The Journal of Experimental Biology 212, 2254-2260
Published by The Company of Biologists 2009
doi:10.1242/jeb.029025

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

Lourdes Cruz-Garcia[1], Alfonso Saera-Vila[2], Isabel Navarro[1], Josep Calduch-Giner[2] and Jaume Pérez-Sánchez[2,*]

[1]Departament de Fisiología, Facultat de Biología, Universitat de Barcelona, 08028 Barcelona, Spain and [2]Instituto de Acuicultura de Torre de la Sal (CSIC), Departamento de Biología, Cultivo y Patología de Especies Marinas, 12595 Ribera de Cabanes, Castellón, Spain

*Author for correspondence (e-mail: jperez@iats.csic.es)

Accepted 20 April 2009

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 20 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 its peak 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) that was apparently mediated by the downregulated expression of PPARβ. In lean fish, TNFα exerted a strong and non-transcriptionally mediated lipolytic action. Alternatively, TNFα would inhibit lipid deposition *via* the downregulated 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.

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 triglyceride-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 shown 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 data, 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 a sensor 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 8×10^{-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 all the above findings into account, the first goal of the present study was to analyze how LPL and HSL are regulated in concert in the adipose tissue of juvenile fish with lean and fat phenotypes in gilthead sea bream. 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.

MATERIALS AND METHODS

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:8 h light:dark) and temperature (19–23°C) at the IATS latitude (40 deg.5′N; 0 deg.10′E). At sampling time, overnight fasted fish were randomly selected and killed by decapitation under anaesthesia (3-aminobenzoic acid ethyl ester, MSS-222; 100 μg ml^{-1}). 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 current EU legislation on the handling of experimental animals.

Adipocyte isolation

Adipocytes were individually isolated from 20 fish as described elsewhere (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% CO_2 in O_2, containing collagenase type II (130 U ml^{-1}) 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 7×10^5 cells ml^{-1}. Cells were counted using a Fuchs-Rosenthal chamber. Aliquots of 1 ml of this final adipocyte suspension were incubated in polypropylene tubes for up to 3 h at 22°C in the absence or presence (100 ng ml^{-1}) of recombinant human TNFα (Sigma-Aldrich, Madrid, Spain).

At the end of the incubation time, the cells were centrifuged at 18,000 ***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 spectrophotometric 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.

RNA extraction and RT procedure

Total RNA from adipose tissue and adipocytes was isolated using the ABI PRISM^{TM} 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^{-1}) and isolated adipocytes (2.8×10^6 cells ml^{-1}). 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 measurements ($A_{260/280}$) and was always within 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 of total RNA was reverse transcribed into a final volume of 100 μl. RT reactions were incubated for 10 min at 25°C and for 2 h at 37°C. Negative control reactions were run without reverse transcriptase.

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 μmol l^{-1} 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 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.

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 (105°C for 3 h) in porous receptacles before Soxhlet extraction with 50 ml diethyl ether at 120°C (Soxhlet 4001046 Auto Extraction Apparatus; Selecta, Barcelona, Spain).

Statistics

Data values were checked for normal distribution and homogeneity of variances, and when necessary arcsin transformation was

Table 1. Gilthead sea bream primer sequences used for real-time PCR

Gene	Accession number		Primer sequence	Position
HSL	EU254478	F	GCT TTG CTT CAG TTT ACC ACC ATT TC	154–179
		R	GAT GTA GCG ACC CTT CTG GAT GAT GTG	275–249
LPL	AY495672	F	GAG CAC GCA GAC AAC CAG AA	500–520
		R	GGG GTA GAT GTC GAT GTC GC	672–691
PPARα	AY590299	F	TCT CTT CAG CCC ACC ATC CC	106–125
		R	ATC CCA GCG TGT CGT CTC C	221–203
PPARβ	AY590301	F	AGG CGA GGG AGA GTG AGG ATG AGG AG	375–400
		R	CTG TTC TGA AAG CGA GGG TGA CGA TGT TTG	562–533
PPARγ	AY590304	F	CGC CGT GGA CCT GTC AGA GC	318–337
		R	GGA ATG GAT GGA GGA GGA GGA GAT GG	420–395
LXRα	FJ502320	F	GCA CTT CGC CTC CAG GAC AAG	476–496
		R	CAG TCT TCA CAC AGC CAC ATC AGG	582–559
TNFα	AJ413189	F	CAG GCG TCG TTC AGA GTC TC	1069–1088
		R	CTG TGG CTG AGA GGT GTG TG	1145–1126
β-actin	X89920	F	TCC TGC GGA ATC CAT GAG A	811–829
		R	GAC GTC GCA CTT CAT GAT GCT	861–841

performed before Student's *t*-test analysis comparing data on growth, adiposity and gene expression in fish with lean and fat characteristics. In isolated adipocyte incubations, lipolytic rates and gene expression after TNFα treatment were normalized to control values (isolated adipocyte incubations without TNFα) and analyzed for statistical significance. All procedures were performed using SPSS v. 14.0 (SPSS, Chicago, IL, USA).

RESULTS

Characteristics of study group

Adiposity and biometric parameters of fish used in the study are shown in Table 2. Two major groups of fish with lean and fat characteristics were considered on the basis of the mesenteric fat index (MFI): (1) the 10 animals with the highest MFI (1.69–2.86%) were clustered in the 'fat group' and (2) the other 10 fish with a reduced MFI (1.01–1.46%) and 10% reduction in the average body mass (M_b) were put in the 'lean group'. Condition factor (fish mass and length^3 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 twofold increase in the absolute amount of liver lipids (mg liver lipids per 100 g body mass) was found in fat *vs* lean fish.

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 resulted in 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 upregulated 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).

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 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 that of controls (Fig. 2B); the group of non-responders (F-NR) was made up of seven fish with a lower body mass (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: (1) the L-R group comprised six fish (316±14.1 g) with a significant increase in lipolysis (>50% above controls) (Fig. 3A) and no detectable effects on the expression of LXRα and PPARs (Fig. 3B); (2) the L-NR group comprised four fish (285±20 g) with a low lipolytic response (less than 20% above

Table 2. Data on growth and adiposity parameters in gilthead sea bream sampled fish

	Fat fish	Lean fish	P[a]
Body mass (g)	341.9±10.8*	308.9±10.1	0.041
Length (cm)	22.8±0.27	22.2±0.22	0.086
K (%)[b]	2.86±0.043	2.82±0.066	0.551
Adipose tissue (g)	7.40±0.74*	4.04±0.28	<0.001
Liver (g)	4.65±0.25	4.05±0.26	0.1
MFI (%)[c]	2.16±0.19*	1.31±0.08	<0.001
HSI (%)[d]	1.35±0.049	1.30±0.058	0.438
Liver lipids (mg/100 g body mass)	95.39±16.90*	51.76±8.75	0.038
Fillet lipids (% wet matter)	4.97±0.21	4.2±0.31	0.129

Asterisks (*) indicate significant differences (P<0.05) between fat and lean groups. Values are means ± s.e.m. (N=10).
[a]P values result from Student's *t*-test.
[b]Condition factor index (K)=(fish mass/length^3)×100.
[c]Mesenteric fat index (MFI)=(mesenteric fat mass/fish mass)×100.
[d]Hepatosomatic index (HSI)=(liver mass/fish mass)×100.

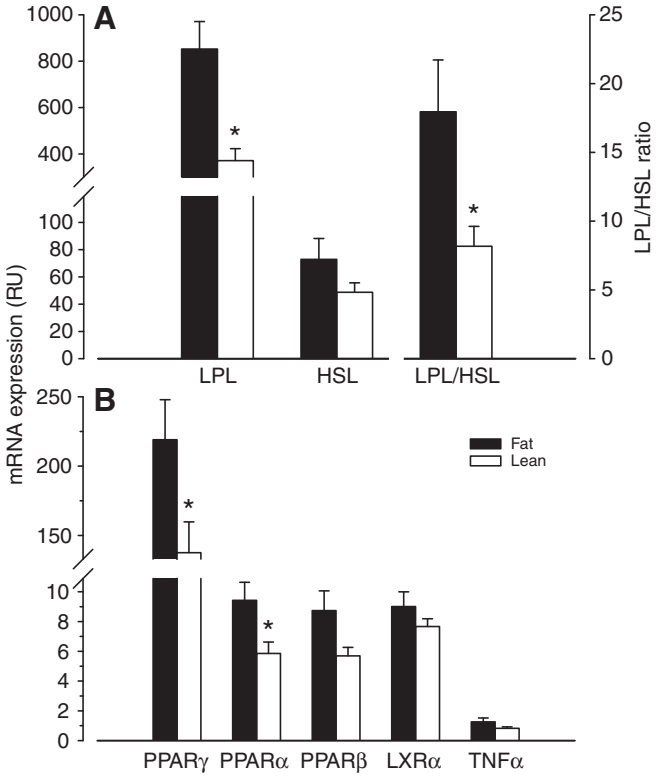

Fig. 1. Gene expression of key lipid enzymes (LPL, HSL), nuclear receptors (PPARα, PPARβ, PPARγ, LXRα) and TNFα in the mesenteric adipose tissue of fat and lean fish. Values are means ± s.e.m. of 8–10 animals and are referred to the highest tissue expression (relative units, RU) using β-actin as a housekeeping gene (delta-delta method). Asterisks (*) indicate significant differences between lean and fat fish (P<0.05, Student's t-test).

controls) (Fig. 3C) accompanied by a significant downregulated expression of PPARγ and LXRα (Fig. 3D).

DISCUSSION

Gilthead sea bream is a highly valuable fish for the Mediterranean aquaculture, with an aquaculture production of 125,355 tonnes in 2007 (www.fao.org). However, a particular problem concerning both health and quality of farmed fish is related to energy regulation and, particularly, lipid homeostasis. In the current study, a relatively high variability in adiposity was present in fish from the same batch and similar mass range, which happens even under improved management and culture conditions (Benedito-Palos et al., 2007; Benedito-Palos et al., 2008). Thus, two groups of fish were clearly separated according to the MFI, which increased in parallel with 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; Sitjà-Bobadilla et al., 2005). However, this liver injury may reflect a wide range of lipid and lipoprotein metabolic disorders, including impaired insulin sensitivity and defects of lipid trafficking and lipoprotein processing. Hence, the current consensus is that lipid metabolic disorders are part of a common pathology that 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 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 upregulated 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 twofold 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 upregulated 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, which are released into the 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 the transcriptional level, and HSL mRNA levels are affected by hibernation, fasting and even severe obesity (Holm et al., 2000). This may also be the case in the present study, and the lack of changes in HSL expression may indicate that the higher MFI of fat fish was primarily due to increased lipid deposition rather than to inhibition of lipolysis. This kind of regulation, in which LPL expression is reduced whereas HSL expression remains at 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). Therefore, the current increase in the expression of PPARγ and PPARα can be viewed as a part of a

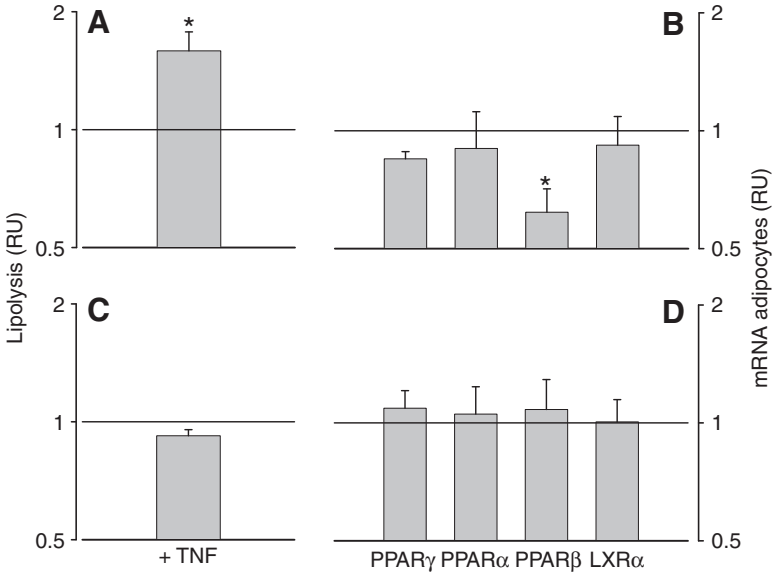

Fig. 2. Effect of recombinant human TNFα on lipolysis (A,C) and gene expression of PPARs (γ, α, β) and LXRα (B,D) in gilthead sea bream adipocytes isolated from fat fish. Two different patterns of TNFα-induced lipolysis are recognised (F-R fish, A,B; F-NR fish, C,D). Lipolysis is represented as the ratio (relative units, RU) of glycerol release in the presence or absence of TNFα (control adipocyte preparations). Gene expression values (RU) are referred to control values (without TNFα) using β-actin as a housekeeping gene. All data are represented as means ± s.e.m. (G1 fish, *N*=3; G2 fish, *N*=7). Values of >1 or <1 indicate an increase or decrease with respect to control values. Asterisks (*) indicate significant differences between control and TNFα groups ($P<0.05$, Student's *t*-test).

counter-regulatory system, which may be tissue- and species-specific.

The isolated adipocyte 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 in 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 with enhanced feed intake. The precise mechanisms underlying the TNFα-induced lipolysis remain to be fully elucidated, although it might be mediated by the downregulated expression of PPARβ. In mammals, this isotope differs from the other two PPAR isotopes by its almost ubiquitous tissue expression, which suggests a general housekeeping role (Kliewer et al., 1994; Schmidt et al., 1992). However, it has subsequently 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, hyperglycemia and dyslipidemia (Seedorf and Aberle, 2007).

In lean fish, dual effects of TNFα were also evidenced in isolated adipocyte 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

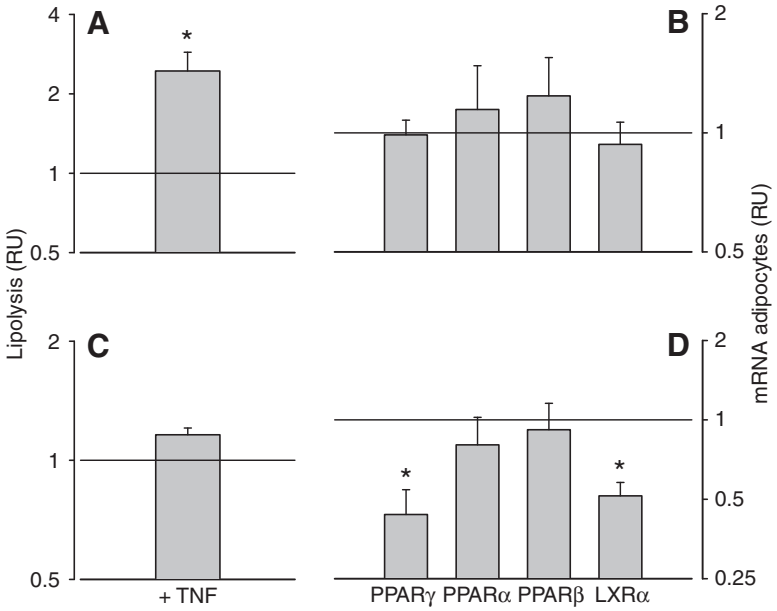

Fig. 3. Effect of recombinant human TNFα on lipolysis (A,C) and gene expression of PPARs (γ, α, β) and LXRα (B,D) in gilthead sea bream adipocytes isolated from lean fish. Two different patterns of TNFα-induced lipolysis are recognised (L-R fish, A,B; L-NR fish, C,D). Lipolysis is represented as the ratio (relative units, RU) of glycerol release in the presence or absence of TNFα (control adipocyte preparations). Gene expression values (RU) are referred to control values (without TNFα) using β-actin as a housekeeping gene. All data are represented as the means ± s.e.m. (G3 fish, *N*=6; G4 fish, *N*=4). Values of >1 or <1 indicate an increase or decrease with respect to control values. Asterisks (*) indicate significant differences between control and TNFα groups ($P<0.05$, Student's *t*-test).

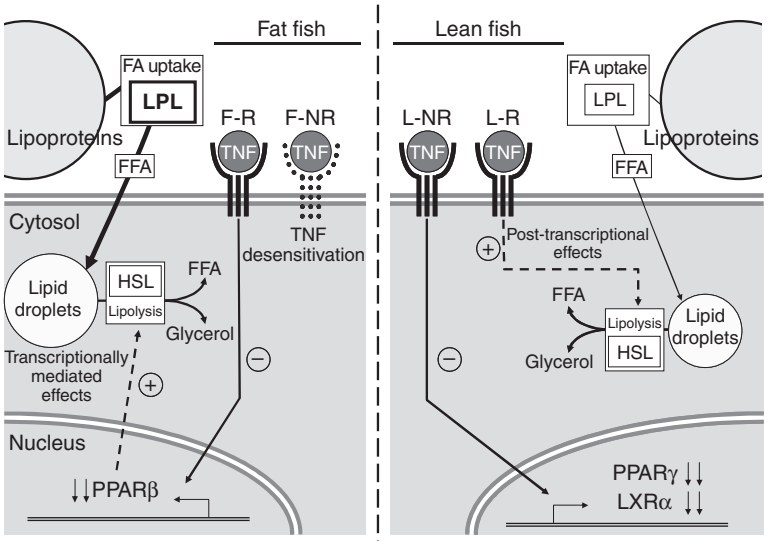

Fig. 4. Schematic drawing of the TNFα-induced lipolysis in fish with fat and lean phenotypes. TNFα-induced lipolysis in fat fish (F-R group) is primarily mediated by the inhibition of PPARβ expression. TNFα desensitisation (F-NR group) might be due to defects in either receptor or intracellular signalling. 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). Abbreviations: FA, fatty acid; FFA, free fatty acid; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase.

suggests an intriguing participation of catecholamine receptors and protein kinases, as recently reviewed in mammals (González-Yanes and Sánchez-Margalet, 2006). Alternatively, in the absence of TNFα-induced lipolysis (L-NR group), the downregulated expression of PPARγ and LXRα suggests that the primary action of 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 upregulates 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 (Chinetti 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 new insights on fish lipid metabolism, addressing the gene expression of some relevant genes for the regulation of adipose tissue mass. The results highlight the increased LPL/HSL expression ratio in fish with fat characteristics and increased MSI. Also, the target genes for TNF-induced lipolysis were different in fish with lean and fat characteristics, which reflects 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 ABBREVIATIONS

HSI	hepatosomatic index
HSL	hormone-sensitive lipase
K	condition factor index
LPL	lipoprotein lipase
LXR	liver X receptor
MFI	mesenteric fat index
PPAR	peroxisome proliferator-activated receptor
RT	reverse transcription
TNFα	tumour necrosis factor-α

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 (CONSOLIDER-INGENIO 2010: Improvement of Aquaculture Production by the Use of Biotechnological Tools) projects. A.S.-V. was the recipient of a Spanish Ph.D. fellowship from the Diputación Provincial de Castellón. L.C. was funded by a Ph.D. 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

Al-Hasani, H. and Joost, H. G. (2005). Nutrition-/diet-induced changes in gene expression in white adipose tissue. *Best Pract. Res. Clin. Endocrinol. Metab.* **19**, 589-603.

Albalat, A., Gómez-Requeni, P., Rojas, P., Médale, F., Kaushik, S., Vianen, G. J., Van den Thillart, G., Gutiérrez, J., Pérez-Sánchez, J. and Navarro, I. (2005a). Nutritional and hormonal control of lipolysis in isolated gilthead seabream (*Sparus aurata*) adipocytes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **289**, R259-R265.

Albalat, A., Liarte, C., MacKenzie, S., Tort, L., Planas, J. V. and Navarro, I. (2005b). Control of adipose tissue lipid metabolism by tumor necrosis factor-α in rainbow trout (*Oncorhynchus mykiss*). *J. Endocrinol.* **184**, 527-534.

Albalat, A., Saera-Vila, A., Capilla, E., Gutiérrez, J., Pérez-Sánchez, J. and Navarro, I. (2007). Insulin regulation of lipoprotein lipase (LPL) activity and expression in gilthead sea bream (*Sparus aurata*). *Comp. Biochem. Physiol.* **148B**, 151-159.

Archer, A., Lauter, G., Hauptmann, G., Mode, A. and Gustafsson, J. A. (2008). Transcriptional activity and developmental expression of liver X receptor (lxr) in zebrafish. *Dev. Dyn.* **237**, 1090-1098.

Barak, Y., Liao, D., He, W., Ong, E. S., Nelson, M. C., Olefsky, J. M., Boland, R. and Evans, R. M. (2002). Effects of peroxisome proliferator-activated receptor δ on placentation, adiposity, and colorectal cancer. *Proc. Natl. Acad. Sci. USA* **99**, 303-308.

Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J. A., Kaushik, S. and Pérez-Sánchez, J. (2007). Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): networking of systemic and local components of GH/IGF axis. *Aquaculture* **267**, 199-212.

Benedito-Palos, L., Navarro, J. C., Sitjà-Bobadilla, A., Gordon Bell, J., Kaushik, S. and Pérez-Sánchez, J. (2008). High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty acid profiles and histological alterations of target tissues. *Br. J. Nutr.* **100**, 992-1003.

Boukouvala, E., Antonopoulou, E., Favre-Krey, L., Diez, A., Bautista, J. M., Leaver, M. J., Tocher, D. R. and Krey, G. (2004). Molecular characterization of three peroxisome proliferator-activated receptors from the sea bass (*Dicentrarchus labrax*). *Lipids* **39**, 1085-1092.

Bouraoui, L., Gutierrez, J. and Navarro, I. (2008). Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (*Oncorhynchus mykiss*). *J. Endocrinol.* **198**, 459-469.

Caballero, M. J., Izquierdo, M. S., Kjorsvik, E., Fernandez, A. J. and Rosenlund, G. (2004). Histological alterations in the liver of sea bream, *Sparus aurata* L., caused by short- or long-term feeding with vegetable oils: recovery of normal morphology after feeding fish oil as the sole lipid source. *J. Fish Dis.* **27**, 531-541.

Calduch-Giner, J. A., Mingarro, M., Vega-Rubín de Celis, S., Boujard, D. and Pérez-Sánchez, J. (2003). Molecular cloning and characterization of gilthead sea bream (*Sparus aurata*) growth hormone receptor (GHR): assessment of alternative splicing. *Comp. Biochem. Physiol.* **136B**, 1-13.

Chawla, A., Repa, J. J., Evans, R. M. and Mangelsdorf, D. J. (2001). Nuclear receptors and lipid physiology: opening the X-Files. *Science* **294**, 1866-1870.

Chinetti, G., Lestavel, S., Bocher, V., Remaley, A. T., Neve, B., Torra, I. P., Teissier, E., Minnich, A., Jaye, M., Duverger, N. et al. (2001). PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat. Med.* **7**, 53-58.

Cruz-Garcia, L., Minghetti, M., Navarro, I. and Tocher, D. R. (2009). Molecular cloning, tissue expression and regulation of liver X Receptor (LXR) transcripotion factors of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. B* **153B**, 81-88.

Desvergne, B., Michalik, L. and Wahli, W. (2006). Transcriptional regulation of metabolism. *Physiol. Rev.* **86**, 465-514.

Diez, A., Menoyo, D., Perez-Benavente, S., Calduch-Giner, J. A., Vega-Rubin de Celis, S., Obach, A., Favre-Krey, L., Boukouvala, E., Leaver, M. J., Tocher, D. R. et al. (2007). Conjugated linoleic acid affects lipid composition, metabolism, and gene expression in gilthead sea bream (*Sparus aurata* L). *J. Nutr.* **137**, 1363-1369.

Farrell, A. P. (2002). Coronary arteriosclerosis in salmon: growing old or growing fase? *Comp. Biochem. Physiol.* **132A**, 723-735.

Fonseca-Alaniz, M. H., Takada, J., onso-Vale, M. I. and Lima, F. B. (2007). Adipose tissue as an endocrine organ: from theory to practice. *J. Pediatr.* **83**, S192-S203.

González-Yanes, C. and Sánchez-Margalet, V. (2006). Signalling mechanisms regulating lipolysis. *Cell. Signal.* **18**, 401-408.

Guilherme, A., Virbasius, J. V., Puri, V. and Czech, M. P. (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat. Rev. Mol. Cell. Biol.* **9**, 367-377.

Harmon, J. S., Rieniets, L. M. and Sheridan, M. A. (1993). Glucagon and insulin regulate lipolysis in trout liver by altering phosphorylation of triacylglycerol lipase. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **265**, R255-R260.

Hazel, J. R. and Sidell, B. D. (2004). The substrate specificity of hormone-sensitive lipase from adipose tissue of the Antarctic fish *Trematomus newnesi. J. Exp. Biol.* **207**, 897-903.

Holm, C., Osterlund, T., Laurell, H. and Contreras, J. A. (2000). Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annu. Rev. Nutr.* **20**, 365-393.

Kern, P. A., Ranganathan, S., Li, C., Wood, L. and Ranganathan, G. (2001). Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am. J. Physiol. Endocrinol. Metab.* **280**, E745-E751.

Kim, M. S., Sweeney, T. R., Shigenaga, J. K., Chui, L. G., Moser, A., Grunfeld, C. and Feingold, K. R. (2007). Tumor necrosis factor and interleukin 1 decrease RXRα, PPARα, PPARγ, LXRα, and the coactivators SRC-1, PGC-1α, and PGC-1β in liver cells. *Metabolism* **56**, 267-279.

Kliewer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J., Umesono, K. and Evans, R. M. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. USA* **91**, 7355-7359.

Leaver, M. J., Boukouvala, E., Antonopoulou, E., Diez, A., Favre-Krey, L., Ezaz, M. T., Bautista, J. M., Tocher, D. R. and Krey, G. (2005). Three peroxisome proliferator-activated receptor isotypes from each of two species of marine fish. *Endocrinology* **146**, 3150-3162.

Leaver, M. J., Bautista, J. M., Björnsson, B. T., Jönsson, E., Krey, G., Tocher, D. R. and Torstensen, B. E. (2008). Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture. *Rev. Fish. Sci.* **16**, 73-94.

Lee, C. H., Olson, P., Hevener, A., Mehl, I., Chong, L. W., Olefsky, J. M., Gonzalez, F. J., Ham, J., Kang, H., Peters, J. M. et al. (2006). PPARδ regulates glucose metabolism and insulin sensitivity. *Proc. Natl. Acad. Sci. USA* **103**, 3444-3449.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. *Methods* **25**, 402-408.

Michelsen, K. G., Harmon, J. S. and Sheridan, M. A. (1994). Adipose tissue lipolysis in rainbow trout, *Oncorhynchus mykiss*, is modulated by phosphorylation of triacylglycerol lipase. *Comp. Biochem. Physiol.* **107B**, 509-513.

Ou, J., Tu, H., Shan, B., Luk, A., Bose-Boyd, R. A., Bashmakov, Y., Goldstein, J. L. and Brown, M. S. (2001). Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *Proc. Natl. Acad. Sci. USA* **98**, 6027-6032.

Peet, D. J., Turley, S. D., Ma, W., Janowski, B. A., Lobaccaro, J. M., Hammer, R. E. and Mangelsdorf, D. J. (1998). Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR α. *Cell* **93**, 693-704.

Peters, J. M., Lee, S. S. T., Li, W., Ward, J. M., Gavrilova, O., Everett, C., Reitman, M. L., Hudson, L. D. and Gonzalez, F. J. (2000). Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β(δ). *Mol. Cell. Biol.* **20**, 5119-5128.

Repa, J. J., Turley, S. D., Lobaccaro, J. M. A., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R. A., Dietschy, J. M. and Mangelsdorf, D. J. (2000). Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* **289**, 1524-1529.

Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., Spiegelman, B. M. and Mortensen, R. M. (1999). PPARγ is required for the differentiation of adipose tissue *in vivo* and *in vitro*. *Mol. Cell* **4**, 611-617.

Ruyter, B., Andersen, Ø., Dehli, A., Östlund Farrants, A. K., Gjøen, T. and Thomassen, M. S. (1997). Peroxisome proliferator activated receptors in Atlantic salmon (*Salmo salar*): effects on PPAR transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and fatty acids. *Biochim. Biophys. Acta* **1348**, 331-338.

Saera-Vila, A., Calduch-Giner, J. A., Gómez-Requeni, P., Médale, F., Kaushik, S. and Pérez-Sánchez, J. (2005). Molecular characterization of gilthead sea bream (*Sparus aurata*) lipoprotein lipase. Transcriptional regulation by season and nutritional condition in skeletal muscle and fat storage tissues. *Comp. Biochem. Physiol.* **142B**, 224-232.

Saera-Vila, A., Calduch-Giner, J. A., Navarro, I. and Pérez-Sánchez, J. (2007). Tumour necrosis factor (TNF)α as a regulator of fat tissue mass in the Mediterranean gilthead sea bream (*Sparus aurata* L.). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **146**, 338-345.

Schmidt, A., Endo, N., Rutledge, S. J., Vogel, R., Shinar, D. and Rodan, G. A. (1992). Identification of a new member of the steroid hormone receptor superfamily that is activated by a peroxisome proliferator and fatty acids. *Mol. Endocrinol.* **6**, 1634-1641.

Seedorf, U. and Aberle, J. (2007). Emerging roles of PPARδ in metabolism. *Biochim. Biophys. Acta* **1771**, 1125-1131.

Shearer, K. D. and Swanson, P. (2000). The effect of whole body lipid on early sexual maturation of 1+ age male chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture* **190**, 343-367.

Silverstein, J. T., Shimma, H. and Ogata, H. (1997). Early maturity in amago salmon (*Oncorhynchus masu ishikawai*): an association with energy storage. *Can. J. Fish. Aquat. Sci.* **54**, 444-451.

Silverstein, J. T., Shearer, K. D., Dickhoff, W. W. and Plisetskaya, E. M. (1998). Effects of growth and fatness on sexual development of chinook salmon (*Oncorhynchus tshawytscha*) parr. *Can. J. Fish. Aquat. Sci.* **55**, 2376-2382.

Sitjà-Bobadilla, A., Mingarro, M., Pujalte, M. J., Garay, E., Alvarez-Pellitero, P. and Pérez-Sánchez, J. (2003). Immunological and pathological status of gilthead sea bream (*Sparus aurata* L.) under different long-term feeding regimes. *Aquaculture* **220**, 707-724.

Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S. and Pérez-Sánchez, J. (2005). Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). *Aquaculture* **249**, 387-400.

Skurk, T., berti-Huber, C., Herder, C. and Hauner, H. (2007). Relationship between adipocyte size and adipokine expression and secretion. *J. Clin. Endocrinol. Metab.* **92**, 1023-1033.

Tebar, F., Soley, M. and Ramírez, I. (1996). The antilipolytic effects of insulin and epidermal growth factor in rat adipocytes are mediated by different mechanisms. *Endocrinology* **137**, 4181-4188.

Tontonoz, P. and Spiegelman, B. M. (2008). Fat and beyond: the diverse biology of PPARγ. *Annu. Rev. Biochem.* **77**, 289.

Warne, J. P. (2003). Tumor necrosis factor α: a key regulator of adipose tissue mass. *J. Endocrinol.* **177**, 351-355.

Wójcicka, G., Jamroz-Wisniewska, A., Horoszewicz, K. and Beltowski, J. (2007). Liver X receptors (LXRs). Part I: structure, function, regulation of activity, and role in lipid metabolism. *Postepy Hig. Med. Dosw.* **61**, 736-759.

Ye, J. (2008). Regulation of PPARγ function by TNF-α. *Biochem. Biophys. Res. Commun.* **374**, 405-408.

Zhang, Y. and Mangelsdorf, D. J. (2002). LuXuRies of lipid homeostasis: the unity of nuclear hormone receptors, transcription regulation, and cholesterol sensing. *Mol. Interv.* **2**, 78-87.

Zhang, Y., Repa, J. J., Gauthier, K. and Mangelsdorf, D. J. (2001). Regulation of lipoprotein lipase by the oxysterol receptors, LXRα and LXRβ. *J. Biol. Chem.* **276**, 43018-43024.

Zhao, S. P. and Dong, S. Z. (2008). Effect of tumor necrosis factor α on cholesterol efflux in adipocytes. *Clin. Chim. Acta* **389**, 67-71.