# Gilthead seabream (*Sparus aurata*) *in vitro* adipogenesis and its endocrine regulation by leptin, ghrelin, and insulin

***Catarina Basto-Silva*[a,b,*,1], *Sara Balbuena-Pecino*[c,1], *Aires Oliva-Teles*[a,b], *Natàlia Riera-Heredia*[c], *Isabel Navarro*[c], *Inês Guerreiro*[a,2], *Encarnación Capilla*[c,2]**

[a] CIIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, 4450-208 Matosinhos, Portugal

[b] FCUP - Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, Ed. FC4, 4169-007 Porto, Portugal

[c] Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain

[1] Shared first authorship

[2] Shared senior authorship

* Corresponding author:

Catarina Basto-Silva

bastosilva.c@gmail.com

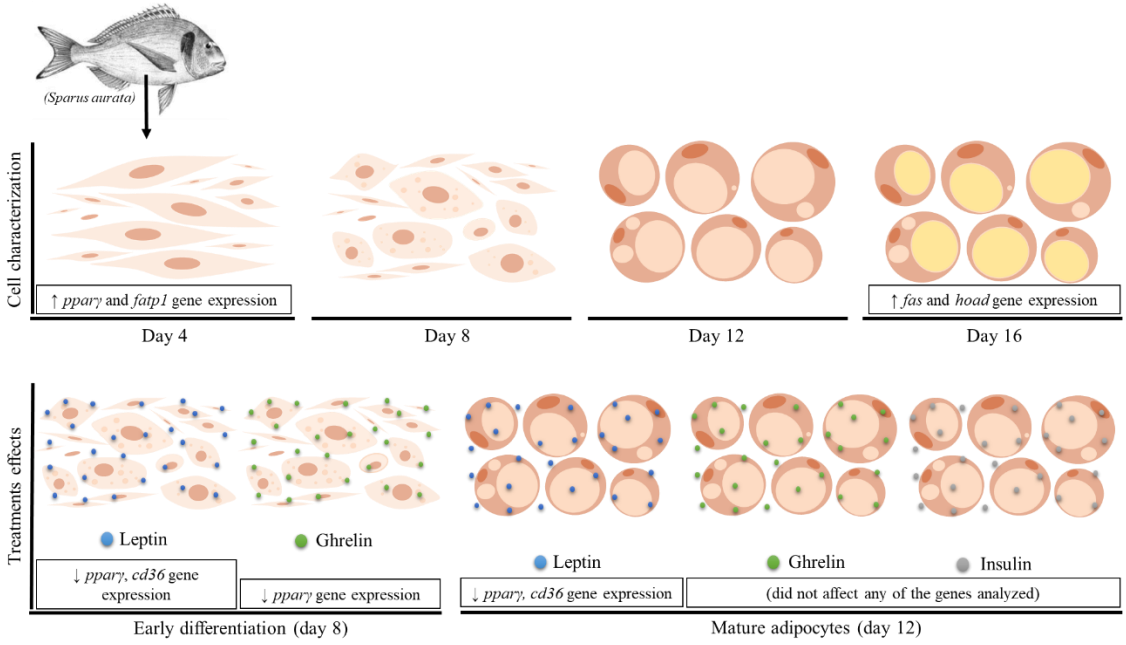
**Abstract**

Leptin, ghrelin, and insulin influence lipid metabolism and thus can directly affect adipose tissue characteristics, modulating the organoleptic quality of aquaculture fish. The present study explored gilthead seabream (*Sparus aurata*) cultured preadipocytes development, and the regulation of adipogenesis by those three hormones. Preadipocytes presented a fibroblast-like phenotype during the proliferation phase that changed to round-shaped with an enlarged cytoplasm filled with lipid droplets after complete differentiation, confirming the characteristics of mature adipocytes. *peroxisome proliferator-activated receptor-γ* (*pparγ*) expression was higher at the beginning of the culture, while *fatty acid synthase* and *3-hydroxyacyl-CoA dehydrogenase* gradually increased with cell maturation. The expression of *lipoprotein lipase-like*, *lysosomal acid lipase* (*lipa*), *fatty acid translocase/cluster of differentiation-36* (*cd36*), and *leptin receptor* (*lepr*) were not affected during cell culture development; and undetectable expression levels were observed for *leptin*. Concerning regulation, leptin~~, and ghrelin~~ inhibited lipid accumulation~~Leptin~~ significantly reduc~~ed~~ing *pparγ* and *cd36* gene expression, both in early differentiating and mature adipocytes, while ghrelin decreased the expression of *pparγ* ~~and *lipa* and increased *lepr* expression, but only~~ in the early differentiating phase but did not reduce intracellular lipid content significantly. Additional insulin past the onset of adipogenesis did not affect lipid accumulation either. In conclusion, at present culture conditions ~~both~~ leptin ~~and ghrelin~~ has~~ve~~ an anti-adipogenic function in differentiating preadipocytes of gilthead seabream~~. In mature adipocytes, leptin~~ and continues exerting ~~an anti-adipogenic~~this role in mature adipocytes, while ghrelin and insulin do not seem to influence adipogenesis progression. A better understanding of leptin, ghrelin, and insulin impact on the adipogenic process could help in the prevention of fat accumulation, improving aquaculture fish production and quality.

**Keywords**

Adipocytes characterization, adipose tissue, lipid metabolism, *pparγ*, *cd36*.

## Graphical Abstract



## 1. Introduction

In fish, t~~T~~he adipose tissue has an important role in whole-organism energy homeostasis, particularly in lipid metabolism, namely by regulating tissue lipogenesis, lipolysis, and β-oxidation (Salmerón, 2018). In mature adipocytes, lipogenesis converts fatty acids (FA) or other substrates (as glucose, amino acids or carbohydrates) from the diet into triglycerides (TG) for long-term storage until later use is required (Weil et al., 2013). During energy requirement periods, lipolysis and β-oxidation pathways are activated promoting the release of FA and glycerol into the blood from where they are captured by cells to provide energy for metabolic processes (Weil et al., 2013; Salmerón, 2018). The adipose tissue grows either by hypertrophy (increase in size by TG storage) and hyperplasia (i.e. adipogenesis), the later occurring by differentiation of precursor cells (Otto and Lane, 2005). The adipogenic process includes two main phases: (i) proliferation, where cells from the stromal vascular fraction divide and are committed to differentiate towards the adipocyte lineage, mainly through the coordination of ~~p~~Peroxisome proliferator-activated receptor-γ (~~p~~Pparγ) and CCAAT/enhancer-binding protein-α (~~c~~C/ebpα), and (ii) differentiation, in which those transcription factors promote the expression of characteristic proteins (as ~~l~~Lipoprotein lipase, ~~l~~Lpl, or ~~f~~Fatty acid translocase/cluster of differentiation 36, ~~c~~Cd36) involved in lipid uptake, transport, synthesis and storage of FA and subsequent adipokines secretion (Rosen and MacDougald, 2006; Salmerón, 2018).

Leptin and ghrelin are two hormones that mainly take part in appetite regulation, but also affect many other processes, such as lipid metabolism, in fish as in mammals (Kim et al., 2008; Liu et al., 2009; Salmerón et al., 2015). Leptin was already described in several fish species, for instance, orange-spotted grouper (*Epinephelus coioides*), pacu (*Piaractus mesopotamicus*) and rainbow trout (*Oncorhynchus mykiss*) (Murashita et al., 2008; Zhang et al., 2013; Volkoff et al., 2017), as being mainly produced in the liver, but also in other tissues, such as adipose tissue, stomach, and intestine (reviewed by Rønnestad et al., 2017). Leptin has been described as a satiety signal, anti-obesogenic hormone, and regulator of the liberation or storage of lipids from tissues (Copeland et al., 2011). In rainbow trout, *in vitro* leptin treatment stimulated lipolysis in adipocytes, supporting an anti-adipogenic role of this hormone (Salmerón et al., 2015). Similar results were

observed by Lu et al. (2012) and Song et al. (2015) in grass carp (*Ctenopharyngodon idellus*) and yellow catfish (*Pelteobagrus fulvidraco*), respectively, where leptin treatment stimulated both, hepatic lipolysis and β-oxidation, while inhibiting~~ed~~ lipogenesis. In both studies, leptin treatment promoted a release of glycerol, a reduction of hepatic lipid content, a decrease of *pparγ* gene and protein expression, and an upregulation of key β-oxidation-related genes, such as *pparα*, and *carnitine palmitoyl transferase-1* (*cpt-1*).

Ghrelin is mainly expressed in the stomach but also in the gastrointestinal tract, pancreas, heart, and hypothalamus, and seems to act mainly as a hunger signal, although differences exist between fish species (Jönsson, 2013; Perelló-Amorós et al., 2018; Bertucci et al., 2019). These authors suggested that ghrelin has species-specific functions in fish, not only in appetite regulation but also concerning other metabolic responses; however, available data regarding its effects on lipid metabolism are still scarce and contradictory. In rainbow trout adipocytes, ghrelin treatment seemed to activate lipid turnover, stimulating the synthesis of TG (i.e. lipogenesis), their mobilization and use (Salmerón et al., 2015), while in Mozambique tilapia (*Oreochromis mossambicus*) long-term ghrelin treatment with micro-osmotic pumps increased liver and muscle total fat content (Riley et al., 2005). ~~Contrarily~~Differently, in *in vivo* studies with rainbow trout and brown trout (*Salmo trutta*), ghrelin did not affect lipid metabolism or deposition (Jönsson et al., 2010; Tinoco et al., 2014).

Insulin acts as a growth promoter and affects lipid metabolism by inducing adipocytes differentiation and increasing adipose fat stores in red sea bream (*Pagrus major*), Atlantic salmon (*Salmo salar*), and large yellow croaker (*Pseudosciaena crocea* R.) (Oku et al., 2006; Sánchez-Gurmaches et al., 2011; Wang et al., 2012). Insulin promoted rainbow trout preadipocyte differentiation and stimulated *lpl* gene expression in proliferating and freshly isolated adipocytes of the same species (Bouraoui et al., 2012; Cruz-Garcia et al. 2015). However, insulin did not seem to increase lipid accumulation during the differentiation phase on rainbow trout (Salmerón et al., 2015). On the other hand, insulin injection promoted *lpl* gene expression in gilthead seabream (*Sparus aurata*) adipose tissue, suggesting also an adipogenic role of insulin in this species (Albalat et al., 2007). Consistently, insulin induced lipid accumulation in primary cultured

preadipocytes of gilthead seabream, as it does in rainbow trout, which suggests that insulin can trigger the process of differentiation of adipocytes also in sparids (Bouraoui et al., 2008; Salmerón et al., 2013).

Gilthead seabream represents about 7% of all marine fish produced in the world (FIGIS, 2019), and has an important economic value for Mediterranean aquaculture. Since hormonal factors, like ghrelin, leptin, and insulin, influence lipid metabolism in a species-specific manner, it is of utmost importance to have a better understanding of their effects on adipocyte cells of gilthead seabream, as this may influence adipose tissue characteristics and consequently hamper fish quality, by affecting both carcass and fillet yields, and organoleptic parameters. Moreover, understanding and increasing knowledge on fish adipose tissue biology has great scientific interest. Thus, the present study aims to contribute to the characterization of adipogenesis and the evaluation of leptin, ghrelin, and insulin effects in the adipogenic process using an *in vitro* primary cell culture model of gilthead seabream preadipocytes.

## 2. Material and methods

### *2.1. Fish maintenance and ethics statement*

Gilthead seabream (*Sparus aurata*) juveniles of approximately 30 g body weight were obtained from Piscimar S.L. (Burriana, Castellón, Spain) and maintained at the animal facilities of the Faculty of Biology at the University of Barcelona (Spain). Fish were kept in 0.4 $m^3$ tanks in a temperature-controlled seawater recirculation system at 23 ± 1ºC, salinity of 36 ± 1 g $L^{-1}$, dissolved oxygen kept near saturation, and a 12 h light/12 h dark photoperiod. Fish were fed *ad libitum* twice daily with a commercial diet (OptiBream, Skretting, Burgos, Spain), and fasted 24 h before performing the cell cultures to avoid contamination from the gastrointestinal tract. Before adipose tissue extraction, fish were anesthetized (MS-222, 0.1 g $L^{-1}$) and subsequently sacrificed by cranial concussion. All animal handling procedures were done by accredited scientists (following FELASA category C recommendations) and approved by the Ethics and Animal Care Committee of the University of Barcelona (certification number CEEA OB34/17), following the European Union, Spanish, and Catalan government-established norms and procedures.

## 2.2. Gilthead seabream cultured preadipocytes: characterization and endocrine regulation

### 2.2.1. Establishment of the preadipocyte primary culture

The establishment of the preadipocyte primary cultures followed the procedure described by Salmerón et al. (2013). For each culture, 6 to 9 gilthead seabream juveniles were used, collecting a pool of 3 g of visceral adipose tissue. In fact, pooling adipose tissue samples from different animals allows to obtain sufficient and homogeneus preparations of precursor cells, not biased by a particular individual condition, to perform at once all the experimental ~~conditions~~ treatments for them to be comparable. Briefly, the extracted tissue was first washed and minced with Krebs-HEPES buffer (pH 7.4) with 1% antibiotic/antimycotic solution and digested for 1 h with type II collagenase (130 UI $mL^{-1}$) in Krebs-HEPES buffer plus 1% BSA at 18ºC with gentle agitation. Next, the cell suspension was filtered through a 100 µm cell strainer, centrifuged (1500 rpm, 10 min) to get rid of mature adipocytes, and the obtained pelleted cells were counted using a Neubauer chamber. Cells were seeded in 1% gelatin-treated 6- or 12-well plates at a final density of $4.3\times10^4$ cells/$cm^2$ in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% of antibiotic/antimycotic solution and 60 mM NaCl (growth medium, GM), and incubated at 23ºC with 2.5% $CO_2$. The medium was changed every 2 days during the whole experiment.

### 2.2.2. Cell culture development characterization

The primary cultured preadipocytes were maintained during 16 days as described in Salmerón et al. (2013), first cultured in GM and then, on day 8, the medium was replaced by a differentiation medium (DM), composed by GM plus 10 µg $mL^{-1}$ porcine insulin (corresponding to 1700 nM), 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) and 0.25 µM dexamethasone. To properly induce adipocyte maturation, 5 µL $mL^{-1}$ of lipid mixture (4.5 mg $ml^{-1}$ cholesterol, 10~~0~~ mg $ml^{-1}$ cod liver oil fatty acids (methyl esters), 25 ~~0~~ mg $ml^{-1}$ polyoxyethylene sorbitan monooleate and 2.~~9~~0 mg $ml^{-1}$ D-α-tocopherol actetate) (L5146, Sigma) were also added to the DM. Four days after

induction of differentiation the medium was changed to GM plus lipid mixture (5 µL $mL^{-1}$) and the cells were maintained on it until the end of the experiment. During the development of the cells, representative images were taken at different times with an Axiovert 40C inverted microscope (Carl Zeiss, Germany) coupled to a Canon EOS 1000D digital camera (Tokyo, Japan). For gene expression characterization, preadipocyte samples were collected at days 4 and 8 (i.e. before the induction of differentiation), and at days 12 and 16 (i.e. mature adipocytes). After being washed with phosphate-buffered saline (PBS), cell samples of two duplicate wells of the 6 well-plates were collected with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) using a cell scraper, transferred to an RNase-free polypropylene tube, and kept at −80°C until performing RNA extraction, as described in 2.4.1. Results are the average of 7 independent adipocyte ~~isolations~~ cultures (n=7).

*2.2.3. Endocrine regulation of adipocytes differentiation*

For the evaluation of the endocrine regulation of the adipogenic process, cells were stimulated at two moments. First, preadipocytes at day 8 were induced to differentiate with DM containing lipid mixture (5 µL $mL^{-1}$), leptin (100 nM), or ghrelin (10 nM). Second, adipocytes at day 12 were treated with GM plus lipid mixture (5 µL $mL^{-1}$), leptin (100 nM), ghrelin (10 nM), or insulin (1000 nM). Insulin was only tested at day 12, since this hormone is *per se* already included in the cocktail used for differentiation at a concentration of 1700 nM (i.e. DM), and thus an additional 1000 nM would not have make a difference according to previous data (Bouraoui et al., 2012). But in fact, it is well-kwown that insulin enhances fish adipocytes differentiation by itself, as reported by several authors (Oku et al., 2006; Sánchez-Gurmaches et al., 2011; Wang et al., 2012). The recombinant rainbow trout leptin (29% of sequence identity with gilthead seabream) used was a kind gift of Dr. Ivar Rønnestad (University of Bergen, Norway), who produced it following the procedure described in Murashita et al. (2008). The synthetic 20 amino-acid octanoylated rainbow trout ghrelin (80% of sequence identity with gilthead seabream) used was a kind gift of Dr. Elisabeth Jönsson (University of Gothenburg, Sweden), who obtained it from the Peptide Institute Inc., Osaka (Japan). The porcine insulin (67% and 88% of sequence identity of insulin

chains A and B respectively, with gilthead seabream sequences) was obtained from Sigma. In all cases, identity of leptin, ghrelin and insulin was verified by Blast and BlastP searches, and the concentrations used were chosen based on previous literature (Salmerón et al., 2015). The DM or GM plus lipid mixture treatments at days 8 and 12, respectively, were used as positive controls since they represent the standard culturing procedure. Six hours after being subjected to the treatments, cells were washed with PBS and, for each condition, two duplicate wells of the 6 well-plates were collected with 1 mL of TRI Reagent Solution using a cell scraper, transferred to an RNase-free polypropylene tube, and kept at −80°C until performing gene expression analyses. Cell samples were obtained from 7 independent experimental adipocyte ~~isolations~~ cultures (n=7). Furthermore, in parallel 12-well plates, cells at day 8 were treated for 72 h with DM or DM plus leptin (100 nM), ghrelin (10 nM), insulin (1000 nM), or lipid mixture (5 µL $mL^{-1}$), as a positive control to evaluate lipid accumulation by Oil Red O (ORO) staining. To corroborate the pro-adipogenic effect of lipid mixture in the current experimental conditions, cells maintained only in DM were used as a negative control. Six independent adipocyte ~~isolations~~ cultures (n=6) were performed.

### *2.3. Oil Red O Staining*

To evaluate leptin, ghrelin, and insulin effects in adipocyte differentiation, after each treatment cells were stained with ORO (O0625, Sigma) as described by Capilla et al. (2011). Cells were fixed with 10% formalin for 1 h and stained with 0.3% ORO diluted in 36% triethyl-phosphate for 2 h. After washing excessive dye with distilled water, representative images of the development of the cells were obtained using an Axiovert 40C inverted microscope coupled to a Canon EOS 1000D digital camera. Then, quantification of the lipid content was done by extraction of the lipids with 2-propanol for 30 min and reading the absorbance at 490 nm in duplicate 96-wells (Tecan Infinite M200, Switzerland). For total protein extraction, cells were then washed with distilled water, stained with Comassie brilliant blue G-250 dye for 1 h, and incubated at 60ºC with 85% propylene glycol (398039, Sigma) during 1 h. Quantification of total protein was obtained from the absorbance measured at 630 nm in duplicate 96-wells using the

same microplate reader. Final TG quantification was calculated as the quotient of the absorbances measured at 490 nm and at 630 nm.

### *2.4. Gene expression*

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

RNA extraction followed the TRI Reagent Solution manufacturer's instructions (Applied Biosystems, Alcobendas, Spain). Total RNA concentration and purity were determined in a NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain). Four-hundred fifty ng of total RNA were used for cDNA synthesis using DNase I enzyme (Life Technologies, Alcobendas, Spain) to remove all genomic DNA, and Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) according to the manufacturer's recommendations. Samples were stored at -20ºC until used.

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

qPCR analyses followed the requirements of MIQE guidelines (Bustin et al., 2009) and were performed in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). All samples were analyzed in duplicate, by adding 2.5 µL of iTaq Universal SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers (Table 1), 1 µL of each cDNA sample at the appropriate dilution, and autoclaved water until a final volume of 5 µL. The qPCR reactions included the activation step (1 cycle of 3 min at 95ºC; followed by 40 cycles of 10 s at 95ºC and 30 s at primer melting temperature); and the amplicon dissociation step (increasing temperature by 0.5ºC every 30 s from 55 to 95ºC). The appropriate cDNA dilution, primers efficiency, and absence of primer-dimers were determined by a dilution curve with a pool of samples. *Ribosomal protein l27* (*rpl27*) and *β-actin* were selected as reference genes since they did not show significant differences between groups ($P>0.05$), and relative expression was calculated following the Pfaffl (2001) method.

### *2.5. Statistical analysis*

All data are presented as mean ± standard error (SE). Data were tested for normality by the Shapiro-Wilk test and homogeneity of variances by the Levene's test. When normality was not verified data were log-transformed. Data on gene profile characterization were analyzed by one-way ANOVA, followed by Tukey's test to determine differences between means. Hormone (leptin, ghrelin, and insulin) effects were assessed ~~with~~ by one-way ANOVA, followed by Dunnett's test ~~Student's *t* test~~. The lipid accumulation effects on gilthead seabream adipocyte cells were evaluated comparing each treatment with the negative control and, the gene expression data were evaluated using the lipid treatment as the positive control. A statistical significance of $P<0.05$ was set for all the statistical tests performed. All statistical analyses were carried out using SPSS 25 software package for Windows (IBM® SPSS® Statistics, New York, USA).

## 3. Results

### *3.1. Characterization of preadipocyte cell culture development*

On day 4 (Fig. 1A), preadipocyte cells showed a triangular fibroblastic shape that became increasingly elongated by day 8 (Fig. 1B). After DM addition, the differentiating adipocyte cells acquired a rounded shape (Fig. 1C) and, its continuous growth promoted the enlargement of the cytoplasm, where lipid droplets could be found, characteristic of a mature adipocyte (Fig. 1D). Concerning transcriptional characterization, the expression of the key adipogenic factor *pparγ* was significantly higher at day 4 of culture development when compared with all other days (Table 2). Similarly, the gene expression of the *fatty acid transport protein 1* (*fatp1*) was higher at the beginning of the culture, at day 4 compared to day 8; whereas on the contrary, *fatty acid synthase* (*fas*) and *3-hydroxyacyl-CoA dehydrogenase* (*hoad*) gene expression significantly increased with adipocyte differentiation. The gene expression of *lipoprotein lipase-like* (*lpl-lk*), *lysosomal acid lipase* (*lipa*), *cd36*, and *leptin receptor* (*lepr*) were not affected during cell culture development. Undetectable levels of expression were observed for *leptin* throughout the whole adipogenic process.

### *3.2. Leptin, ghrelin, and insulin effects on adipocyte differentiation*

Lipid accumulation in adipocyte cells measured using ORO staining was significantly inhibited by leptin ~~and ghrelin~~ treatment~~s~~ (Fig. 2C~~, D~~, F), while ghrelin and insulin treatments had no effect (Fig. 2D, E, F) when compared to the negative control cells, induced to differentiate only with the DM, containing the usual hormonal cocktail but not lipid mixture (Fig. 2A, F). The addition of lipid mixture to the DM consistently promoted the highest lipid accumulation on adipocyte cells (Fig. 2B, F), confirming its effectiveness as a positive control.

During the initial preadipocyte differentiation (at day 8), leptin promoted a decrease in *pparγ* and *cd36* gene expression, while ghrelin also downregulated *pparγ* ~~and *lipa*~~ expression ~~and increased *lepr*~~ (Table 3). The mRNA levels of all other genes analyzed (namely *fas*, *lpl-lk*, *lipa*, *hoad*, ~~and~~ *fatp1*, and *lepr*) were not affected by any of the hormonal treatments.

When the hormonal treatments were applied in more advanced stages of adipocyte differentiation (at day 12), leptin also caused a decrease of both *pparγ* and *cd36* transcript levels, while ghrelin and insulin did not further affect any of the genes analyzed (Table 4).

## 4. Discussion

In the present study, the morphological changes of adipocytes during culture development followed the same pattern previously reported by Salmerón et al. (2013) for primary cultured preadipocytes of the same species. Namely, with preadipocyte cells showing a fibroblast appearance during the proliferation phase, and mature adipocytes presenting a rounded shape and a larger cytoplasm with lipids accumulated after complete differentiation. Similar morphological evolution was also reported for cultured adipocytes of other fish species like Atlantic salmon (Vegusdal et al., 2003), rainbow trout (Bouraoui et al., 2008), large yellow croaker (Wang et al., 2012), and grass carp (Liu et al., 2015).

The transcriptional characterization during gilthead seabream *in vitro* adipogenesis initiated in Salmerón et al. (2016) has been extended in the present study. As previously reported, the key transcription factor of adipogenesis, *pparγ*, showed higher gene expression during the cell proliferation phase, evidencing its importance only up to the onset of adipocyte differentiation (Salmerón et al., 2016). However, in other fish species, such as Atlantic salmon, rainbow trout,

and large yellow croaker, *pparγ* gene expression seemed to be longer promoted during the adipocyte differentiation process (Vegusdal et al., 2003; Bouraoui et al., 2008; Wang et al., 2012), while in red sea bream *pparγ* gene expression was not affected during adipogenesis (Oku and Umino, 2008). Nevertheless, also in rainbow trout, a recent detailed analysis during the early differentiation phase (days 7 to 11) showed a *pparγ* expression profile similar to the current one, with a transient upregulation and a subsequent abrupt decrease within 24 h after induction of differentiation by the addition of a DM (Riera-Heredia et al., unpublished observations). Thus, Ppparγ seems to have a critical role in early adipogenesis, but more studies should be done for a better understanding of its specific function during this process in fish.

Similar to what was previously observed in red sea bream and grass carp (Oku and Umino, 2008; Liu et al., 2015), in the present study *fas* gene expression increased during adipogenesis. This was expected, since in the adipocytes Ffas participates in *de novo* lipogenesis for fat storage (Wang et al., 2012). However, in our previous study in gilthead seabream, *fas* gene expression gradually decreased during adipocyte differentiation (Salmerón et al., 2016), suggesting a negative feedback mechanism, due to the high availability of FA in the culture medium. Such negative feedback was also shown in Atlantic salmon preadipocytes treated with palmitic acid (Bou et al., 2016). In that study, it was observed a decrease of aAcetyl-CoA carboxylase expression, and consequently in the malonyl-CoA production needed for palmitate synthesis through fas action. Although in the present study such negative feedback was not detected, at least regarding *fas* expression, in primary fetal rat calvarial cultured cells, palmitate treatment reduced the expression of *pparγ* (Yeh et al., 2014), which could explain the observed decrease in *pparγ* gene expression in the present study. In fact, the upregulation of *fas* expression may lead to increased production of palmitate, which in turn might cause a reduction in *pparγ* gene expression.

The lLpl is a key enzyme involved in lipid deposition and metabolism and has been recognized as a lipogenesis marker, being an indicator of preadipocytes differentiation (Weil et al., 2013). In previous studies, *lpl* gene expression increased during adipocytes differentiation in Atlantic salmon (Todorčević et al., 2008), rainbow trout (Bouraoui et al., 2012), large yellow croaker (Wang et al., 2012), and grass carp (Liu et al., 2015). Previously, also in gilthead seabream, *lpl*

expression gradually increased during adipocytes differentiation, although a major decrease was observed during the proliferation phase and upon adipogenic induction (Salmerón et al., 2016). In the present study, the gene expression of *lpl-lk* was was also evaluated. Lpl-lk is an exclusive fish lineage isoform of Ll~~l~~pl, that was found in zebrafish (*Danio rerio*), tuna (*Thunnus orientalis*), and red sea bream, in addition to gilthead seabream (Benedito-Palos et al., 2013). The correlation between Ll~~l~~pl and Ll~~l~~pl-lk metabolic regulation seems to be tissue-specific. While in skeletal muscle *lpl* and *lpl-lk* had different expression responses (Benedito-Palos et al., 2013), in the liver both lipases were up-regulated in fasted fish in comparison to fed fish (Benedito-Palos et al., 2014). In the present study, *lpl-lk* mRNA levels were not affected by cell development, suggesting a different regulation for both isoforms in these conditions. Nonetheless, *lpl* and *lpl-lk* gene expression patterns and specific functions during adipocyte development in gilthead seabream still need to be better elucidated.

Lipa is essential for TG hydrolysis in lysosomes (Du et al., 2001); however, its effects in fish adipogenesis remain unclear. In *lipa*-deficient adult mice, a significant reduction of white and brown adipose tissues was observed, suggesting that this enzyme has important roles in adipocyte differentiation, lipid metabolism or fat mobilization (Du et al., 2001). However, data of the present study indicated that *lipa* gene expression did not change during adipocytes development, suggesting that this enzyme may not participate in the adipogenic pathway, at least in the cell culture times studied.

In Atlantic salmon, *aAcyl-~~C~~coA dehydrogenase* expression, an enzyme involved in mitochondrial β-oxidation, decreased at later stages of adipocyte differentiation (Todorčević et al., 2008), leading the authors to conclude that preadipocytes have a higher capacity for FA β-oxidation, while mature cells are more specialized in lipid storage. However, in the present study the gene expression of *hoad*, another enzyme involved in mitochondrial β-oxidation, increased during adipocyte culture development. In agreement with these observations, ~~h~~Hoad presence in adipose tissue was also reported for a few fish species, including gilthead seabream (Polakof et al., 2011; Bou et al., 2017; Sánchez-Moya et al., 2020), suggesting that it may have an important role both in adipogenesis and fully mature adipocytes.

The gene expression during adipocyte development of two membrane-associated FA transporters: *fatp1* and *cd36*, was also analyzed. According to Sánchez-Gurmaches et al. (2012), in rainbow trout Ff~~f~~atp1 is mainly produced in the adipose tissue, while ~~c~~Cd36 is expressed at a higher level in the liver although it is also expressed in the adipose tissue. Both, in Atlantic salmon and rainbow trout adipocytes, *fatp1* transcript levels were induced during adipogenesis, in parallel to lipid accumulation (Todorčević et al., 2008; Sánchez-Gurmaches et al., 2012), whereas *cd36* expression was not affected along the process (Sánchez-Gurmaches et al., 2012). Similarly, in the present study, *cd36* gene expression remained unaltered during adipocyte differentiation; however, differently to what was observed in previous studies, *fatp1* gene expression decreased during adipogenesis. This seems to indicate that differences may exist between species in the regulation of FA transporters expression throughout cell differentiation, which is in agreement with the complex regulation of these transporters in fish (Sánchez-Gurmaches et al., 2011; 2012). Although Vegusdal et al. (2003) and Salmerón et al. (2015) described an increase of *leptin* expression during adipocyte cell differentiation in Atlantic salmon and rainbow trout, respectively, in the present study, undetectable expression levels were observed for *leptin* during *in vitro* development of gilthead seabream adipocytes. Similar results were also found *in vivo* in the same species (Basto-Silva et al. ~~in preparation~~unpublished observations), where *leptin* expression in the adipose tissue was not detected, suggesting that leptin may be none or poorly produced by gilthead seabream adipocytes, although in the same study, *leptin* mRNA was detected in brain and liver. Indeed, while in mammals the adipose tissue is the major producer of leptin (Harris, 2014), in fish, leptin is mainly expressed and produced in the liver (Zhang et al., 2013; Volkoff et al., 2017).

Nonetheless, the presence of a lepr in the adipose tissue was already reported for a few fish species, such as Atlantic salmon (Rønnestad et al., 2010), rainbow trout (Gong et al., 2013), orange-spotted grouper (Zhang et al., 2013), and Nile tilapia (*Oreochromis niloticus*) (Shpilman et al., 2014). The present study confirmed, for the first time in gilthead seabream adipocyte cells, the expression of a *lepr*. Although, Chisada et al. (2014) suggested that this hormone modulates lipogenesis in adult medaka (*Oryzias latipes*), the lepr relevance during adipogenesis is not

completely understood for gilthead seabream. In the present study, *lepr* expression was unaltered during adipocyte differentiation and, mRNA levels of *leptin* were undetectable, raising doubts about the regulation of seabream adipose tissue growth and metabolism by leptin. ~~n~~Nevertheless, as previously mentioned, in another *in vivo* trial from our group also in gilthead seabream (Basto-Silva et al., unpublished observations), although *leptin* was neither detected in the adipose tissue, maybe due to very low levels of expression, it was found in brain and liver~~its sole presence~~, supporting~~s the possible existence of autocrine or paracrine~~ a role for leptin ~~regulation~~ in adipocytes regulation.

Concerning the endocrine regulation of the adipogenic process, in the present study leptin treatment significantly reduced *pparγ* and *cd36* gene expression, both in early differentiating and mature adipocytes, suggesting an anti-adipogenic role of this hormone. These data are also supported by the lower accumulation of lipids in the leptin-treated gilthead seabream cells. Similarly, leptin treatment reduced intracellular TG content and *pparγ* gene expression in yellow catfish hepatocytes (Song et al., 2015) and decreased *lpl* and *fatp1* gene expression during rainbow trout adipocytes differentiation (Salmerón et al., 2015). Although in the present study a trend was also noticed for a decrease in *lpl* and *fatp1* gene expression, due to the high variability between samples this decrease was not statistically significant. These results are in agreement with the anti-adipogenic and anti-obesogenic actions of leptin described in mammals (Friedman and Halaas, 1998). Also in fish, intracerebroventricular and intraperitoneal injections of leptin inhibited feed intake (Murashita et al., 2008; Won et al., 2012), suggesting a decrease of energy intake which in turn could be converted into adipose tissue.

In rainbow trout, ghrelin seemed to influence adipogenesis, promoting simultaneously the synthesis of TG and their mobilization into adipocytes, accelerating lipid turnover (Salmerón et al., 2015). Similar results were observed in Mozambique tilapia, where long-term ghrelin treatment with micro-osmotic pumps promoted an increase of liver and muscle lipid content (Riley et al., 2005). However, different results were obtained in previous *in vivo* studies in rainbow and brown trout. In rainbow trout, Jönsson et al. (2010) did not observe significant differences in mesenteric adipose stores and liver or muscle lipid content between the control and

412 the ghrelin-treated fish after a 14-days treatment period. In brown trout, a ghrelin intraperitoneal
413 injection did not affect lipid metabolism or deposition, since the hepatosomatic index, TG content
414 and Ll~~l~~pl activity in liver and muscle were not affected when compared with control fish (Tinoco
415 et al., 2014). In the present study, although ghrelin treatment significantly decreased the gene
416 expression of the key adipogenic transcription factor *pparγ* ~~and *lipa* gene expression~~ in gilthead
417 seabream preadipocytes, significant effects on ~~together with the lower~~ lipid accumulation during
418 the differentiation phase, compared to the control condition were not observed. Moreover, the
419 lack of significant effects on the expression of any of the genes analyzed in mature cultured
420 adipocytes, suggested that ghrelin does not affect adipogenesis progression in this species~~its~~
421 ~~regulatory effect might only be of relevance at an early stage~~. Notwithstanding, further studies
422 would be required to confirm this hypothesis as ~~G~~ghrelin effects on adipogenesis remain
423 controversial, both in fish and in mammals, since its effect appears to be influenced not only by
424 the life cycle phase of the adipocytes, but also by the ghrelin concentration applied. For instance,
425 in a mouse 3T3-L1 preadipocyte line, a $10^{-6}$ M ghrelin treatment inhibited differentiation but
426 promoted the proliferation step (Zhang et al., 2004), while a $10^{-7}$ M to $10^{-15}$ M ghrelin treatment
427 induced both proliferation and differentiation (Liu et al., 2009).
428 In fish, as in mammals (Géloën et al., 1989; Zhou et al., 2009), insulin promotes lipid
429 accumulation and adipogenesis-related genes expression during differentiation in several species,
430 such as red sea bream, Atlantic salmon, or large yellow croaker (Oku et al., 2006; Sánchez-
431 Gurmaches et al., 2011; Wang et al., 2012). However, in the present study, lipid accumulation
432 and the differentiation step were not affected when additional 1000 nM insulin was added to the
433 cells, which were already exposed to 1700 nM insulin present in the DM hormonal cocktail.
434 Similar results were also reported in this species by Salmerón et al. (2013), which concluded that
435 the differentiation could be triggered by insulin, but once switched on by a DM containing
436 hormones and a lipid mixture, insulin did not further induce lipid synthesis and accumulation.
437 Accordingly, Bouraoui et al. (2012) also reported in rainbow trout adipocytes that the extra
438 addition of a 1 µM insulin did not affect *lpl* gene expression nor lipid content levels. Despite this,
439 in the same study, a combination of 1 µM insulin plus 1 µM troglitazone, an anti-diabetic agent

that enhances insulin sensitivity, increased the lipid content in the cells, leading the authors to conclude that the combination of various adipogenic factors can lead to an optimal medium to induce adipocyte differentiation in rainbow trout. Thus, a better understanding of the influence of insulin in the adipogenic process, as well as its interactions with other factors, may help to understand the mechanisms of fish adipose tissue growth.

## 5. Conclusions

*In vitro* cultured preadipocytes and mature adipocytes of gilthead seabream exhibited a normal morphological evolution. The gene expression of *pparγ*, *fas*, *hoad*, and *fatp1* was affected during culture development, while *lpl-lk*, *lipa*, *cd36*, and *lepr* remained unaltered. ~~Both, l~~Leptin ~~and ghrelin~~ appeared to have an anti-adipogenic function~~s~~ in gilthead seabream differentiating preadipocytes while ghrelin had a minor effect only downregulating *pparγ*. In mature adipocytes, leptin seemed to continue exerting its anti-adipogenic role, while ghrelin and insulin did not further affect adipogenesis progression. Notwithstanding, a better understanding of leptin, ghrelin, and insulin influences in the adipogenic process, either in this as in other species, could help the prevention of fat accumulation, improving aquaculture fish production and quality.

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## Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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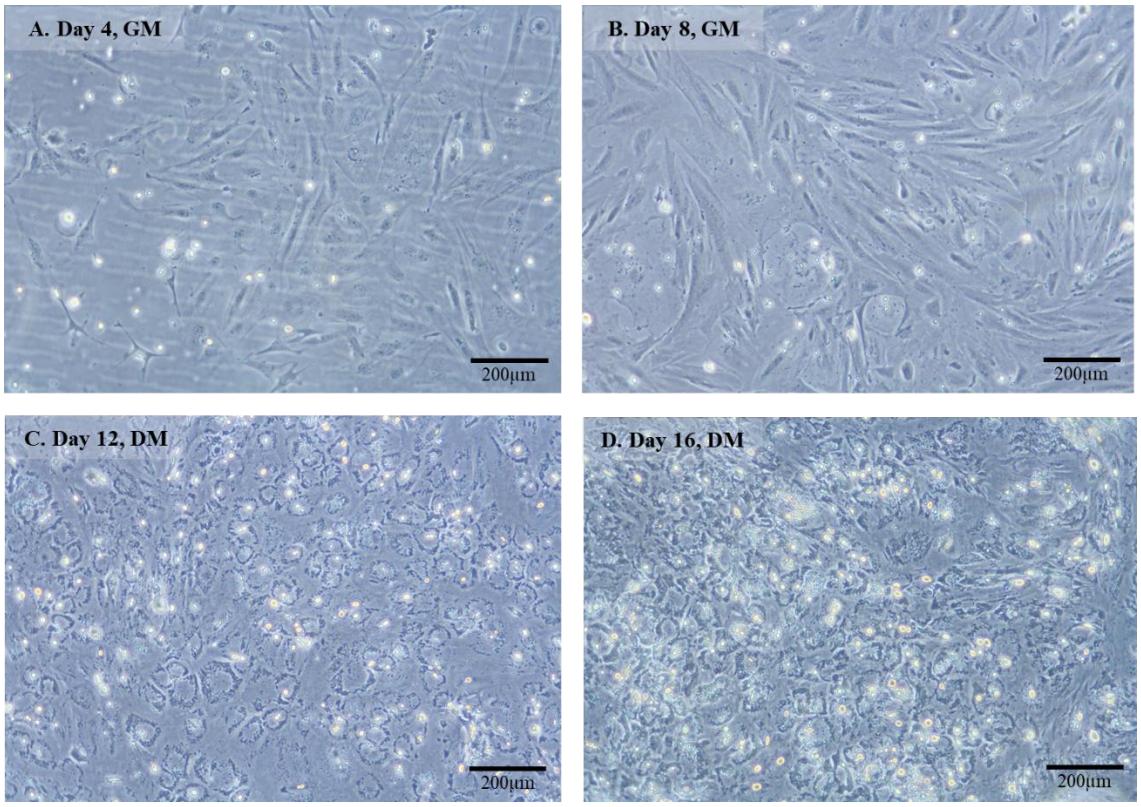
643 **Figures and tables**




644

645 **Figure 1.** Representative phase-contrast images of gilthead seabream preadipocyte cells growing
646 in growth medium (GM), at day 4 (**A**) and day 8 (**B**); and adipocytes in differentiation medium
647 (DM), at day 12 (**C**) and day 16 (**D**). Magnification 10x.

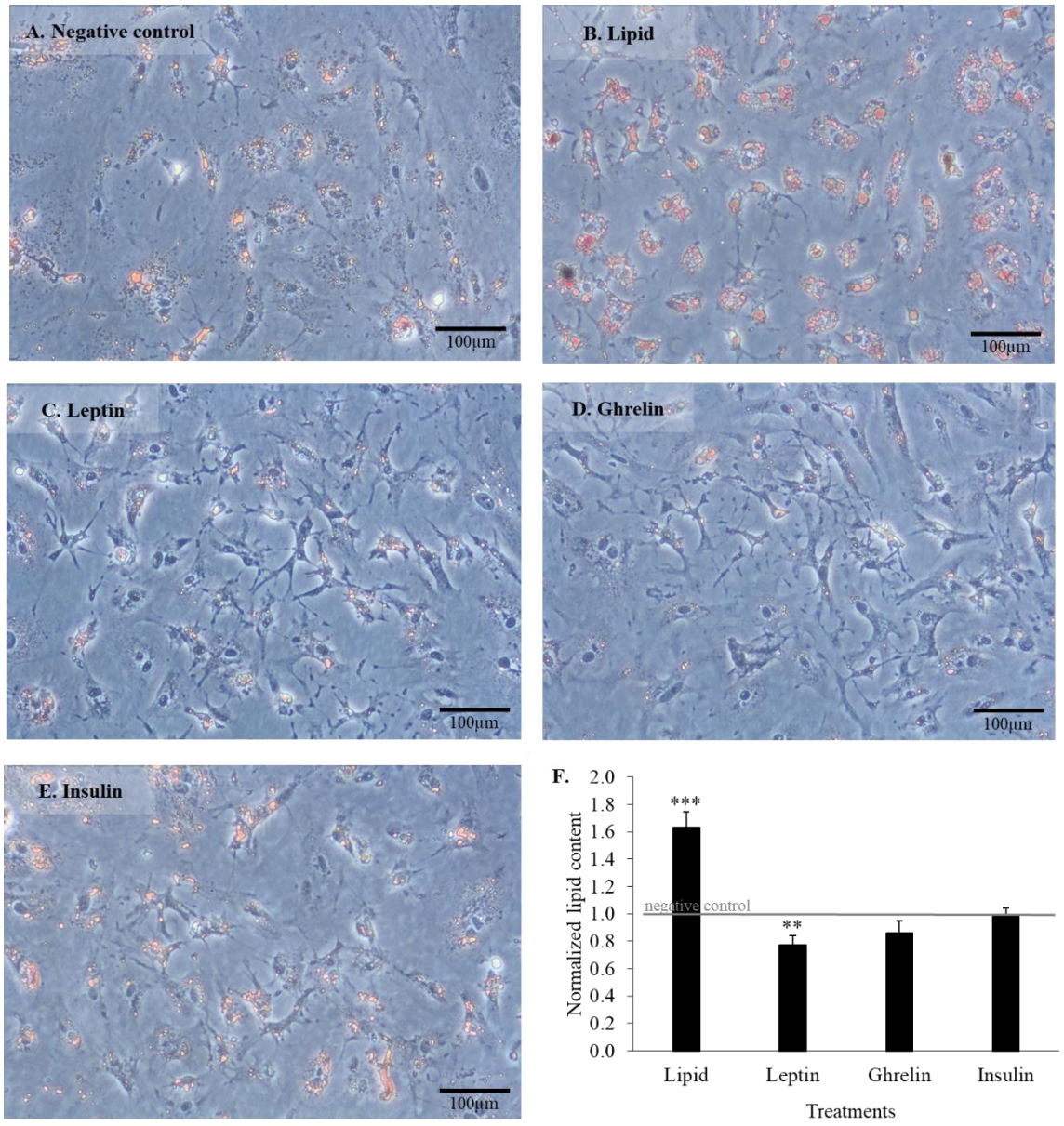



**Figure 2.** Lipid, leptin, ghrelin, and insulin effects on lipid accumulation in gilthead seabream adipocyte cells. Representative phase-contrast images of gilthead seabream adipocyte cells treated at day 8 with only differentiation medium (DM) as negative control (**A**), 5 µL $mL^{-1}$ lipid mixture (**B**) 100 nM leptin (**C**), 10 nM ghrelin (**D**), or 1000 nM insulin (**E**) for 72 h and stained with Oil red O. Magnification 20x. (**F**) Quantification of lipid content normalized by protein content and expressed as fold change respect to the negative control treatment (grey line). Data are presented as means (n = 6) and standard error (SE). Results were analyzed by one-way ANOVA, followed by Dunnett's test. Significant differences between the negative control and each one of the treatments tested are indicated by **P ≤ 0.051; ***P ≤ 0.001.

658 **Table 1.** Genes and primers used for qPCR.

| Gene | Sequence (5´- 3´) | Accession nº | Tm (ºC) | Efficiency (%) |
|---|---|---|---|---|
| *Transcription factor* | | | | |
| *pparγ* | **F:** CGCCGTGGACCTGTCAGAGC<br>**R:** GGAATGGATGGAGGAGGAGGAGATGG | AY590304 | 66 | 97.9 |
| *Lipogenesis markers* | | | | |
| *fas* | **F:** TGGCAGCATACACACAGACC<br>**R:** CACACAGGGCTTCAGTTTCA | AM952430 | 60 | 97.0 |
| *lpl-lk* | **F:** CAGAGATGGAGCCGTCACTCAC<br>**R:** TCTGTCACCAGCAGGAACGAATG | JQ390609 | 60 | 93.0 |
| *Lipolysis marker* | | | | |
| *lipa* | **F:** TACTACATCGGACACTCTCAAGGAAC<br>**R:** GTGGAGAACGCTATGAATGCTATCG | JQ308831 | 60 | 94.0 |
| *β-oxidation marker* | | | | |
| *hoad* | **F:** GAACCTCAGCAACAAGCCAAGAG<br>**R:** CTAAGAGGCGGTTGACAATGAATCC | JQ308829 | 60 | 95.3 |
| *Fatty acid transporters* | | | | |
| *cd36* | **F:** GTCGTGGCTCAAGTCTTCCA<br>**R:** TTTCCCGTGGCCTGTATTCC | Riera-Heredia et al. (2019) | 60 | 94.0 |
| *fatp1* | **F:** CAACAGAGGTGGAGGGCATT<br>**R:** GGGGAGATACGCAGGAACAC | Riera-Heredia et al. (2019) | 60 | 102.0 |
| *Appetite regulation-related* | | | | |

| | | | | |
|---|---|---|---|---|
| *leptin* | **F:** TCTCTTCGCTGTCTGGATTCCTGGAT<br>**R:** CTCCTTCTTGCTCTGTAGCTCTT | KP822924 | 60 | - |
| *lepr* | **F:** GGCGGAACTGATTCTACTCTG<br>**R:** AGTATCGGACCTCGTATCTCA | MG570178 | 60 | 111.0 |
| *Reference genes* | | | | |
| *β-actin* | **F:** TCCTGCGGAATCCATGAGA<br>**R:** GACGTCGCACTTCATGATGCT | X89920 | 60 | 102.0 |
| *rpl27* | **F:** AAGAGGAACACAACTCACTGCCCCAC<br>**R:** GCTTGCCTTTGCCCAGAACTTTGTAG | AY188520 | 68 | 100.2 |
| *ef1α* | **F:** CTTCAACGCTCAGGTCATCAT<br>**R:** GCACAGCGAAACGACCAAGGGGA | AF184170 | 60 | 84.3 |

F: forward; R: reverse; Tm: melting temperature; *pparγ*: *peroxisome proliferator-activated receptor-γ*; *fas*: *fatty acid synthase*; *lpl-lk*: *lipoprotein lipase like*; *lipa*: *lysosomal acid lipase*; *hoad*: *3-hydroxyacyl-CoA dehydrogenase*; *cd36*: *fatty acid translocase/cluster of differentiation 36*; *fatp1*: *fatty acid transport protein 1*; *lepr*: *leptin receptor*; *β-actin*: *beta-actin*; *rpl27*: *ribosomal protein L27*; *ef1α*: *translation elongation factor 1 alpha*.

**Table 2.** Normalized gene expression profile in gilthead seabream adipocytes during culture development.

| | Days | | | |
|---|---|---|---|---|
| | 4 | 8 | 12 | 16 |
| *pparγ* | 0.00432 ± 0.00055$^{b}$ | 0.00201 ± 0.00029$^{a}$ | 0.00154 ± 0.00017$^{a}$ | 0.00236 ± 0.00029$^{a}$ |
| *fas* | 0.039 ± 0.006$^{a}$ | 0.089 ± 0.033$^{ab}$ | 0.102 ± 0.028$^{ab}$ | 0.139 ± 0.040$^{b}$ |
| *lpl-lk* | 0.00088 ± 0.00042 | 0.00091 ± 0.00038 | 0.00129 ± 0.00044 | 0.00071 ± 0.00025 |
| *lipa* | 0.036 ± 0.010 | 0.030 ± 0.005 | 0.032 ± 0.007 | 0.036 ± 0.007 |
| *hoad* | 0.180 ± 0.041$^{a}$ | 0.220 ± 0.066$^{a}$ | 0.358 ± 0.071$^{ab}$ | 4.121 ± 3.527$^{b}$ |
| *cd36* | 0.269 ± 0.048 | 0.498 ± 0.076 | 0.606 ± 0.137 | 0.359 ±0.063 |
| *fatp1* | 0.042 ± 0.007$^{b}$ | 0.020 ± 0.003$^{a}$ | 0.024 ± 0.006$^{ab}$ | 0.028 ± 0.004$^{ab}$ |
| *lepr* | 0.00047 ± 0.00020 | 0.00086 ± 0.00014 | 0.00059 ± 0.00014 | 0.00073 ± 0.00010 |

Preadipocyte cells (days 4 and 8) and mature adipocyte cells (days 12 and 16). At day 8, after preadipocyte cells collection, a differentiation medium (DM) was used to promote cell differentiation. Values are presented as means (n = 7) ± standard error (SE). Results were analyzed by one-way ANOVA, followed by Tukey's test. Values with different superscripts are significantly different (P<0.05). Transcription factor: *pparγ*, *peroxisome proliferator-activated receptor-γ*; lipogenesis markers: *fas*, *fatty acid synthase*; and *lpl-lk*, *lipoprotein lipase like*; lipolysis marker: *lipa*, *lysosomal acid lipase*; β-oxidation marker: *hoad*, *3-hydroxyacyl-CoA dehydrogenase*; fatty acid transporters: *cd36*, *fatty acid translocase/cluster of differentiation 36*; and *fatp1*, *fatty acid transport protein 1*; appetite regulation-related gene: *lepr*, *leptin receptor*.

673 **Table 3.** Normalized gene expression in gilthead seabream preadipocyte cells at day 8 after 6 h

674 of lipid mixture (5 µL $mL^{-1}$), leptin (100 nM) and ghrelin (10 nM) treatments.

| | Treatments | | |
|---|---|---|---|
| | Lipid | Leptin | Ghrelin |
| *pparγ* | 0.0087 ± 0.0023 | 0.0039 ± 0.0003* | 0.0036 ± 0.0005* |
| *fas* | 0.059 ± 0.004 | 0.045 ± 0.008 | 0.040 ± 0.010 |
| *lpl-lk* | 0.0132 ± 0.0101 | 0.0073 ± 0.0020 | 0.0068 ± 0.0035 |
| *lipa* | 0.074 ± 0.003 | 0.082 ± 0.014 | 0.056 ± 0.007* |
| *hoad* | 0.105 ± 0.015 | 0.096 ± 0.017 | 0.087 ± 0.011 |
| *cd36* | 0.368 ± 0.057 | 0.146 ± 0.020** | 0.209 ± 0.076 |
| *fatp1* | 0.0022 ± 0.0005 | 0.0010 ± 0.0000 | 0.0013 ± 0.0002 |
| *lepr* | 0.00007 ± 0.00001 | 0.00016 ± 0.00006 | 0.00010 ± 0.00001* |

675 Values are presented as means (n = 7) ± standard error (SE). Results were analyzed by one-way

676 ANOVA, followed by Dunnett's test. Significant differences between the lipid (= positive

677 control) and each one of the treatments tested are indicated by *P ≤ 0.05~~; **P ≤ 0.01~~. Transcription

678 factor: *pparγ*, *peroxisome proliferator-activated receptor-γ*; lipogenesis markers: *fas*, *fatty acid*

679 *synthase*; and *lpl-lk*, *lipoprotein lipase like*; lipolysis marker: *lipa*, *lysosomal acid lipase*; β-

680 oxidation marker: *hoad*, *3-hydroxyacyl-CoA dehydrogenase*; fatty acid transporters: *cd36*, *fatty*

681 *acid translocase/cluster of differentiation 36*; and *fatp1*, *fatty acid transport protein 1*; appetite

682 regulation-related gene: *lepr*, *leptin receptor*.

683 **Table 4.** Normalized gene expression in gilthead seabream adipocyte cells at day 12 after 6 h of

684 lipid mixture (5 µL $mL^{-1}$), leptin (100 nM), ghrelin (10 nM) and insulin (1000 nM) treatments.

| | Treatments | | | |
|---|---|---|---|---|
| | Lipid | Leptin | Ghrelin | Insulin |
| *pparγ* | 0.0043 ± 0.0006 | 0.0019 ± 0.0004** | 0.0045 ± 0.0011 | 0.0029 ± 0.0003 |
| *fas* | 0.081 ± 0.014 | 0.067 ± 0.012 | 0.087 ± 0.017 | 0.072 ± 0.011 |
| *lpl-lk* | 0.043 ± 0.024 | 0.009 ± 0.004 | 0.017 ± 0.007 | 0.057 ± 0.034 |
| *lipa* | 0.087 ± 0.010 | 0.086 ± 0.015 | 0.077 ± 0.007 | 0.079 ± 0.019 |
| *hoad* | 0.181 ± 0.038 | 0.105 ± 0.027 | 0.173 ± 0.042 | 0.139 ± 0.018 |
| *cd36* | 0.406 ± 0.099 | 0.124 ± 0.027* | 0.218 ± 0.032 | 0.216 ± 0.058 |
| *fatp1* | 0.0019 ± 0.0003 | 0.0012 ± 0.0001 | 0.0027 ± 0.0006 | 0.0017 ± 0.0003 |
| *lepr* | 0.00016 ± 0.00004 | 0.00006 ± 0.00002 | 0.00016 ± 0.00005 | 0.00015 ± 0.00002 |

685 Values are presented as means (n = 7) ± standard error (SE). Results were analyzed by one-way

686 ANOVA, followed by Dunnett's test. Significant differences between the lipid (= positive

687 control) and each one of the treatments tested are indicated by *P ≤ 0.05~~; **P ≤ 0.01~~.

688 Transcription factor: *pparγ, peroxisome proliferator-activated receptor-γ*; lipogenesis markers:

689 *fas, fatty acid synthase*; and *lpl-lk, lipoprotein lipase like*; lipolysis marker: *lipa, lysosomal acid*

690 *lipase*; β-oxidation marker: *hoad, 3-hydroxyacyl-CoA dehydrogenase*; fatty acid transporters:

691 *cd36, fatty acid translocase/cluster of differentiation 36*; and *fatp1, fatty acid transport protein 1*;

692 appetite regulation-related gene: *lepr, leptin receptor*.