Targets for TNFα-induced lipolysis in gilthead sea bream (*Sparus aurata* L.) adipocytes isolated from lean and fat juvenile fish

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Short title: TNF-induced lipolysis in fish adipocytes

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Summary. The present study aimed to analyze adiposity heterogeneity and the role of liver X receptor (LXRα) and peroxisome proliferator-activated receptors (PPARs) as targets of tumour necrosis factor-α (TNFα) in gilthead sea bream (Sparus aurata L.). The screening of twenty fish at the beginning of the warm season identified two major groups with fat and lean phenotypes. Fat fish showed increased liver and mesenteric fat depots. This increased adiposity was concurrent in the adipose tissue to enhanced expression of lipoprotein lipase (LPL), whereas mRNA levels of the hormone sensitive lipase (HSL) remained almost unchanged. The resulting LPL/HSL ratio was thereby highest in fat fish, which suggests that this group of fish has not reached their peak of fat storage capacity. This is not surprising given the increased expression of PPARγ in the absence of a counter-regulatory raise of TNFα. However, this lipolytic cytokine exerted dual effects in primary adipocyte cultures that differ within and between lean and fat fish. One set of fat fish did not respond to TNFα treatment, whereas a second set exhibited a lipolytic response (increased glycerol release) which was apparently mediated by the down-regulated expression of PPARβ. In lean fish, TNFα exerted a strong and non-transcriptionally mediated lipolytic action. Alternatively, TNFα would inhibit lipid deposition via the down-regulated expression of adipogenic nuclear factors (PPARγ and LXRα). TNFα targets are therefore different in fish with lean and fat phenotypes, which is indicative of the complex network involved in the regulation of fish lipid metabolism.

Key words: lipoprotein lipase, hormone sensitive lipase, PPARα, PPARβ, PPARγ, LXRα
1. Introduction

Fish growth is a very complex process that responds to a variety of nutritional, environmental and genetic factors. Life-history decisions are, however, not fixed and depend on critical size and energy sufficiency at a specific stage “opportunity window” several months prior to transformation itself. Thus, the decision in salmonids to become smolt or sexually mature (Shearer and Swanson, 2000; Silverstein et al., 1997; Silverstein et al., 1998) is linked to growth and fat deposition in mid summer and spring. In Mediterranean fish, the replenishment of body fat stores is also dictated by the seasonal calendar, but current aquaculture practices in European sea bass and gilthead sea bream largely increase fat deposition in fat storage tissues leading to production and indirect selection of specimens with fatty characteristics. At the same time the individual variability in fish adiposity is relatively high and a major goal for the Mediterranean aquaculture is to explore the different lipostat mechanisms operating in farmed fish. For instance, liver steatosis can be induced in gilthead sea bream by high feeding ratios (Sitjà-Bobadilla et al., 2003) and partial or total replacement of fish meal and fish oil with alternative vegetable sources (Benedito-Palos et al., 2008; Caballero et al., 2004; Sitjà-Bobadilla et al., 2005). The underlying mechanisms are not well understood, but the enhanced removal of plasma TG-rich lipoproteins by hepatic lipoprotein lipase (LPL) explains, at least in part, the increased liver fat deposition and hypotriglyceridaemic effect of plant protein diets (Saera-Vila et al., 2005).

The hormone sensitive lipase (HSL) is the principal mediator of regulated lipolysis in body fat stores (González-Yanes and Sánchez-Margalet, 2006), and its regulation and substrate specificity have been addressed in salmonids (Harmon et al., 1993; Michelsen et al., 1994) and Antarctic fish (Hazel and Sidell, 2004), respectively. Thus far, HSL sequences are not available in fish, but recently the gilthead sea bream HSL has been characterized using RT-PCR approaches, and its nucleotide sequence has been introduced into Genbank with the accession number EU254478. There is now also increasing interest in defining the involvement of tumour necrosis factor-α (TNFα) in the regulation of fish lipid metabolism. This pro-inflammatory cytokine affects many aspects of adipocyte function, and its lipolytic action has been demonstrated in rainbow trout and gilthead sea bream adipocytes (Albalat et al., 2005b; Saera-Vila et al., 2007). Recent studies have also evidenced that TNFα inhibits the differentiation of rainbow trout preadipocytes (Bouraoui et al., 2008). This, together with the high expression level
of TNFα in the fat storage organs of gilthead sea bream (Saera-Vila et al., 2007), makes this cytokine a good candidate for playing a key role in reducing the adipose tissue mass. Nevertheless, the regulation and mode of action of TNFα remains mostly unexplored in cultured fish (Albalat et al., 2005b).

In mammals, TNFα regulates the expression of peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) that belong to the nuclear hormone receptor superfamily with a crucial role in lipid and lipoprotein metabolism (Desvergne et al., 2006; Kim et al., 2007). Three PPAR isotypes (α, β and γ) exist in all the vertebrate species studied to date, including Atlantic salmon (Ruyter et al., 1997), European sea bass (Boukouvala et al., 2004) and gilthead sea bream (Diez et al., 2007; Leaver et al., 2005). Each PPAR isotope is the product of a separate gene and has a distinct tissue distribution and specific function as sensors of dietary fatty acids (Chawla et al., 2001). Likewise, two isoforms of LXR exist in mammals (LXRα and LXRβ) and they are involved in the regulation of cholesterol homeostasis and fatty acid synthesis (Ou et al., 2001; Peet et al., 1998; Repa et al., 2000). Expression of LXRα is highest in liver and intestine but is also detected in macrophages, adipose tissue, kidney, lung and spleen, whereas LXRβ is ubiquitously expressed (Zhang and Mangelsdorf, 2002). Recently, LXR cDNA sequences have also been reported in zebrafish and salmonids (Archer et al., 2008; Cruz-Garcia et al., 2009). As far as we know LXRs have not been characterized in typically marine fish, but searches in the AQUAFIRST gilthead sea bream database (www.sigenae.org/aquafirst) identified as LXRα (E-value 8e-60) a contig of 792 bp in length. This sequence has been introduced into Genbank (FJ502320) and the deduced amino acid sequence for the C-terminal region (142 amino acids) shares 95-97%, 85% and 80% identity with fish, chicken and mammalian counterparts, respectively.

Taking in mind all the above findings, the first goal of the present study was to analyze in gilthead sea bream how LPL and HSL are regulated in concert in the adipose tissue of juvenile fish with lean and fat phenotypes. Secondly, the role of PPARs and LXRα as targets for the TNFα-induced lipolysis was monitored in freshly isolated adipocytes to evidence the existence of different mechanisms regulating the size of the adipose tissue.
2. Materials and methods

2.1 Animals and experimental procedures

Two year-old gilthead sea bream (*Sparus aurata* L.) were reared from fingerlings in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS, Spain). The experimental set-up was conducted in May-June under natural conditions of light (16 h. light: 8 h. dark) and temperature (19-23°C) at the IATS latitude (40º 5’ N; 0º 10’ E). At sampling time, overnight fasted fish were randomly selected and killed by decapitation under anaesthesia (3-aminobenzoic acid ethyl ester, MSS 222; 100µg/ml). Liver and whole right fillets (denuded of skin and bone) were excised, frozen in liquid nitrogen and stored at – 80°C until analyses. Mesenteric adipose tissue was also excised, a small piece was frozen, and the remaining tissue was processed for adipocyte isolation. All procedures were carried out according to national (Consejo Superior de Investigaciones Científicas, Institute of Aquaculture Torre de la Sal Review Board) and the current EU legislation on the handling of experimental animals.

2.2 Adipocyte isolation

Adipocytes were individually isolated from 20 fish as described elsewhere by Albalat et al. (2005a). Briefly, mesenteric adipose tissue was cut into thin pieces and incubated in a shaking water bath at 18°C for 60 min. with Krebs-Hepes buffer (pH 7.4) pre-gassed with 5% CO₂ in O₂, containing collagenase type II (130 U/ml) and 1% bovine albumin serum (BSA). The cell suspension was filtered through a double layer of nylon cloth and then washed three times by flotation. Cells were carefully resuspended in Krebs-Hepes buffer containing 2% BSA at a density of 7·10⁵ cells/ml. Cells were counted using a Fuchs-Rosenthal chamber. Aliquots of 1 ml of this final adipocyte suspension were incubated in polypropylene tubes up to 3 h. at 22°C in the absence or presence (100 ng/ml) of recombinant human TNFα (Sigma-Aldrich, Madrid, Spain).

At the end of the incubation time, the cells were centrifuged at 18000 g for 2 min. at 4°C and 300 µl of medium were placed into perchloric acid to give a final concentration of 7% (v/v). Perchloric acid was neutralised for the measurement of glycerol concentration as an index of lipolysis using a spectophotometric method (Tebar...
et al., 1996). The remaining medium was removed and lysis reagent was added for RNA extraction (see below). All products were obtained from Sigma-Aldrich. Control and experimental conditions were conducted in triplicate for each animal and cell preparation.

2.3 RNA extraction and RT procedure

Total RNA from adipose tissue and adipocytes was isolated using the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). Briefly, tissue and cell samples were homogenized with a guanidine-detergent lysis reagent at given ratios for adipose tissue (25 mg tissue/ml) and isolated adipocytes (2.8·10⁶ cells/ml). The reaction mixture was treated with proteinase K, and RNA purification was achieved by passing the lysates through a purification tray containing an application-specific membrane. Wash solutions containing DNAse were applied, and total RNA was eluted into a 96-well PCR plate. The RNA purity was checked by absorbance measures (A₂₆₀/₂₈₀) and always entered into the ideal range (1.9-2.1). Reverse transcription (RT) with random decamers was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems). For this purpose, 500 ng total RNA were reverse transcribed into a final volume of 100 μl. RT reactions were incubated for 10 min. at 25 ºC and 2 h. at 37 ºC. Negative control reactions were run without reverse transcriptase.

2.4 Real-time PCR assays

Transcript measurements of lipid enzymes (LPL, HSL), lipid transcription factors (PPARα, PPARβ, PPARγ, LXRα) and lipolytic cytokines (TNFα) were made using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA) as described elsewhere (Calduch-Giner et al., 2003). Briefly, diluted RT reactions were used for PCR reactions in 25 μl volume. Each PCR-well contained a SYBR Green Master Mix (Bio-Rad), and specific primers at a final concentration of 0.9 μM were used to obtain amplicons of 77-192 bp in length (Table 1). β-actin was used as housekeeping gene, and the efficiency of PCR reactions for the target and the reference gene varied between 92% and 96%, respectively. The dynamic range of standard curves (serial dilutions of RT-PCR reactions) spanned five orders of magnitude, and the
amount of product in a particular sample was determined by interpolation of the cycle threshold (C_t) value. The specificity of reaction was verified by analysis of melting curves and by electrophoresis and sequencing of PCR amplified products. Reactions were performed in triplicate and the fluorescence data acquired during the extension phase were normalized to β-actin by the delta-delta method (Livak and Schmittgen, 2001). No significant changes in β-actin expression were found within individuals and cell preparations.

2.5 Lipid determinations

 Freeze-dried samples of liver and flesh were used for lipid content determinations. As established in routine procedures, sample aliquots of 0.5 g were desiccated (105º C for 3 h.) in porous recipients before Soxhlet extraction with 50 ml diethyl ether at 120 ºC (Soxhlet 4001046 Auto extraction apparatus; Selecta, Barcelona, Spain).

2.6 Statistics

 Data values were checked for normal distribution and homogeneity of variances, and when necessary arcsin transformation was performed before Student t-test analysis comparing data on growth, adiposity and gene expression in fish with lean and fat characteristics. In isolated adipocytes incubations, lipolytic rates and gene expression after TNFα-treatment were normalized to control values (isolated adipocytes incubations without TNFα) and analyzed for statistical significance. All procedures were performed using the SPSS package version 14.0 (SPSS Inc, Chicago, USA).
3. Results

3.1 Characteristics of study group

Adiposity and biometric parameters of fish used in the study are shown in Table 1. Two major groups of fish with lean and fat characteristics were considered on the basis of the mesenteric fat index (MFI): a) the ten animals with highest MFI (1.69-2.86%) were clustered in the “fat group”; b) the other 10 fish with a reduced MFI (1.01-1.46%) and 10% reduction on the average body weight were put in the “lean group”. Condition factor (weight and length³ quotient) and fillet lipid levels (% wet matter) did not differ significantly between groups, although the observed values were slightly higher in the fat than in the lean group. In the same way, a two fold increase in the absolute amount of liver lipids (mg liver lipids per 100 g body weight) was found in fat vs lean fish.

3.2 Gene expression profile of adipose tissue

The expression pattern of LPL and HSL in adipose tissue is shown in Fig. 1A. The fat group showed a higher level of LPL transcripts than the lean group (P<0.05), which also presented an increased LPL/HSL ratio (P<0.05) in the absence of major changes in HSL expression. The expression level of PPARγ and PPARα was also significantly up-regulated in the fat group (P<0.05). Conversely, the expression pattern of PPARβ, LXRα and TNFα did not vary significantly with the change of growth parameters and body adiposity (Fig. 1B).

3.3 In vitro mediated effects of TNFα in isolated adipocytes

The TNFα-induced effects were tested in isolated adipocytes from the two fish groups. The analyzed response included measurements of glycerol release and mRNA levels of PPARs and LXRα. Both in fat and lean fish two patterns of adipocyte response were identified on the basis of the TNFα-induced lipolysis (glycerol release). In fish with fat characteristics, the group of TNFα-responders (F-R) was composed of three fish (380.6±19.5 g) with an enhanced lipolysis (more than 40% over controls, adipocytes without TNFα ) (Fig. 2A) in which expression of PPARβ was significantly lower than
controls (Fig. 2B); b) the group of non responders (F-NR) was made up of seven fish with a lowest body weight (324.3±6.7 g) and no apparent effects of TNFα on lipolysis (Fig. 2C) and transcriptional activity were observed (Fig. 2D). Adipocytes from lean fish also showed two different patterns of response corresponding to L-R and L-NR fish: a) the L-R group was comprised of six fish (316±14.1 g) with a significant increase in lipolysis (more than 50% over controls) (Fig. 3A) and no detectable effects on the expression of LXRα and PPARs (Fig. 3B); b) the L-NR group was constituted by four fish (285±20 g) with a low lipolytic response (less than 20% over controls) (Fig. 3C) accompanied of a significant down-regulated expression of PPARγ and LXRα (Fig. 3D).

4. Discussion

Gilthead sea bream is a highly valuable fish for the Mediterranean aquaculture with a production of 107,620 tonnes in 2006 (www.fao.org). However, a particular problem concerning both health and quality of farmed fish is related to energy regulation and, particularly, to lipid homeostasis. In the current study, a relatively high variability in adiposity was present in fish from the same batch and similar weight range, which happens even under improved management and culture conditions (Benedito-Palos et al., 2007; 2008). Thus, two groups of fish were clearly separated according to the mesenteric fat index, which increased in parallel with the liver fat deposition. Our current understanding of the mechanisms by which an excess of lipid deposition progresses to hepatic steatosis is limited in gilthead sea bream and fish in general (Sitjà-Bobadilla et al., 2003; 2005). However, this liver injury may reflect a wide range of lipid and lipoprotein metabolic-disorders, including impaired insulin sensitivity and defects on lipid trafficking and lipoprotein processing. Hence, the current consensus is that lipid-metabolic disorders are part of a common pathology which has not been properly defined in cultured fish, although it is becoming more and more evident with the currently intensive production systems (Farrel 2002).

TNFα is synthesized and secreted from adipocytes and, hence, is in a key position to play a paracrine/autocrine role in the control of the fat adipose mass. Several clinical studies have reported increased levels of TNFα in the blood of obese patients with signs of insulin resistance or dyslipidemia (Kern et al., 2001; Skurk et al., 2007).
However, conflicting results using human and mouse obese models indicate that TNFα expression is increased only in the more extreme forms of obesity (Warne, 2003). In these cases, the stimulated TNFα production acts on the adipocyte to shift lipid metabolism from lipid accumulation towards lipid mobilisation (Fonseca-Alaniz et al., 2007; Guilherme et al., 2008; Skurk et al., 2007). The anti-adipogenic and lipolytic effects of TNFα have also been demonstrated in fish (Albalat et al., 2005b; Bouraoui et al., 2008), and interestingly the expression of TNFα is seasonally up-regulated in gilthead sea bream with the replenishment of liver and mesenteric fat depots (Saera-Vila et al., 2007). Thus, in the present study, the lack of changes in TNFα expression with the increase in MFI suggests that fat fish have not reached their peak of fat storage capacity and continue increasing the size of their body fat depots. Consistent with this, the expression of LPL, a key limiting enzyme of tissue fatty acid uptake, was two fold higher in fat fish than in lean fish. This enzyme modulation is not surprising given the conservation of TNFα regulatory elements in the proximal 5´-flanking region of gilthead sea bream LPL (Saera-Vila et al., 2007). This agrees with the observation that LPL activity and expression are up-regulated by insulin treatment in the adipose tissue of gilthead sea bream (Albalat et al., 2007). Moreover, experimental evidence indicates that the age-related changes in the tissue-specific profile of LPL may drive the redistribution of fat depots from mesenteric adipose tissue to skeletal muscle (Saera-Vila et al., 2007).

HSL is the rate-limiting step for the breakdown of stored triglycerides to glycerol and fatty acids that are released to plasma to be used as metabolic fuels in other tissues. HSL activity has been well characterized in the Antarctic fish *Trematomus newnesi* (Hazel and Sidell, 2004) but, as far as we know, there have been no reported expression studies in fish tissues until now. In mammals, the short-term regulation of HSL is carried out by reversible phosphorylation and translocation to the surface lipid droplets in response to catecholamines and other lipolytic hormones. However, the long-term regulation takes place at transcriptional level, and HSL mRNA levels are affected by hibernation, fasting and even severe obesity (Holm et al., 2000). This may be also the case of the present study, and the lack of changes in HSL expression may indicate that the higher MFI of fat fish was primarily due to the increased lipid deposition rather than to inhibition of lipolysis. This kind of regulation, in which LPL expression is reduced whereas HSL expression remains as in control levels, has also been found in other physiological situations such as lactation in rats (Holm et al., 2000).
As expected, both in this and previous gilthead sea bream studies (Diez et al., 2007; Leaver et al., 2005), PPARγ is the most highly expressed PPAR isotope in the adipose tissue. Moreover, the expression of PPARγ was enhanced in the group of fat fish, which agrees with the idea that this nuclear factor is the master regulator of adipocyte differentiation that stimulates the expression of adipogenic enzymes such as LPL and adipocyte fatty acid binding protein (Rosen et al., 1999; Tontonoz and Spiegelman, 2008). When comparing fat and lean phenotypes, we also found a similar expression pattern for the other two PPAR isotopes, although a significant increase in transcript levels was only reported for PPARα. Earlier studies in fish indicate that the expression of PPARα is increased by fasting and the tissue oxidative capacity (Leaver et al., 2005; Leaver et al., 2008). Thereby, the current increase in the expression of PPARγ and PPARα can be viewed as a part of a counter-regulatory system, which may be tissue- and species-specific.

The isolated adipocytes system also evidenced a complex lipid metabolic network and most fish with fat characteristics (F-NR group) were refractory to TNFα-induced lipolysis. In this group of fish we also failed to detect any transcriptional effect on LXR and PPARs. This lack of response can be viewed as a steady state with an enhanced refractoriness to lipolytic TNFα action. This is not surprising given the aforementioned increase of the overall LPL/HSL ratio in fish with fat characteristics. At the same time, however, some fish with fat characteristics (F-R group) were sensitive to TNFα-induced lipolysis, which can be interpreted as a selective advantage or adaptive response to limit the size increase of the adipose tissue mass. This agrees with the biggest size of this group of fish, which was probably associated to an enhanced feed intake. The precise mechanisms underlying the TNFα-induced lipolysis remain to be fully elucidated, although it might be mediated by the down regulated expression of PPARβ. In mammals, this isotope differs from the other two PPAR isotopes by its almost ubiquitous tissue expression, which suggested a general housekeeping role (Kliewer et al., 1994; Schmidt et al., 1992). However, it has meanwhile become clear that this is not true, and analyses in a PPARβ null mouse model (db/db) demonstrate that PPARβ-deficiency is associated with multiple developmental and metabolic abnormalities, including demyelization and diminished adipose tissue mass (Barak et al., 2002; Peters et al., 2000). Moreover, PPARβ-specific agonists improve insulin sensitivity, suppress hepatic glucose output and inhibit free fatty acid release from adipocytes in the db/db mouse (Lee et al., 2006), which indicates that high-affinity
PPARβ ligands would be useful drugs to effectively target insulin resistance, hyperglucemia and dyslipidemia (Seedorf and Aberle, 2007).

In lean fish, dual effects of TNFα were also evidenced in isolated adipocytes cultures. In this case, most fish (L-R group) were highly sensitive to TNFα action, but the induced lipolysis was not transcriptionally mediated by PPARs and LXRα. This observation suggests an intriguing participation of catecholamine receptors and protein kinases as recently reviewed in mammals by González-Yanes and Sánchez-Margalet (2006). Alternatively, in the absence of TNFα-induced lipolysis (L-NR group), the down-regulated expression of PPARγ and LXRα suggests that the primary action TNFα on adipocytes from lean fish would be the inhibition of lipid deposition rather than the enhancement of lipid mobilization. Indeed, PPARγ is clearly involved in the activation of lipogenic enzymes and adipocyte differentiation, and its expression is reduced by TNFα in human hepatoma Hep3B cell line (Kim et al., 2007) and mice adipose tissue (Ye, 2008). Experimental evidence also indicates that LXRα up-regulates the expression of lipogenic genes such as acetyl-CoA carboxylase, fatty acid synthase and LPL (Al-Hasani and Joost, 2005; Zhang et al., 2001). Moreover, PPARγ-agonists increase LXRα expression in different experimental models such as primary cultures of human and murine macrophages, 3T3-L1 adipocyte cells and rat epidymal adipose tissue (Chineti et al., 2001; Wójcicka et al., 2007). Conversely, PPARγ and LXRα mRNA levels are significantly reduced by TNFα treatment in rabbit adipocytes, and their decrease is accompanied by a reduced cholesterol efflux as the result of a transcriptional cascade mediated by LXRα (Chawla et al., 2001; Zhao and Dong, 2008).

In summary, we present here new insights on fish lipid metabolism addressing the gene expression of some relevant genes for the regulation of the adipose tissue mass. The results highlighted the increased LPL/HSL expression ratio in fish with fat characteristics and increased MSI. Also the target genes for the TNF-induced lipolysis were different in fish with lean and fat characteristics, which reflected the different metabolic capabilities and/or mechanisms operating in lean and fat fish to limit the size increase of the adipose tissue mass (see Fig. 4 for schematic drawing). Thus, the TNFα-induced lipolysis in adipocytes from fat fish was transcriptionally mediated by the reduced expression of PPARβ, whereas other signalling pathways enhancing lipolysis (post-transcriptional mediated effects) or inhibiting adipogenesis (PPARγ- and LXRα-mediated effects) orchestrated the TNFα-mediated effects in lean fish. The practical consequences of these findings remain to be explored but they open new research
opportunities for genetics and comparative physiologists (e.g. linkage studies of fish adiposity and allele polymorphism in TNFα and associated target genes).

**List of symbols and abbreviations**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Bwt.</td>
<td>Body weight</td>
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<td>C_t</td>
<td>Cycle threshold</td>
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<tr>
<td>EU</td>
<td>European Union</td>
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<tr>
<td>h.</td>
<td>Hour</td>
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<td>HSI</td>
<td>Hepatosomatic index</td>
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<td>HSL</td>
<td>Hormone sensitive lipase</td>
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<td>K</td>
<td>Condition factor index</td>
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<td>LPL</td>
<td>Lipoprotein lipase</td>
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<td>LXR</td>
<td>Liver X receptor</td>
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<td>MFI</td>
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<tr>
<td>min.</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
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<td>RU</td>
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<td>RT</td>
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<tr>
<td>TNFα</td>
<td>Tumour necrosis factor-α</td>
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**Acknowledgements**

This work was funded by EU (FOOD-CT-2006-16249; Sustainable Aquafeeds to Maximise the Health Benefits of Farmed Fish for Consumers, AQUAMAX), and Spanish (CONSORTER-INGENIO 2010: Improvement of aquaculture production by the use of biotechnological tools) projects. AS-V was the recipient of a Spanish PhD fellowship from the Diputación Provincial de Castellón. LC was funded by a PhD fellowship (BES-2005-9566) from the Ministerio de Educación y Ciencia (project AGL-2004-06319-C02-02). The authors are grateful to M.A. González for the excellent technical assistance in molecular analysis.
References


Legends of Figures

Figure 1. Gene expression of key lipid enzymes (LPL, HSL), nuclear receptors (PPARα, PPARβ, PPARγ, LXRα) and TNFα in the mesenteric adipose tissue of fat and lean fish. Values are the mean ± s.e.m. of 8-10 animals and are referred to the highest tissue expression (relative units, RU) using β-actin as a housekeeping gene (delta-delta method). Asterisk (*) indicates significant differences between lean and fat fish (P<0.05, Student t-test).

Figure 2. Effect of recombinant human TNFα on lipolysis (A, C) and gene expression of PPARs (γ, α, β) and LXRα (B, D) in gilthead sea bream adipocytes isolated from fat fish. Two different patterns of TNFα-induced lipolysis are recognised (F-R fish, A-B; F-NR fish, C-D). Lipolysis is represented as the ratio (relative units, RU) of glycerol release in the presence or absence of TNFα (control adipocyte preparations). Gene expression values (relative units, RU) are referred to control values (without TNFα) using β-actin as a housekeeping gene. All data are represented as the mean ± s.e.m. (G1 fish, n=3; G2 fish, n = 7). Values > 1 or < 1 indicate an increase or decrease respect to control values. Asterisk (*) indicates significant differences between control and TNFα groups (P<0.05, Student t-test).

Figure 3. Effect of recombinant human TNFα on lipolysis (A, C) and gene expression of PPARs (γ, α, β) and LXRα (B, D) in gilthead sea bream adipocytes isolated from lean fish. Two different patterns of TNFα-induced lipolysis are recognised (L-R fish, A-B; L-NR fish, C-D). Lipolysis is represented as the ratio (relative units, RU) of glycerol release in the presence or absence of TNFα (control adipocyte preparations). Gene expression values (relative units, RU) are referred to control values (without TNFα) using β-actin as a housekeeping gene. All data are represented as the mean ± s.e.m. (G3 fish, n=6; G4 fish, n = 4). Values > 1 or < 1 indicate an increase or decrease respect to control values. Asterisk (*) indicates significant differences between control and TNFα groups (P<0.05, Student t-test).

Figure 4. Schematic drawing for the TNFα induced lipolysis in fish with fat and lean phenotypes. TNFα–induced lipolysis in fat fish (F-R group) is primarily mediated by
the inhibition of PPARβ expression. TNFα desensitivation (F-NR group) might be due
to defects in either receptor or intracellular signalling. TNFα–induced lipolysis in fish
with lean phenotypes (L-R) can be post-transcriptionally mediated. Alternatively,
inhibitory effects on adipogenic factors (PPARγ, LXRα) may also contribute to limit
the increase of the adipose tissue mass (L-NR group).
Table 1. Gilthead sea bream primer sequences used for real-time PCR.

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<td>PPAR γ</td>
<td>AY590304</td>
<td>F CGC CGT GGA CCT GTC AGA GC</td>
<td>318-337</td>
</tr>
<tr>
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<td></td>
<td>R GGA ATG GAT GGA GGA GGA GGA GAT GG</td>
<td>420-395</td>
</tr>
<tr>
<td>LXR α</td>
<td>FJ502320</td>
<td>F GCA CTT CGC CTC CAG GAC AAG</td>
<td>476-496</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CAG TCT TCA CAC AGC CAC ATC AGG</td>
<td>582-559</td>
</tr>
<tr>
<td>TNFα</td>
<td>AJ413189</td>
<td>F CAG GCG TCG TTC AGA GTC TC</td>
<td>1069-1088</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CTG TGG CTG AGA GGT GTG TG</td>
<td>1145-1126</td>
</tr>
<tr>
<td>β-actin</td>
<td>X89920</td>
<td>F TCC TGC GGA ATC CAT GAG A</td>
<td>811-829</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R GAC GTC GCA CTT CAT GAT GCT</td>
<td>861-841</td>
</tr>
</tbody>
</table>
Table 2. Data on growth and adiposity parameters in gilthead sea bream sampled fish. Asterisk (*) indicates significant differences (P<0.05) between fat and lean groups. Values are the mean ± SEM (n = 10).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fat Fish</th>
<th>Lean Fish</th>
<th>P (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>341.9 ± 10.8*</td>
<td>308.9 ± 10.1</td>
<td>0.041</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>22.8 ± 0.27</td>
<td>22.2 ± 0.22</td>
<td>0.086</td>
</tr>
<tr>
<td>K (%)(^2)</td>
<td>2.86 ± 0.043</td>
<td>2.82 ± 0.066</td>
<td>0.551</td>
</tr>
<tr>
<td>Adipose tissue (g)</td>
<td>7.40 ± 0.74*</td>
<td>4.04 ± 0.28</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>4.65 ± 0.25</td>
<td>4.05 ± 0.26</td>
<td>0.1</td>
</tr>
<tr>
<td>MFI (%)(^3)</td>
<td>2.16 ± 0.19*</td>
<td>1.31 ± 0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HSI (%)(^4)</td>
<td>1.35 ± 0.049</td>
<td>1.30 ± 0.058</td>
<td>0.438</td>
</tr>
<tr>
<td>Liver lipids (mg/100 g Bwt.)</td>
<td>95.39 ± 16.90*</td>
<td>51.76 ± 8.75</td>
<td>0.038</td>
</tr>
<tr>
<td>Fillet lipids (% wet matter)</td>
<td>4.97 ± 0.21</td>
<td>4.2 ± 0.31</td>
<td>0.129</td>
</tr>
</tbody>
</table>

\(^1\)P values result from Student t-test.
\(^2\)Condition factor index (K) = (fish wt./length\(^3\)) x 100
\(^3\)Mesenteric fat index (MFI) = (mesenteric fat wt./fish wt.) x 100
\(^4\)Hepatosomatic index (HSI) = (liver wt/fish wt.) x 100
Fig. 1
Fig. 2

A. Lipolysis (RU) with TNF:

![Bar chart showing lipolysis with and without TNF](chartA.png)

B. mRNA adipocytes (RU) with different factors:

![Bar chart showing mRNA adipocytes with different factors](chartB.png)

C. Lipolysis (RU) with + TNF:

![Bar chart showing lipolysis with + TNF](chartC.png)

D. mRNA adipocytes (RU) with PPAR γ, α, β, LXR α:

![Bar chart showing mRNA adipocytes with different PPAR and LXR factors](chartD.png)
Fig. 3
Lipid droplets

FAT FISH
FA uptake

LPL

FFA

Lipoproteins

F-R

F-NR

TNF

LPL

HSL

Lipolysis

Glycerol

FFA

Lipid droplets

Transcriptionally mediated effects

PPAR β

Cytosol

Nucleus

Post-transcriptional effects

F-R

F-NR

TNF desensitivation

TNF

FFA

Lipid droplets

PPAR γ

LXR α

LEAN FISH

FA uptake

LPL

Lipoproteins

Post-transcriptional effects

L-NR

L-R

Lipolysis

Glycerol

Lipid droplets

Transcriptionally mediated effects