen. To simplify, all the different anomalies evaluated have been indicated in a single animal, although none of the specimens had more than one malformation at a time. CT, control; DNT, dental; JW, jaw; LD, lordosis; OPC, operculum. The LDA and LDN samples in the malformed animal correspond to a region of the vertebral column with or without visual anomaly, respectively, in an LD animal. In the case of the animals showing a lordosis–scoliosis–kyphosis (LSK) malformation, the vertebral column sample was taken from the affected area

position of the malformation. All bone samples were immediately frozen using liquid nitrogen and stored in the freezer at −80°C until RNA extraction.

## 2.2 | RNA extraction and cDNA synthesis

Total RNA was extracted using Tri Reagent (Life Technologies) following the manufacturer's instructions, quantified using a NanoDrop2000 spectrophotometer (Thermo Scientific), and the quality analysed by 1% (w/v) agarose gel electrophoresis. One µg of total RNA per sample was DNase‐treated (Life Technologies) and used to synthesize first‐strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) following the manufacturer's recommendations.

## 2.3 | Quantitative real‐time PCR (qPCR)

The qPCR assays were conducted according to the requirements of the MIQE guidelines (Bustin et al., 2009) using a CFX384™ Real‐Time System (Bio‐Rad). Prior to the analyses, specificity of the reaction, absence of primer–dimer formation, as well as identification of the appropriate cDNA working dilution for each assay were determined by running a dilution curve with a pool of samples. Reactions were performed in triplicate and contained cDNA, iQ SYBR Green Supermix (Bio‐Rad) and 250 nM (final concentration) of forward and reverse primers (Table 1). The protocol consisted on 1 cycle of 3 min at 95°C and 40 cycles of 10 s at 95°C and 30 s at 60–68°C (primer dependent, see Table 1), followed by an amplicon dissociation analysis from 55 to 95°C with a 0.5°C increase every 30 s. SYBR green

**TABLE 1** Primers used for real-time quantitative PCR

| Gene | Primer sequence (5′–3′) | Tm (°C) | Acc. Num. | Reference |
|---|---|---|---|---|
| *β-actin* | F: TCCTGCGGAATCCATGAGA<br>R: GACGTCGCACTTCATGATGCT | 60 | X89920 | Vélez et al. (2014) |
| *col1a1* | F: GAGATGGCGGTGATGTGGCGGAGTC<br>R: GCCTGGTTTGGCTGGATGAAGAGGG | 68 | DQ324363 | Rosa, Tiago, Dias, Cancela, and Laize |
| *ctsk* | F: AGCGAGCAGAACCTGGTGGAC<br>R: GCAGAGTTGTAGTTGGGGTCGTAG | 60 | DQ875329 | Salmerón, Navarro, Johnston, Gutiérrez, |
| *dlx2a* | F: CGGAGCGATCTCGTATAGCC<br>R: ACACGGGGATGCTTTTCGAT | 60 | SRR278741_isotig04863 | Present study |
| *dlx3b* | F: CTGCCACAAGTGATCAGGAA<br>R: CTGCAGAGCAAACAGTCGAG | 60 | SRR278741_isotig06751 | Present study |
| *dlx5a* | F: CTCCAAGAGCCAACGAGTGT<br>R: AGCCTACAGCCACAAAGCAT | 60 | SRR278741_isotig08598 | Present study |
| *dlx6* | F: GCTCTGTGCCGTCTAACCTT<br>R: TACCCTACCCCTACGTGAGC | 60 | SRR278741_isotig14107 | Present study |
| *ef1α* | F: CTTCAACGCTCAGGTCATCAT<br>R: GCACAGCGAAACGACCAAGGGGA | 60 | AF184170 | Salmerón, Acerete, Gutiérrez, Navarro, and |
| *fib1a* | F: CGGTAATAACTACAGAATCGGTGAG<br>R: CGCATTTGAACTCGCCCTTG | 60 | FG262933 | Vieira, Pinto, Guerreiro, and Power (2012) |
| *mgp* | F: TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC<br>R: CGGGCGGATAGTGTGAAAAATGGTTAGTG | 68 | AY065652 | Rosa et al. (2010) |
| *mmp9* | F: ATTCAGAAGGTGGAGGGAGCG<br>R: CATTGGGGACACCACCGAAGA | 60 | AM905938 | Vieira et al. (2013) |
| *ocn* | F: TCCGCAGTGGTGAGACAGAAG<br>R: CGGTCCGTAGTAGGCCGTGTAG | 60 | AF048703 | Pinto, Ohresser, and Cancela (2001) |
| *on* | F: AGGAGGAGGTCATCGTGGAAGAGCC<br>R: GTGGTGGTTCAGGCAGGGATTCTCA | 68 | AY239014 | Rosa et al. (2010) |
| *op* | F: AAAACCCAGGAGATAAACTCAAGACAACCCA<br>R: AGAACCGTGGCAAAGAGCAGAACGAA | 68 | AY651247 | Rosa et al. (2010) |
| *osx* | F: CAGTCAGGGATTCAGCAACA<br>R: GGTGAAGGAGCCAGTGTAGG | 60 | ERR22591_isotig06993 | Present study |
| *rpl27a* | F: AAGAGGAACACAACTCACTGCCCCAC<br>R: GCTTGCCTTTGCCCAGAACTTTGTAG | 68 | AY188520 | Rosa et al. (2010) |
| *runx2* | F: ACCCGTCCTACCTGAGTCC<br>R: AGAAGAACCTGGCAATCGTC | 60 | JX232063 | Vieira et al. (2012) |
| *tnap* | F: CATCGCAACCCTTTTCACAGTCACCCG<br>R: AACAGTGCCCAAACAGTGGTCCCATTAGC | 68 | AY266359 | Rosa et al. (2010) |
| *trap* | F: CTTAATCGTTGCCATCCCTGTG<br>R: CTCCCATCTGCTCTGCTACTTTG | 60 | FM147928 | Vieira et al. (2013) |

*Note*: Tm: melting temperature, Acc. Num.: Gen Bank accession number. Beta-actin (*β-actin*); collagen type 1 alpha-1 (*col1a1*); cathepsin K (*ctsk*); distal-less homeobox 2a, 3b, 5a and 6 (*dlx2a*, *dlx3b*, *dlx5a* and *dlx6*); elongation factor 1-alpha (*ef1α*); fibronectin 1a (*fib1a*); matrix Gla protein (*mgp*); matrix metalloproteinase 9 (*mmp9*); osteocalcin (*ocn*); osteonectin (*on*); osteopontin (*op*); osterix (*osx*); ribosomal protein L27a (*rpl27a*); Runt-related transcription factor (*runx2*); tissue non-specific alkaline phosphatase (*tnap*); tartrate-resistant acid phosphatase (*trap*).

fluorescence was recorded during the annealing-extending phase of cycling. Expression results were normalized to the geometric mean of ribosomal protein L27a (*rpl27a*) and elongation factor 1-alpha (*ef1a*) as they were the most stable of the reference genes tested according to the GeNorm algorithm implemented in the CFX Manager Software (Bio-Rad). Target transcript abundance was analysed using

the delta-delta method (Pfaffl, 2001) with the same CFX Manager Software (Bio-Rad). In addition to showing the quantitative gene expression results of each malformation with the corresponding CT samples, in order to compare the overall changes in gene expression among malformed tissues, a heat map was created. The data from the deformities in the different structures, vertebral column (VC; combining LD and LSK), OPC, DNT and JW, were analysed relative to their corresponding CT samples and standardized (i.e., standard score normalization), and a heat map with these values was then generated using PermutMatrix version 1.9.3 (Caraux & Pinloche, 2005).

### 2.4 | Statistical analyses

Statistical analyses of all parameters were performed in SPSS Statistics version 20 (IBM, Armonk, NY, USA). Normality was analysed according to the Shapiro–Wilk test and homogeneity of variance according to Levene's test. Statistical differences among vertebral column groups (CT, LD and LSK) were assessed by one-way ANOVA, followed by Tukey's *post hoc* test. Statistical differences between malformed OPC, DNT and JW with their respective CT group and between the LDA and LDN regions and each one of them with the CT column were assessed using Student's *t* test. Significant differences were considered at $p < 0.05$ for all statistical tests performed. Data are presented as mean ± standard error of the mean (*SEM*). Significant differences are indicated with different letters or asterisks.

## 3 | RESULTS

Relative gene expression from vertebra bone was analysed in CT, LD and LSK fish. As indicated in the Material and Methods section, LDN samples from non-affected column regions of LD animals were extracted in addition to the malformed LDA fragments and evaluated separately. Despite that tissue was visually normal, the pattern of expression of the different genes analysed in LDN samples was similar to that of the LDA samples and significantly different from that of the CT animals (Table 2). Therefore, expression data from both LDN and LDA column samples of the lordotic specimens were combined and presented together (named as LD) in Figure 3, where comparisons were made with the CT and LSK specimens.

Regarding ECM components, a decrease in their expression was found in the vertebral column along with the increased severity of the malformation types for all the genes studied (Figure 3a). *col1a1* and matrix Gla protein (*mgp*), presented a significantly lower expression in LSK when compared to CT fish, with LD values being intermediate, while *ctsk* showed only significantly lower levels of expression in LD compared to CT animals. In the case of *op*, *tnap* and *mmp9*, transcript expression was significantly lower in both deformed bones than in CT ones. Finally, *ocn* presented the clearest differences, since the three conditions were significantly different, being this gene more expressed in CT animals than in LD, and in those more than in the fish presenting the LSK syndrome that showed the lowest levels.

On the other hand, the mRNA levels of most of the transcription factors analysed were similar among the three animal groups (Figure 3b). Only LD fish showed a significantly higher expression of *runx2* compared with the CT specimens, but those had a significantly higher expression of distal-less homeobox 5a (*dlx5a*) compared to both LD and LSK fish.

When the relative gene expression of all these molecules was tested in OPC-affected fish compared to CT animals, significant differences were not seen but some tendencies were observed (Figure 4a,b). Thus, fibronectin 1a (*fib1a*), *runx2* and distal-less homeobox 2a (*dlx2a*) appeared to increase in deformed OPC samples.

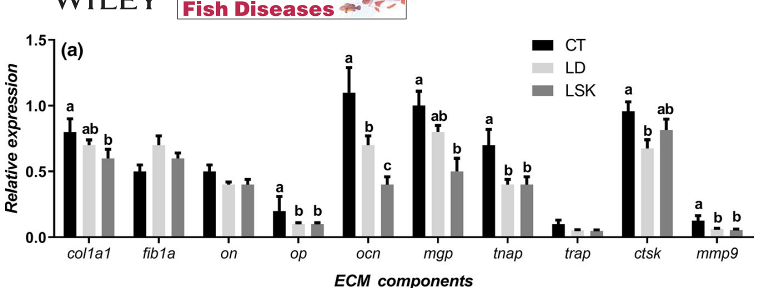
In the DNT tissue (Figure 5a,b), *dlx2a* was the only gene differentially expressed, being lower in the malformed fish compared to the CT one, while the rest of the genes analysed only showed tendencies, like *on* and distal-less homeobox 6 (*dlx6*), which were less expressed in malformed than in CT fish.

Considering the JW (Figure 6a,b), great variability was observed among samples and significant differences were not found. Nevertheless, the most expressed genes in the malformed tissue compared to CT samples were *fib1a*, *runx2* and *dlx6*.

**TABLE 2** Relative gene expression of ECM components and transcription factors in vertebral column samples of gilthead sea bream

| Gene | LDN | LDA | Gene | LDN | LDA |
|---|---|---|---|---|---|
| *col1a1* | 0.91 ± 0.07 | 0.89 ± 0.07 | *ctsk* | 0.68 ± 0.10* | 0.72 ± 0.11 |
| *fib1a* | 1.06 ± 0.14 | 1.46 ± 0.19* | *mmp9* | 0.48 ± 0.07* | 0.52 ± 0.08* |
| *on* | 0.77 ± 0.04* | 0.90 ± 0.08 | *runx2* | 1.60 ± 0.21* | 1.57 ± 0.13* |
| *op* | 0.25 ± 0.03* | 0.38 ± 0.07* | *osx* | 1.04 ± 0.11 | 1.02 ± 0.18 |
| *ocn* | 0.59 ± 0.10 | 0.73 ± 0.09 | *dlx2a* | 1.68 ± 0.34* | 1.98 ± 0.34* |
| *mgp* | 0.77 ± 0.07 | 0.63 ± 0.10* | *dlx3b* | 0.89 ± 0.10 | 0.95 ± 0.15 |
| *tnap* | 0.60 ± 0.10* | 0.57 ± 0.06* | *dlx5a* | 0.78 ± 0.10* | 0.65 ± 0.08* |
| *trap* | 0.55 ± 0.10* | 0.51 ± 0.06* | *dlx6* | 1.46 ± 0.18* | 1.77 ± 0.32* |

*Note*: The samples correspond to a region of the vertebral column without visual anomaly (LDN, $n$ = 7–8) or with evident lordosis (LDA, $n$ = 8). Results are shown as the mean ± *SEM* normalized by the control (CT) specimens. Asterisks indicate significant differences with CT normal fish ($p < 0.05$). Significant differences were not found between the LDN and LDA groups ($p < 0.05$).



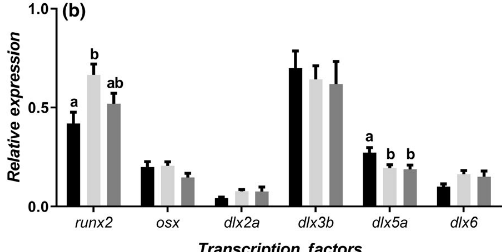




**FIGURE 3** Relative gene expression of (a) ECM components and (b) transcription factors in gilthead sea bream vertebral column fragments of control animals (CT, *n* = 7–8) or specimens with lordosis (LD, *n* = 14–16) or lordosis–scoliosis–kyphosis (LSK, *n* = 10–12). Results are shown as the mean ± *SEM*. Different letters indicate significant differences among groups ($p < 0.05$)

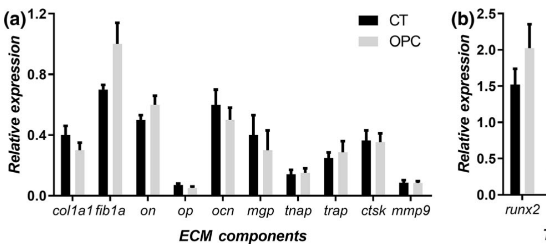
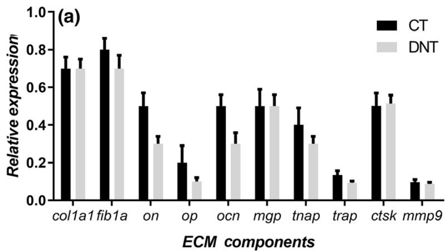



**FIGURE 4** Relative gene expression of (a) ECM components and (b) transcription factors in gilthead sea bream control (CT, *n* = 10–11) or specimens with an operculum deformity (OPC, *n* = 6–7). Results are shown as the mean ± *SEM*. Different letters indicate significant differences between groups ($p < 0.05$)



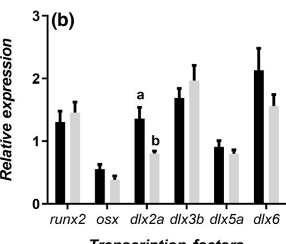




**FIGURE 5** Relative gene expression of (a) ECM components and (b) transcription factors in gilthead sea bream control (CT, *n* = 9–11) or specimens with a dental deformity (DNT, *n* = 8). Results are shown as the mean ± *SEM*. Different letters indicate significant differences between groups ($p < 0.05$)

Finally, a comparative study was carried out analysing all tissue deformities with respect to their corresponding CT samples (Figure 7). Different behaviours were seen depending on the origin of the tissue and the type of molecule evaluated. Regarding ECM components, whereas in the JW most genes were upregulated (red), in the vertebral column (VC), OPC and DNT almost all genes were downregulated (green) with the exception of *fib1a* (Figure 7a). On the other hand, transcription factors were mainly upregulated (red) in the VC, OPC and JW deformed tissues in comparison with the CT samples, especially *runx2*, which also appeared increased in the DNT bone (Figure 7b).

## 4 | DISCUSSION

In the present study, for the first time in gilthead sea bream, the gene expression profile of osteogenic ECM molecules and transcription factors in fish presenting different skeletal malformations (LD,

**FIGURE 6** Relative gene expression of (a) ECM components and (b) transcription factors in gilthead sea bream control (CT, $n = 4–9$) or specimens with a jaw deformity (JW, $n = 2–6$). Results are shown as the mean ± *SEM*. Different letters indicate significant differences between groups ($p < 0.05$)

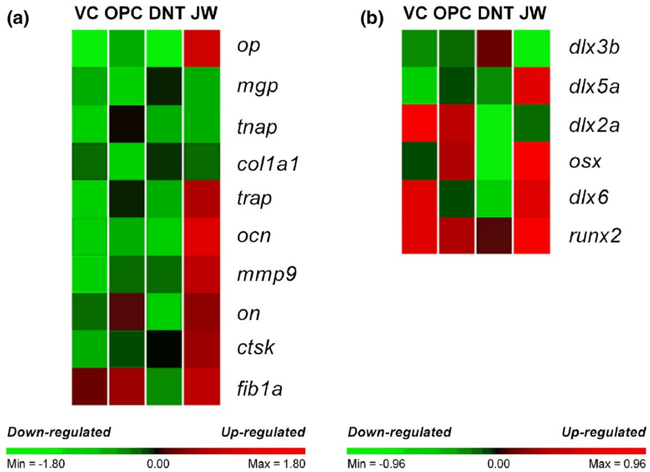



**FIGURE 7** Heat maps presenting the changes in (a) ECM components and (b) transcription factors gene expression in gilthead sea bream with different malformations. Gene expression was first calculated relative to the geometric mean of ribosomal protein L27a (*rpl27a*) and elongation factor 1-alpha (*ef1a*) and then was standardized following a standard score normalization against the corresponding control (CT) samples. Shades of red and green, respectively, indicate the highest and lowest expression levels, as specified in the scale bar at the bottom of the figure. For simplicity and because the changes were always in the same direction, the vertebral column data include combined the LD and LSK fish (VC, $n = 12–16$). Operculum (OPC, $n = 6–7$), dental (DNT, $n = 8$) and jaw (JW, $n = 2–6$)

LSK, OPC, DNT and JW) was evaluated in comparison with CT specimens, with the objective of trying to link specific gene patterns with anomalies. Although the data were not so outstanding for the cranial malformations, in the case of the vertebral centra, the changes in expression of several genes appeared to be related to the type of anomaly (LD or LSK).

The coordinated and sequential expression of specific osteogenic molecules is required for the proper development of the skeleton in *S. aurata* (Riera-Heredia et al., 2018). This expression pattern is susceptible to be altered by genetic factors, and heritability of certain types of malformations has been reported in this species (Navarro et al., 2009; Negrín-Báez et al., 2015). In our current findings, LD and LSK fish had upregulated *runx2* expression, but otherwise, *col1a1*, *op*, *ocn*, *mgp*, *tnap* and *mmp9* were downregulated compared to the CT specimens, supporting that altered expression of these genes might be linked to the appearance of the skeletal anomaly. Moreover, abiotic factors such as nutrition and temperature (Gisbert, Ortiz-Delgado, & Sarasquete, 2008), or a combination of factors (genotype + environment) can also modify gene expression, leading to increased incidence of skeletal malformations. For example, a temperature drop challenge has been demonstrated in gilthead sea bream to have an impact on impairing bone responsiveness (Mateus et al., 2017; Riera-Heredia et al., 2018). In Atlantic salmon, embryonic rearing at an elevated water temperature modifies the expression of several osteogenic genes and is associated with a higher number of vertebral deformities once the fish reach the juvenile state (Ytteborg, Baeverfjord, Torgersen, Hjelde, & Takle, 2010).

Furthermore, lordotic animals had visually an affected as well as a non-affected region in their vertebral column; although using other analytical methods (i.e., radiography or histology), it is plausible considering that other tissue defects along the whole column might have been identified. In Atlantic salmon, up to four vertebral regions have been described with different morphology and gene expression

profiles during normal development (Kacem, Meunier, & Bagliniere, 1998) and, also, that site-specific deformities could develop differentially within a particular life stage or environmental condition (i.e., sea water vs. freshwater), maybe due to this patterning (revised by Fjelldal et al., 2012). In our study, recording of the specific location of the malformation in lordotic gilthead sea bream was not performed, but in all cases, the gene expression pattern of LDN samples was similar to the LDA. This result demonstrated that the presence of a vertebral anomaly affects at a transcriptional level not only locally the malformed part of the tissue, but also the expression pattern of the entire column. Similarly, Boursiaki et al. (2019) observed recently that the presence of scoliosis in gilthead sea bream is more frequent in the caudal region, but in those malformed fish, both the caudal and abdominal vertebrae were significantly shorter when compared to the vertebrae of a normal fish. In other studies, with *Salmonidae* species, malformed vertebrae have been shown to have comparable mineral content to normal vertebrae, even if differences existed at an earlier stage of the malformation process (Fjelldal et al., 2012). Overall, these data support our findings on gene expression in LD animals despite the location of the malformation in the column. Therefore, although we cannot know whether the altered expression observed is the cause of the malformation and not a consequence, our results suggest that the genes identified (or at least their combined expression profiles) are potential candidates as markers to tag and identify possible defects in bone maturation resulting in malformed ossified tissue in gilthead sea bream.

The relevance of the specific ECM genes that were found downregulated in our malformed fish has been demonstrated in mammalian studies. For example, the human genetic disorder osteogenesis imperfecta, associated with a mutation in *col1a1*, induces severe skeletal deformities or mobility problems (Steiner, Adsit, & Basel, 2005). In zebrafish, a similar mutation causes defects in bone growth (Fisher et al., 2003). Taken into account that 90% of the bone ECM is composed of collagen, specifically type I, the fact that in our study the most severe skeletal malformation (SLK) showed significantly the lowest transcript levels of *col1a1* indicates a likely possible problem for that tissue to produce a normal ECM. Together with COL1A1, among the non-collagenous ECM proteins required for bone matrix mineralization, the downregulation of *on*, *op* and *ocn* upon temperature treatments has been associated with a restrained differentiation of Atlantic salmon cultured osteoblasts (Ytteborg, Vegusdal, et al., 2010) and a defect in the late maturation of osteoblasts in mammals (Boskey, 1992; Termine et al., 1981). In fact, Delany et al. (2000) reported that *on*-null mice display decreased trabecular bone volume and stiffness than wild-type mice. Similarly, in *ocn*-KO mouse maturation of mineral crystals in the bones is compromised (Boskey et al., 1998). Furthermore, inappropriate calcification, osteomalacia or soft bones can be caused by alterations in the function of the phosphatases system in mice (Millán, 2013). This could be also speculated as causative of vertebral malformations in gilthead sea bream, since a significantly downregulated expression of *tnap* was also found in LD and LSK fish in comparison with CT specimens.

Skeletal growth is controlled by facilitating bone formation and limiting bone degradation, which is mediated by osteoclasts through the action of CTSK, TRAP and MMP9. *ctsk* and *trap* gene expression in goldfish (*Carassius auratus*) scales correlated with the need of calcium during the reproductive season (Azuma et al., 2007), while *mmp9* was upregulated during tissue remodelling in the regenerating fin of the teleost fish *Poecilia latipinna* (Rajaram, Murawala, Buch, Patel, & Balakrishnan, 2016). Altered expression of these osteoclast markers has not been studied in fish yet in relation to skeletal anomalies, but in humans and in mice, a mutation in *ctsk* has been reported to cause supernumerary teeth due to reduced osteoclast activity (Helfrich, 2005). In our study, in CT fish, the significantly higher levels of *ctsk* expression found compared to LD, and those of *mmp9* compared to LD and LSK specimens, could thus suggest a balanced bone formation and resorption ratio in these non-affected fish, although more research would be required to confirm this hypothesis.

In addition to well-adjusted bone turnover for proper skeletal development, recruitment of new osteoblast cells is also important. Runx2 is one of the key factors that has been identified in zebrafish during skeletal tissue development (Flores, Lam, Crosier, & Crosier, 2006) and more recently also in gilthead sea bream (Vieira et al., 2013). As in mammals (Liu & Lee, 2013), upregulation of *runx2* gene expression in fish with a column deformity supposes a mismatch between the developmental stage and the bone status leading to a fragile tissue due to higher presence of non-mature osteoblasts (Ytteborg, Vegusdal, et al., 2010). To this end, the expression of *runx2* has been demonstrated to be clue in the lineage determination of mesenchymal stem cells (MSCs) towards osteoblasts in vitro. In this sense, in bone-derived MSCs from gilthead sea bream, cell fate was modulated downregulating *runx2* (Riera-Heredia, Lutfi, Gutiérrez, Navarro, & Capilla, 2019), thus compromising the bone tissue integrity, as previously seen in mammals (Berendsen & Olsen, 2014). Similarly, in lordo-kyphotic Senegalese sole (*Solea senegalensis*), condensation of MSCs or transdifferentiation of pre-osteoblastic cells to the chondrocyte lineage has been hypothesized (Cardeira, Bensimon-Brito, Pousão-Ferrerira, Cancela, & Gavaia, 2012).

Other factors involved in osteoblasts differentiation and proliferation belong to the family of the distal-less homeobox proteins (DLX) (Bendall & Abate-Shen, 2000; Komori, 2006; Marie, 2008). In zebrafish, DLX5 is present in the developing visceral skeleton and during scale regeneration (Thamamongood et al., 2012; Verreijdt et al., 2006). The present results reflected a significant downregulation of this gene in LD and LSK specimens compared to CT fish, indicating again an association between the presence of these deformities and changes in the gene expression of tissues formed at the onset of development. Regarding this, morpholino-mediated knock-down of *dlx5a/6a* in zebrafish showed a failure of cleithrum formation (Heude, Shaikho, & Ekker, 2014). Moreover, DLX2a has been related to dental development in mammals (Zhao et al., 2000) and zebrafish (Borday-Birraux et al., 2006). Despite being an early developmental gene in the latter species (Verreijdt et al., 2006), our results in gilthead sea bream showed a significant decrease in the expression of *dlx2a* in malformed DNT specimens compared to their

corresponding CT fish, suggesting a possible imprinted failure effect during development.

Overall, the major differences in gene expression associated with a skeletal malformation in the gilthead sea bream of the present study have been found in the vertebral column. Contrarily, the other malformed structures analysed (OPC, DNT and JW) did not show significant changes in gene expression patterns. Development of skeletal anomalies is a complex process that may be explained not only in terms of gene transcription alteration, but also due to other types of factors, which can also interact with each other. In fact, although in gilthead sea bream, skeletal deformities, such as LD, LSK, lack of OPC or vertebral fusion, have been related to genetic components and inheritance (García-Celdrán et al., 2015; Navarro et al., 2009; Negrín-Báez et al., 2015), some other authors have proved that rearing conditions, stress or inadequate feeding can also be leading to deformities in skeletal structures (Koumoundouros, Oran, Divanach, Stefanakis, & Kentouri, 1997; Prestinicola et al., 2013). In this sense, Prestinicola et al. (2013) demonstrated that it is possible to lower the severe skeletal anomaly incidence and the meristic count variability of dermal bones of reared gilthead sea bream juveniles by lowering stocking densities. Furthermore, synchronicity between bone and muscle growth is required for proper musculoskeletal development (Vélez et al., 2018; Ytteborg, Torgersen, Baeverfjord, & Takle, 2012). Hence, enhanced muscular growth induced by hyperthermia or activity from too intense water currents was significantly associated with increased vertebral deformities in Atlantic salmon (Ytteborg, Baeverfjord, et al., 2010) and European sea bass (Divanach, Papandroulakis, Anastasiadis, Koumoundouros, & Kentouri, 1997) respectively. Furthermore, in mammals, the bone-produced OCN has been reported as crucial for the adaptation of the skeletal muscle to exercise (Mera et al., 2016), suggesting that the crosstalk between bone and muscle tissues is as a factor to be taken into account to fully understand the causes behind fish skeletal malformations.

To sum up, in our study, for vertebral column deformities (LD and LSK), the pattern of expression of genes related to ECM maturation and mineralization as *col1a1*, *op*, *ocn*, *mgp* and *tnap*, as well as those involved in bone resorption like *ctsk* and *mmp9*, seems to be key to discern between normal and potentially malformed fish. Contrarily, in OPC, DNT and JW malformations, different expression profiles had not been observed, making difficult to point to possible gene markers for these anomalies. Notwithstanding, besides having observed clear affectations in specific bones concomitantly to alterations in the relative expression of some genes involved in bone turnover, it would be necessary to analyse other batches of fish from different hatcheries, before drawing final conclusions, in addition to performing more detailed analyses to further characterize the different pathologies. Thus, more studies are necessary to confirm whether the gene expression profiles proposed can be markers of bone development to detect possible malformations to assure optimized production of fish, which would be of real interest for the aquaculture industry.

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## CONFLICT OF INTEREST

The authors have declared no conflict of interest.

## AUTHOR CONTRIBUTIONS

E.C. conceptualized the study; N.R-H., E.J.V., I.N. and E.C. performed the sampling; N.R-H. performed the laboratory analyses; N.R-H. and E.C. analysed and interpreted the data; J.G., I.N. and E.C. acquired funding; N.R-H., I.N. and E.C. drafted and critically reviewed the manuscript. All authors read and approved the final manuscript.

## DATA ACCESSIBILITY

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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