CBP manuscript 18988 revised - Part A

Changes in adipocyte cell size, gene expression of lipid metabolism markers, and lipolytic responses induced by dietary fish oil replacement in gilthead sea bream (*Sparus aurata* L.)

Lourdes Cruz-Garcia[1], Joan Sánchez-Gurmaches[1], Lamia Bouraoui[1], Alfonso Saera-Vila[2], Jaume Pérez-Sánchez[2], Joaquim Gutiérrez[1], Isabel Navarro[1]

([1]) *Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona, 08028 Barcelona, Spain*

([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*

***Corresponding author:** Lourdes Cruz-Garcia, Faculty of Biology, Department of Physiology, University of Barcelona, Avda Diagonal 645, Barcelona, Spain 08028
Tel.: +34 93 4039636, Fax: +34 934110358
Email address: lourdescruzg@gmail.com.

Key words: Adipocyte cell size, CLA, Glucose uptake, Hormone-sensitive lipase, Lipolysis, Lipoprotein lipase, Liver X receptor, Vegetable oils

Abbreviations: t10,c12-CLA; *trans*-10, *cis*-12 conjugated linoleic acid; 66VO, 66% vegetable oil diet; c9,t11-CLA, *cis*-9, *trans*-11 conjugated linoleic acid; BSA, bovine serum albumin; DPM, desintegrations per minute; FO, fish oil; HSI, hepatosomatic index; HSL, hormone-sensitive lipase; LA, linoleic acid; LPL, lipoprotein lipase; LXR, Liver X receptor; MFI, mesenteric fat index; NEFAs, non-esterified fatty acids; PUFA, polyunsaturated fatty acids; RU, relative units; TNFα, Tumour necrosis factor-α; VO, vegetable oil

ABSTRACT

The effects of fish oil (FO) substitution by 66% vegetable oils in a diet with already 75% vegetable protein (66VO) on adipose tissue lipid metabolism of gilthead sea bream were analysed after a 14-month feeding trial. In the last three months of the experiment, a FO diet was administrated to a 66VO group (group 66VO/FO) as a finishing diet. Hormone-sensitive lipase

(HSL) activity was measured in adipose tissue and adipocyte size, and HSL, lipoprotein lipase and liver X receptor gene expression in isolated adipocytes, on which lipolysis and glucose uptake experiments were also performed. Lipolysis was measured after incubation with tumour necrosis factor-α (TNFα), linoleic acid, and two conjugated linoleic acid isomers. Glucose uptake was analysed after TNFα or insulin administration. Our results show that FO replacement increased lipolytic activity and adipocyte cell size. The higher proportion of large cells observed in the 66VO group could be involved in their observed lower response to fatty acid treatments and lower insulin sensitivity. The 66VO/FO group showed a moderate return to the FO conditions. Therefore, FO replacement can affect the morphology and metabolism of gilthead sea bream adipocytes which could potentially affect other organs such as the liver.

1. INTRODUCTION

World aquaculture production has increased significantly in the last 50 years, with an average annual growth rate of 6.1 percent in volume between 2004 and 2006 (FAO, 2009). The high demand for fish meal to maintain aquaculture production is reaching a critical point (Tacon and Metian, 2008). The study of alternative sources of fish oil (FO) and fish meal for the vegetable components of fish feed has become important for the sustainability of aquaculture (Watanabe, 2002). However, vegetable oils (VO) are characterized by a low ratio of omega-3/omega-6 fatty acids, a lack of cholesterol and the presence of phytosterols. Although the VO has a lower ratio of n-3/n-6 fatty acids, different blends of vegetable oils are used trying to obtain a fatty acid composition more similar to a FO diet by means of the use of vegetable oils with a high n-3/n-6 ratio like linseed oil (Turchini et al., 2009). Particularly in gilthead sea bream, there is now experimental evidence that the large-scale replacement of marine feedstuffs with ingredients of plant origin alters the tissue-specific profile of fatty acids (Benedito-Palos et al., 2008; 2009, 2010) and results in changes in tissue body fat allocation (Saera-Vila et al., 2005) and lipogenic/lipolytic rates (Albalat et al., 2005a; Bouraoui et al., *In Press*). These observations in gilthead sea bream and other fish species (Drew et al., 2007; Menoyo et al., 2005; Todorčević et al., 2009; Torstensen et al., 2008; Turchini et al., 2009) have prompted the study of the lipid metabolism in fish in order to obtain a quality product in farmed fish fed alternative diets.

The mesenteric adipose tissue is an organ of fat accumulation in fish which, together with the muscle and liver, controls the lipid homeostasis and energetic balance of the animal (Jobling and Johansen, 2003; Sheridan and Kao, 1998). Adipose tissue stores lipids and provides energy from lipid stores. Triglycerides, which come from the diet, are hydrolyzed by lipoprotein lipase (LPL) and the fatty acids released are taken up by the adipocytes and accumulated in droplet form. In

response to energy demands, hormone-sensitive lipase (HSL), after phosphorylation by protein kinase A, can access the lipid droplet and hydrolyze the triglycerides into glycerol and fatty acids (Lafontan and Langin, 2009). In fish as in mammals, the development of adipose tissue is a continuous process which includes the hypertrophy of existing adipocytes and the proliferation of new ones. These processes are known to be affected by diet in mammals, but how dietary changes affect the capacity for enlargement of adipocytes or the differentiation of new ones is poorly understood in fish.

Adipose tissue also acts as an endocrine organ secreting adipokines which act as potent messengers to distant organs such as the liver and muscle to maintain the body's energy balance (Gregor and Hotamisligil, 2007). The pro-inflammatory cytokine tumour necrosis factor α (TNFα) is secreted by adipose tissue, among others, and in mammals it is known to act as an adiposity limiting factor and to lead to obesity-induced insulin resistance (Nieto-Vazquez et al., 2008). TNFα also acts by limiting the fat deposits in gilthead sea bream mesenteric adipose tissue (Saera-Vila et al., 2007), it inhibits adipocyte differentiation in rainbow trout (Bouraoui et al., 2008) and it stimulates lipolysis in rainbow trout adipocytes (Albalat et al., 2005b). It has been suggested that excessive lipid accumulation in the liver or steatosis is due in part to the increased hepatic uptake of fatty acids released from adipose tissue with enhanced lipolysis (Benedito-Palos et al., 2008). Therefore, changes in adipose tissue metabolism could affect other organs in fish.

The present study is part of multidisciplinary research performed on gilthead sea bream and uses the same diets and animals as in the study of Benedito-Palos et al. (2009). That study reports that FO replacement is reflected in the fatty acid profile of the fish fillet, while a final FO refeed (finishing diet) restores the profile of the FO diet in the fish fillet of animals fed with different degrees of FO substitution (Benedito-Palos et al., 2009). Such results confirm that it is possible to partially substitute the FO in a plant protein based diet without affecting growth, while changing the muscle fatty acid profile. Nevertheless, although this diet did not produce apparent damage to the intestinal and hepatic architecture, the hepatosomatic index (HSI) was increased in animals fed the 66VO diet. This suggests an increase in hepatic lipid content, as previously observed in gilthead sea bream fed vegetable diets (Benedito-Palos et al., 2008). Therefore, the objective of the present study is to analyse the effect of vegetable dietary treatment (66VOdiet) and a finishing diet on the lipid metabolism of adipose tissue, which besides being a fat reservoir, also regulates the energy balance in the organism and could be involved in dysregulation of this lipid metabolism. We hypothesize that dietary vegetable oil and subsequent changes in tissue fatty acid composition can affect the metabolism of adipocytes and proliferation of new cells. To this end, we focused on the effects of diet on adipocyte cell size,

adipose tissue lipolytic activity and gene expression of lipolytic/accumulation markers in response to hormones and fatty acids in isolated adipocytes.

2. MATERIAL AND METHODS

2.1 Animals and diets

Juvenile gilthead sea bream (*Sparus aurata L.*) of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were acclimated to laboratory conditions at the Institute of Aquaculture Torre de la Sal (IATS) for 20 days before the study. After this initial acclimation period, fish of 18 g mean initial body weight were distributed into six fibreglass tanks (3000 L each) in groups of 150 fish each, where they were kept from July 2006 to September 2007 (as described in Benedito-Palos et al. (2009)). Fish were fed either a FO reference diet or a blend of VO (17:58:25 of rapeseed oil: linseed oil: palm oil) which replaced 66% of the FO (66 VO diet). Table 1 shows the composition of the diets (for more details on diet composition see Benedito-Palos et al., 2009).

Twelve weeks before the end of the trial, two tanks with animals fed until then with the 66VO diet were switched to the FO diet (66VO/FO group). The fish in the other 4 tanks continued to be fed with the FO or 66VO diet, as they had been until that time. At sampling, 18 fish per dietary treatment were randomly selected and killed by a blow to the head. Mesenteric adipose tissue was excised, and a piece was frozen for enzymatic activity analysis. The remaining tissue was processed for adipocyte isolation

2.2 Adipocyte isolation and lipolysis measurement

Adipocytes were isolated and pooled from six fish for each independent lipolysis experiment performed on each dietary group (n=3), 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 ºC 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) and 1% bovine serum albumin (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 $7 \cdot 10^5$ cells/mL. 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 ºC in the absence or presence (100 ng/mL) of recombinant human TNFα (Sigma-Aldrich, Madrid, Spain), linoleic acid (LA, 100 μM; Matreya, USA), *trans*-10, *cis*-12 conjugated linoleic acid (t10,c12-CLA, 100 μM; Matreya), *cis*-9, *trans*-11 conjugated linoleic acid (c9,t11-CLA; Matreya).

At the end of the incubation time, 300 µL of the medium was placed in perchloric acid to give a final concentration of 7% (v/v). The perchloric acid was neutralized 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 a lysis reagent was added for RNA extraction (see below). Control and experimental groups were studied in triplicate for each experiment.

2.3 RNA isolation and cDNA synthesis

Total RNA was extracted from the adipocytes by the TriReagent method (Sigma-Aldrich). The quantity and quality of the isolated RNA was determined by spectrophotometry with ND-1000 Nanodrop (Labtech Int., UK). For cDNA synthesis, 500 ng of RNA, 3 µL of a blend 2:1 random hexamers (600 µM)/oligo-dT (50 µM), 2 µL dNTP (10 mM), 0.5 µL of reverse transcriptase (20 U/µL), and 0.5 µL of RNAse inhibitor (40 U/µL) were mixed with the kit buffer in a final volume of 20 µL (Transcriptor first strand cDNA synthesis kit, Roche, Germany), and incubated at 50 ºC for 60 min, followed by 85 ºC for 5 min to inactivate the enzymes.

2.4 Real-time PCR assays

PCR measurements were performed applying the primers at 0.5 µM with one fortieth of the cDNA synthesis reaction and SYBR-green PCR mix (BioRad, Spain) in a total volume of 20 µL. The RT-PCR primer sequences for target genes (LXR, LPL and HSL) and reference gene (β-actin) are shown in Table 2. Reactions were performed in a MyiQ PCR Detection System (Bio-Rad, Spain) in duplicate, and the fluorescence data acquired during the extension phase were normalized to β-actin by the delta-delta method (Livak and Schmittgen, 2001).

2.5 Glucose uptake assay

In the isolated adipocytes, glucose transport was determined as previously described (Capilla et al., 2004). Adipocytes ($2.5 \cdot 10^5$ cells/mL) were incubated in Krebs buffer in the presence or absence of recombinant human insulin (100 nM, Sigma-Aldrich) or recombinant human TNFα (100 ng/mL, Sigma-Aldrich) for 30 min at 22 °C in a shaking water bath. Subsequently, radiolabelled 2-deoxy-D-[^{3}H] glucose (0.8 µCi) was added, and transport was stopped after 2 h with cytochalasin B. The transport assay was terminated by transferring a 200-µL aliquot of the cell suspension into small polyethylene microcentrifuge tubes containing 150 µL of dinonylphthalate. Cells and buffer were separated by centrifugation at 16,000 g for 2 min. The upper phase, which contained the adipocytes, was collected and subjected to liquid scintillation counting. All uptake data were corrected by substracting the extracellularly and cell-associated radioactivity background by means of measuring the non-specific uptake in cell pre-treated with

cytochalasin B.

2.6 Cell analysis

Adipocytes from mesenteric adipose tissue of animals fed with FO, 66VO and 66VO/FO diets were isolated. Four different pictures from each isolation procedure (n=3 for each experimental group) were taken of the preparations of isolated adipocytes in a Fuchs Rosenthal counting chamber using a digital camera coupled to a microscope (Olympus BX51). All the pictures were acquired using a 10x objective. The cell diameters were measured using image analysis software (analySYS®, Soft Imaging System).

2.7 HSL activity assay

HSL assays are based on the measurement of the release of [^{3}H]oleic acid from tri[^{3}H]olein, which is a measurement of the activated form of HSL influenced by phosphorylation by pKA. The HSL activity assay was performed as previously described (Fredrikson et al., 1981; Østerlund et al., 1996) with minor modifications. Tissue samples were homogenized in three volumes of 0.25 M sucrose buffer containing 1 mM EDTA, 1 mM dithioerythreitol, 20 μg/mL antipain and leupeptin, pH 7.4. Infranatants were obtained by centrifugation at 110,000 g for 45 min at 4 ºC. The protein content of the infranatant was measured by the Bradford method (Bradford, 1976). The tri[^{3}H]olein substrate was emulsified with phospholipids by sonication, and BSA was used as the fatty acid acceptor. Samples of 10 μL of adipose infranatants were incubated in triplicate for 2 h at 18 ºC with 100 μL of 5 mM of tri[^{3}H]olein substrate and enzyme dilution buffer (total volume 200 μL). Hydrolysis was stopped by the addition of 3.25 mL of methanol/chloroform/heptane (10:9:7), followed by 1.1 mL of 0.1 M potassium carbonate/0.1 M boric acid (pH 10.5). The mixture was centrifuged for 20 min at 1,100 g and 1 mL of the upper phase was collected and subjected to liquid scintillation counting. Radioactivity was measured with a TRICARB 2100 β-counter (Packard Bioscience Company, Meriden, CT, USA). The disintegrations per minute (DPM) were referred to protein content to compare the HSL activity between groups.

2.8 Plasma parameters

Plasma glucose concentration was determined by a glucose oxidase colorimetric method (Spinreact, Spain). Plasma-free fatty acids and triglycerides were analysed using commercial enzymatic methods (Spinreact, Spain).

2.9 Statistical analysis

Statistical analysis was performed using the SigmaStat software. Data are presented as means ± standard error of the mean (S.E.M). Results were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test. A significance of $p<0.05$ was applied to all statistical tests performed.

3. RESULTS

3.1 Effect of diet on biometrics and plasma metabolites

Biometric parameters were measured at the sampling time of the gilthead sea bream fed with FO, 66VO and 66VO/FO diets (Table 3). Liver weight and HSI values were higher in 66VO and 66VO/FO groups than in the FO group. However, the 66VO/FO group showed a tendency to recover the liver weight of the FO group. No significant differences were found in adipose tissue weight or mesenteric fat index (MFI) between groups.

Plasma glucose and triglycerides did not show any differences between the groups. However, the non-esterified fatty acids (NEFAs) levels were lower in the 66VO/FO group than in 66VO.

3.2 Cell size distribution

The cell size of each experimental group was divided into four ranges: 1-20 μm, 20-60 μm, 60-100 μm and over 100 μm (Fig. 1). The adipocytes in the 1-20 μm range were the most abundant in all groups. However, the FO group showed smaller adipocytes, with a considerably higher proportion of cells in the 1-20 μm range than the 66VO group; while the value for the 66VO/FO group was between those of the FO and 66VO groups. The 66VO group had the biggest cells, with a high frequency of cells in the ranges 20-60 μm and 60-100 μm. Conversely, FO group showed smallest cells, and the 66VO/FO had intermediate values in general between the other diet groups.

3.3 Effect of diet on hormone-sensitive lipase activity

The HSL activities in mesenteric adipose tissue of the gilthead sea bream fed with FO, 66VO and 66VO/FO diets are shown in Fig. 2. The HSL activity was higher in adipose tissue from the 66VO and 66VO/FO groups than in that from the FO group. However, the 66VO/FO group showed a lower induction of HSL activity than the 66VO group did.

3.4 Basal lipolysis levels and the effect of incubation with TNFα, LA, c9,t11-CLA and t10,c12-CLA on lipolysis.

The measurements of basal lipolysis levels in adipocytes from animals fed FO, 66VO and 66VO/FO showed that the animals fed vegetable oils have higher basal lipolysis levels than the animals fed a FO diet (Fig. 3A).

The cells incubated with the different treatments showed that the lipolysis level was increased in the FO group with the LA and the t10,c12-CLA (Fig. 3B). The 66VO group adipocyte lipolysis was stimulated with TNFα (Fig. 3C), meanwhile, the 66VO/FO group showed lipolysis induction by LA but not by t10,c12-CLA (Fig. 3D).

Stimulation with TNFα tended to increase lipolysis in the 66VO/FO group, but it was not significantly different from the control. Therefore, the 66VO/FO group seems to present an intermediate response to the treatments between FO and 66VO groups (Fig. 3).

Comparisons of the effect of the treatments described below between the diet groups showed that the induction of LA and t10,c12-CLA in the FO group is significant in comparison with the 66VO group (Fig. 3B and 3C). However, the induction of lipolysis mediated by TNFα in the 66VO group is not significantly different from 66VO and 66VO/FO groups.

3.5 LPL, HSL and LXR expression profile in isolated adipocytes from animals fed with FO, 66VO and 66VO/FO diets.

The LPL, HSL and LXR gene expressions were analysed in the adipocytes from the three diet groups (incubated in BSA-Krebs buffer only). The LPL did not show any change between diets, but HSL and LXR expressions were differently regulated in FO, 66VO and 66VO/FO groups (Fig. 4A, B, C). HSL gene transcription showed induction in the 66VO and 66VO/FO groups, with a pattern similar to that of HSL activity (Fig. 2). Conversely, LXR was downregulated in the 66VO and 66VO/FO groups.

3.6 *Effect of incubation with TNFα, LA, c9,t11-CLA and t10,c12-CLA on LPL, HSL and LXR gene expression in isolated adipocytes from animals fed with FO, 66VO and 66VO/FO diets.*

The LPL gene expression in the adipocytes from FO group was upregulated by LA and t10,c12-CLA (Fig. 4D). In the 66VO and 66VO/FO groups, the LPL gene expression was not regulated by any treatment (Fig. 4E, F). In contrast, HSL showed a slight upregulation with LA and t10,c12-CLA in the FO group (Fig. 4D), but these tendencies were not significant. However, the 66VO/FO group showed upregulation of HSL by LA (Fig. 4F). Neither TNFα nor fatty acids changes HSL mRNA levels in the 66VO adipocytes (Fig. 4E). The LXR gene expression showed downregulation and upregulation by LA in the FO and 66VO/FO groups respectively (Fig. 4D, F).

The differences in gene expression levels between the three genes were also observed in each

280 experimental group (Fig. 4D, E, F). Data of gene expression are expressed in relation to the gene
281 with lowest level of expression. The results show that the differences of expression between
282 LPL and HSL are reduced in the 66VO and 66VO/FO because the HSL is expressed at higher
283 levels in these groups (Fig. 4 A) but the LPL expression is not modulated by diet (Fig. 4C).
284 Differences are also observed in the LXR expression profile regarding to the other genes (Fig.
285 4D, E, F).
286 The effect of the treatment in the different diets was also analysed (Fig. 4G). The upregulation
287 of LPL expression by LA observed in the FO group was also maintained when this effect is
288 compared between diets. However the TNFα and c9,t11-CLA did not show significant
289 differences in LPL expression in each experimental group (in comparison with controls). When
290 the effects of these treatments are compared between diets, both showed higher induction of
291 LPL in the FO group than in the 66VO and 66VO/FO groups. The effect of t10,c12-CLA
292 treatment on LPL expression showed differences between FO and 66VO/FO. These results
293 demonstrate that the LPL expression was more affected by the treatments in the control group
294 (FO) than in 66VO and 66VO/FO groups.
295 The HSL expression analyses after the treatments did not show differences between the dietary
296 groups (Fig.4G).
297 The LXR expression analyses showed differences after the LA treatment between the FO and
298 66VO/FO groups as previously observed in comparison with their controls in both groups (Fig.
299 4D, F).
300
301 *3.7 Basal levels of glucose uptake and effects of insulin and TNFα on glucose uptake of isolated*
302 *adipocytes from gilthead sea bream fed with FO, 66VO and 66VO/FO diets.*
303 The basal levels of glucose uptake did not show differences between dietary groups (Fig. 5A).
304 However, after the treatment with insulin only the FO group adipocytes revealed an
305 upregulation of glucose uptake (Fig. 5B, C and D). TNFα did not regulate glucose uptake in
306 adipocytes from any of the three experimental groups.
307
308 **4. DISCUSSION**
309
310 In the present study, although the fish showed good growth performance (Benedito-Palos et al.,
311 2009), the HSI of the animals fed the 66VO diet was greater than that of the FO group, which
312 agrees with the fat accumulation previously observed (Benedito-Palos et al., 2008). This finding
313 indicates that the animals could present a certain degree of lipid metabolism dysregulation and
314 we focus on the morphological, enzymatic activity and gene expression changes of adipose

315 tissue produced by FO replacement in diets with already 75% plant protein together with
316 possible recovery through FO refeeding. Analysing these alterations may make it possible to
317 understand the different lipid metabolism changes produced in other tissues.
318 Gilthead sea bream fed the 66VO diet showed a ~67% increase in HSL activity compared to the
319 FO group. The group with the finishing diet (66VO/FO) also had high levels of HSL activity
320 (~41% increase with regard to the FO group) but slightly lower than the 66VO group. Therefore,
321 the adipose tissue from the 66VO and 66VO/FO groups seems to have more lipolytic activity
322 than that from the FO group. Previous studies of gilthead sea bream have shown that plant
323 components increase basal lipolysis levels in adipocytes (Albalat et al., 2005a) as well as it has
324 been observed in the measurements of the basal lipolysis levels in isolated adipocytes from
325 animals fed vegetable oils (66VO and 66VO/FO groups). This is in agreement with the present
326 results showing enhanced HSL activity. Furthermore, HSI and liver weight values were higher
327 in the 66VO and 66VO/FO groups, suggesting that the fatty acids released from adipose tissue
328 could produce lipid accumulation in the liver, as previously suggested (Benedito-Palos et al.,
329 2008), as has been described in humans (Browning and Horton, 2004). Hepatic triglyceride
330 accumulation is a consequence of human obesity and the derived insulin resistance, which
331 produces a release of fatty acids coming from the increased adipose mass and HSL activity. In
332 turn, this may lead to lipotoxicity in the liver (Browning and Horton, 2004; Lewis et al., 2002).
333 Moreover, liver lipid deposition with vegetable diets has also been related to the increase of
334 lipogenesis caused by the decrease of n-3 polyunsaturated fatty acids (PUFA) (Arzel et al.,
335 1994; Robaina et al., 1998) or alterations of β-oxidation (Izquierdo et al., 2003). However,
336 previous studies of gilthead sea bream showed that the inclusion of VO in the diet did not
337 modify hepatic lipogenic activity (Bouraoui et al., *In Press*). Therefore, these results seem to
338 suggest that the liver lipids come from the adipose tissue.
339 Fat accumulation in the adipose tissue of gilthead sea bream is regulated seasonally, with a
340 tendency for fat deposits to increase during spring, favoured by the upregulation of LPL (Saera-
341 Vila et al., 2005). Subsequent high levels of TNFα expression appear to act as a limiting factor
342 of adiposity. In mammals, it has been reported that TNFα production is linked to adipocyte size
343 which thus limits lipid accumulation (Skurk et al., 2007). An increase in adipokine secretion
344 (e.g., TNFα, IL-6 and IL-8), low cell sensitivity, different patterns of protein expression,
345 accumulation of fatty acids in non-adipose tissue and insulin resistance have all been described
346 as related to adipocyte enlargement in mammals (Blüher et al., 2004; Östman et al., 1975; Skurk
347 et al., 2007). In gilthead sea bream, FO replacement resulted in an increase in adipose cell size,
348 with a partial recovery in the 66VO/FO group. However, the MFI and total mesenteric adipose
349 tissue did not show any differences between groups. Therefore, the adipose tissue undergoes

morphological changes through the enlargement of the cells with the VO diet. The increased cell size in the 66VO and 66VO/FO groups could be related to adipocyte metabolic changes. In mammals, a positive correlation between cell size and increased lipolysis rates, and HSL activity and expression has been demonstrated (Berger and Barnard, 1999; Farnier et al., 2003; Gregor and Hotamisligil, 2007). In keeping with this, high HSL activity and mRNA expression was found in the 66VO and 66VO/FO groups, which also had larger cells. However, LPL transcription levels did not show any differences between the groups, in agreement with previous studies of rat adipocytes, in which the increase in adipocyte size induced LPL activity but not its expression (Farnier et al., 2003).

The enlargement of adipocytes and increased lipolysis reflect a defective storage capacity in adipose tissue deposits, as occurs in humans during situations of lipodystrophy or obesity, although such deficiency develops differently (Slawik and Vidal-Puig, 2006). Furthermore, hypertrophy of adipocytes is considered a marker of failure in the mechanism of preadipocyte recruitment to develop new (and smaller) adipocytes (Medina-Gomez and Vidal-Puig, 2005). The capacity of adipose tissue to expand through hyperplastic changes is critical for accommodating changes in the availability of energy and type of diet. Studies *in vitro* of salmon adipocytes indicate that incubation with different fatty acids induces different degrees of lipid filling as cellular triglycerides, supporting our conclusion that the composition of dietary fatty acids can affect adipocyte enlargement (Todorčević et al., 2008).

However, LXR gene expression showed downregulation with the incorporation of VO into the diet and the FO refeed did not lead to a recovery of LXR gene expression. LXR is a transcription factor recently described in gilthead sea bream and salmonids (Cruz-Garcia et al., 2009a; Cruz-Garcia et al., 2009b) and it is known in mammals to be involved in cholesterol, fatty acid and glucose metabolism (Mitro et al., 2007; Repa et al., 2000; Schultz et al., 2000; Steffensen and Gustafsson, 2004). In salmonids the LXR transcription is downregulated in the liver by VO diets (Cruz-Garcia et al., 2009a) as it is in gilthead sea bream adipocytes from the 66VO group. LXR is a cholesterol sensor (Steffensen and Gustafsson, 2004) and can be regulated by fatty acids (Pawar et al., 2002). In this way, the higher proportion of n-6 fatty acids and less n-3 PUFA, the presence of phytosterol or, most probably, the small proportion of cholesterol in the 66VO diet, could be some of the causes of the LXR downregulation. Nevertheless, from our results we cannot elucidate which is the precise cause of the changes in LXR expression. In mammals, the increase in adipocyte size produces an inhibition in cholesterol-sensing (de Ferranti and Mozaffarian, 2008), so this could also be one of the factors involved in the downregulation of LXR in the 66VO and 66VO/FO groups.

The enlargement of adipocytes decreases the sensitivity of mammalian cells to insulin action

385 and lipolytic stimulators such as isoproterenol, and makes the cells less dynamic in their
386 responses (Frayn et al., 2007; Jolly et al., 1978; Östman et al., 1975; Smith, 1971). This lack of
387 response associated with the cell morphology seems to be reflected in the incubations with fatty
388 acids of isolated adipocytes from gilthead sea bream. CLAs, omega-6 fatty acids and isomers of
389 linoleic acid, have been widely studied in mammals (Campbell and Kreider, 2008; Churruca et
390 al., 2009; Evans et al., 2002). The t10,c12-CLA prevents the development of adiposity, reduces
391 the triglyceride content in adipocytes and increases lipolysis (Chung et al., 2005). This last
392 effect of t10,c12-CLA, upregulating lipolysis, is reflected in the gilthead sea bream adipocytes
393 from the FO group. However c9,t11-CLA did not modulate the basal lipolysis in the adipocytes
394 from any of the three diet groups as it has been demonstrated to do in mammals (Chung et al.,
395 2005). The FO group showed an induction of lipolysis with LA and t10,c12-CLA, but the 66VO
396 group did not, while the 66VO/FO group responded to LA, but not to t10,c12-CLA. The lower
397 response to treatments of the 66VO group adipocytes is also observed in the gene expression of
398 LPL, HSL and LXR after fatty acid incubations. This different response to the fatty acids in the
399 66VO adipocytes could be related to the different dietary fatty acid composition. It has been
400 observed in mammals that the variation of the fatty acid composition in the diet produce changes
401 in the fatty acid profile of the tissue affecting the adipocyte metabolism. The changes in the
402 adipocyte metabolism can affect the efficiency of substrate accumulation (Su and Jones, 1993),
403 the permeability of the cell membrane, the ability to recognize extracellular molecules like
404 hormones (Hagve, 1988) and the hyperplasia of the tissue (Launy et al., 1972; Soriguer Escofet
405 et al., 1996). Therefore, this lack of response to the treatments observed in the lipolytic levels
406 and gene expression in adipocytes from animals fed wih the VO diet might be associated to the
407 changes in the fatty acid profile of the tissue.
408 The lipolytic response to TNFα in gilthead sea bream has been demonstrated to show
409 differences linked in part to the degree of fish adiposity (Cruz-Garcia et al., 2009b). The relation
410 between TNFα and adipose tissue has been linked to adipocyte size in mammals, and TNFα has
411 been considered a limiting regulator of adipocyte hypertrophy in fish, as in mammals (Saera-
412 Vila et al., 2007; Skurk et al., 2007). Therefore, it is not surprising that the 66VO group
413 adipocytes, with a larger size than those of the other groups, when incubated with TNFα
414 increased their lipolysis level to prevent further hypertrophy, whereas the FO and 66VO/FO
415 group adipocytes did not.
416 At the same time, the hormonal regulation of glucose uptake showed an upregulation by insulin
417 in the FO group but the 66VO and 66VO/FO groups showed no response. In mammals, a lack of
418 response to insulin and consequently a reduction in glucose uptake has been correlated with fat
419 cell size, as observed in the characteristic disorder of insulin resistance defined in mammals (de

Ferranti and Mozaffarian, 2008; Maffeis et al., 2007).

In conclusion, in the present study we demonstrate that FO replacement changes the lipid metabolism through altering the adipocyte cell size, lipolytic rates and response to fatty acids and hormones. These effects can be moderately reversed by means of a final finishing diet with a FO diet. These metabolic changes can potentially affect other organs such as the liver. However, the total adipose tissue content was not modified by FO replacement. Therefore, the same content of adipose tissue between the different groups jointly with an increase of the cell size in animals fed vegetable oils suggest that the vegetable oils might be affect the hyperplasia of the tissue and consequently producing a hypertrophy of the cells and an adipose tissue metabolism alteration. The lack or reduction in the hyperplasia of the tissue has been associated to changes in the dietary fatty acid profile in humans (Garaulet et al., 2006). Therefore, the different fatty acids profile present in the vegetable oils could be the main factor responsible of the alterations observed in the adipose tissue in the present study. Further study of the effects of dietary FO replacement on adipose tissue could help clarify how this tissue, besides acting as a fat reservoir, contributes to maintaining energy balance and metabolic health.

ACKNOWLEDGEMENTS

LC was funded by a PhD fellowship (BES-2005-9566) from the Spanish *Ministerio de Educación y Ciencia* (project AGL-2004-06319-C02-02). This work was supported by the *Generalitat de Catalunya* Aquaculture R&D and Innovation Reference Network (XRAq) and the *Comisión Internacional de Ciencia y Tecnología* of Spain (CICYT).

REFERENCES

Albalat, A., Gómez-Requeni, P., Rojas, P., Médale, F., Kaushik, S., Vianen, G.J., Van den Thillart, G., Gutiérrez, J., Pérez-Sánchez, J., Navarro, I., 2005a. Nutritional and hormonal control of lipolysis in isolated gilthead seabream (*Sparus aurata*) adipocytes. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289, R259-265.

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

Benedito-Palos, L., Navarro, J.C., Bermejo-Nogales, A., Saera-Vila, A., Kaushik, S., Pérez-Sánchez, J., 2009. The time course of fish oil wash-out follows a simple dilution model in gilthead sea bream (*Sparus aurata L.*) fed graded levels of vegetable oils. Aquaculture 288, 98-105.

Benedito-Palos, L., Navarro, J.C., Kaushik, S., Pérez-Sánchez, J., 2010. Tissue-specific robustness of fatty acid signatures in cultured gilthead sea bream (*Sparus aurata* L.) fed practical diets with a combined high replacement of fish meal and fish oil. J. Anim. Sci. 88, 1759-1770.

Benedito-Palos, L., Navarro, J.C., Sitjà-Bobadilla, A., Gordon Bell, J., Kaushik, S., 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.

Berger, J.J., Barnard, R.J., 1999. Effect of diet on fat cell size and hormone-sensitive lipase activity. J. Appl. Physiol. 87, 227-232.

Blüher, M., Wilson-Fritch, L., Leszyk, J., Laustsen, P.G., Corvera, S., Kahn, C.R., 2004. Role of insulin action and cell size on protein expression patterns in adipocytes. J. Biol. Chem. 279, 31902-31909.

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

Bouraoui, L., Sánchez-Gurmaches, J., Cruz-Garcia, L., Gutiérrez, J., Benedito-Palos, L., Pérez-Sánchez, J., Navarro, I., *In Press*. Effect of dietary fish meal and fish oil replacement on lipogenic and lipoprotein lipase activities and plasma insulin in gilthead sea bream (*Sparus aurata*). Aquacult. Nutr. doi: 10.1111/j.1365-2095.2009.00706.x.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Browning, J.D., Horton, J.D., 2004. Molecular mediators of hepatic steatosis and liver injury. J. Clin. Invest. 114, 147-152.

Campbell, B., Kreider, R.B., 2008. Conjugated linoleic acids. Curr. Sports Med. Rep. 7, 237-241.

Capilla, E., Diaz, M., Albalat, A., Navarro, I., Pessin, J.E., Keller, K., Planas, J.V., 2004. Functional characterization of an insulin-responsive glucose transporter (GLUT4) from fish adipose tissue. Am. J. Physiol. Endocrinol. Metab. 287, E348-357.

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

Cruz-Garcia, L., Saera-Vila, A., Navarro, I., Calduch-Giner, J., Pérez-Sánchez, J., 2009b. Targets for TNFalpha-induced lipolysis in gilthead sea bream (*Sparus aurata* L.) adipocytes isolated from lean and fat juvenile fish. J. Exp. Biol. 212, 2254-2260.

Chung, S., Brown, J.M., Sandberg, M.B., McIntosh, M., 2005. Trans-10,cis-12 CLA increases adipocyte lipolysis and alters lipid droplet-associated proteins: role of mTOR and ERK signaling. J. Lipid Res. 46, 885-895.

Churruca, I., Fernández-Quintela, A., Portillo, M.P., 2009. Conjugated linoleic acid isomers: Differences in metabolism and biological effects. Biofactors 35, 105-111.

de Ferranti, S., Mozaffarian, D., 2008. The perfect storm: Obesity, adipocyte dysfunction, and metabolic consequences. Clin. Chem. 54, 945-955.

Drew, M.D., Ogunkoya, A.E., Janz, D.M., Kessel, A.G.V., 2007. Dietary influence of replacing fish meal and oil with canola protein concentrate and vegetable oils on growth performance, fatty acid composition and organochlorine residues in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 267, 260-268.

Evans, M.E., Brown, J.M., McIntosh, M.K., 2002. Isomer-specific effects of conjugated linoleic acid (CLA) on adiposity and lipid metabolism. J. Nutr. Biochem. 13, 508-516.

FAO, 2009. The State of World Fisheries and Aquaculture 2008. Food and Agriculture Organization of the United Nations (FAO), Rome.

Farnier, C., Krief, S., Blache, M., Diot-Dupuy, F., Mory, G., Ferre, P., Bazin, R., 2003. Adipocyte functions are modulated by cell size change: potential involvement of an integrin/ERK signalling pathway. Int. J. Obes. Relat. Metab. Disord. 27, 1178-1186.

Frayn, K.N., Tan, G.D., Karpe, F., 2007. Adipose tissue: a key target for diabetes pathophysiology and treatment? Horm. Metab. Res. 39, 739-742.

Fredrikson, G., Strålfors, P., Nilsson, N.O., Belfrage, P., 1981. Hormone-sensitive lipase of rat adipose tissue. Purification and some properties. J. Biol. Chem. 256, 6311-6320.

Garaulet, M., Hernandez-Morante, J.J., Lujan, J., Tebar, F.J., Zamora, S., 2006. Relationship between fat cell size and number and fatty acid composition in adipose tissue from different fat depots in overweight/obese humans. Int. J. Obes. (Lond) 30, 899-905.

Gregor, M.F., Hotamisligil, G.S., 2007. Thematic review series: Adipocyte Biology. Adipocyte stress: the endoplasmic reticulum and metabolic disease. J. Lipid Res. 48, 1905-1914.

Hagve, T.A., 1988. Effects of unsaturated fatty acids on cell membrane functions. Scand. J. Clin. Lab. Invest. 48, 381-388.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L., Rosenlund, G., 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. Aquacult. Nutr. 9, 397-407.

Jobling, M., Johansen, S.J.S., 2003. Fat distribution in Atlantic salmon *Salmo salar* L. in relation to body size and feeding regime. Aquacult. Res. 34, 311-316.

Jolly, S.R., Lech, J.J., Menahan, L.A., 1978. Influence of genetic obesity in mice on the lipolytic response of isolated adipocytes to isoproterenol and ACTH-(1-24). Horm. Metab. Res. 10, 223-227.

Lafontan, M., Langin, D., 2009. Lipolysis and lipid mobilization in human adipose tissue. Prog. Lipid Res. 48, 275 - 297.

Launy, M., Richard, M., Alavioni, R., 1972. Excess of linoleic acid and incorporation of radioactive precursors and RNA of adipose ce!ls. Nutr. Rep. Int. 5, 339-348.

Lewis, G.F., Carpentier, A., Adeli, K., Giacca, A., 2002. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. Endocr. Rev. 23, 201-229.

Livak, K.J., 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.

Maffeis, C., Silvagni, D., Bonadonna, R., Grezzani, A., Banzato, C., Tatò, L., 2007. Fat Cell Size, Insulin Sensitivity, and Inflammation in Obese Children. J. Pediatr. 151, 647-652.

Medina-Gomez, G., Vidal-Puig, A., 2005. Gateway to the metabolic syndrome. Nat. Med. 11, 602-603.

Menoyo, D., Lopez-Bote, C.J., Obach, A., Bautista, J.M., 2005. Effect of dietary fish oil substitution with linseed oil on the performance, tissue fatty acid profile, metabolism, and oxidative stability of Atlantic salmon. J. Anim Sci. 83, 2853-2862.

Mitro, N., Mak, P.A., Vargas, L., Godio, C., Hampton, E., Molteni, V., Kreusch, A., Saez, E., 2007. The nuclear receptor LXR is a glucose sensor. Nature 445, 219-223.

Nieto-Vazquez, I., Fernandez-Veledo, S., de Alvaro, C., Lorenzo, M., 2008. Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. Diabetes 57, 3211-3221.

Østerlund, T., Danielsson, B., Degerman, E., Contreras, J.A., Edgren, G., Davis, R.C., Schotz, M.C., Holm, C., 1996. Domain-structure analysis of recombinant rat hormone-sensitive lipase. Biochem. J. 319 411-420.

Östman, J., Backman, L., Hallberg, D., 1975. Cell size and the antilipolytic effect of insulin in human subcutaneous adipose tissue. Diabetologia 11, 159-164.

Pawar, A., Xu, J., Jerks, E., Mangelsdorf, D.J., Jump, D.B., 2002. Fatty acid regulation of liver X receptors (LXR) and peroxisome proliferator-activated receptor alpha (PPARalpha) in HEK293 cells. J. Biol. Chem. 277, 39243-39250.

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

552 Saera-Vila, A., Calduch-Giner, J.A., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sánchez, J.,
553 2005. Molecular characterization of gilthead sea bream (*Sparus aurata*) lipoprotein lipase.
554 Transcriptional regulation by season and nutritional condition in skeletal muscle and fat storage
555 tissues. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 142, 224-232.
556 Saera-Vila, A., Calduch-Giner, J.A., Navarro, I., Pérez-Sánchez, J., 2007. Tumour necrosis factor (TNF-
557 alpha) as a regulator of fat tissue mass in the Mediterranean gilthead sea bream (*Sparus aurata* L.).
558 Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 146, 338-345.
559 Schultz, J.R., Tu, H., Luk, A., Repa, J.J., Medina, J.C., Li, L., Schwendner, S., Wang, S., Thoolen, M.,
560 Mangelsdorf, D.J., Lustig, K.D., Shan, B., 2000. Role of LXRs in control of lipogenesis. Genes
561 Dev. 14, 2831-2838.
562 Sheridan, M.A., Kao, Y.-H., 1998. Regulation of Metamorphosis-Associated Changes in the Lipid
563 Metabolism of Selected Vertebrates. Am. Zool. 38, 350-368.
564 Skurk, T., Alberti-Huber, C., Herder, C., Hauner, H., 2007. Relationship between adipocyte size and
565 adipokine expression and secretion. J. Clin. Endocrinol. Metab. 92, 1023-1033.
566 Slawik, M., Vidal-Puig, A.J., 2006. Lipotoxicity, overnutrition and energy metabolism in aging. Ageing
567 Res. Rev. 5, 144-164.
568 Smith, U., 1971. Effect of cell size on lipid synthesis by human adipose tissue in vitro. J. Lipid Res. 12,
569 65-70.
570 Soriguer Escofet, F.J., Esteva de Antonio, I., Tinahones, F.J., Pareja, A., 1996. Adipose tissue fatty acids
571 and size and number of fat cells from birth to 9 years of age--a cross-sectional study in 96 boys.
572 Metabolism 45, 1395-1401.
573 Steffensen, K.R., Gustafsson, J.-Å., 2004. Putative metabolic effects of the liver X receptor (LXR).
574 Diabetes 53, S36-S42.
575 Su, W., Jones, P.J., 1993. Dietary fatty acid composition influences energy accretion in rats. J. Nutr. 123,
576 2109-2114.
577 Tacon, A.G.J., Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially
578 compounded aquafeeds: Trends and future prospects. Aquaculture 285, 146-158.
579 Tebar, F., Soley, M., Ramírez, I., 1996. The antilipolytic effects of insulin and epidermal growth factor
580 in rat adipocytes are mediated by different mechanism. Endocrinology. 137, 4181-4188.
581 Todorčević, M., Kjær, M.A., Djakovic, N., Vegusdal, A., Torstensen, B.E., Ruyter, B., 2009. N-3
582 HUFAs affect fat deposition, susceptibility to oxidative stress, and apoptosis in Atlantic salmon
583 visceral adipose tissue. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 152, 135 - 143.
584 Todorčević, M., Vegusdal, A., Gjøen, T., Sundvold, H., Torstensen, B.E., Kjaer, M.A., Ruyter, B., 2008.
585 Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; effects
586 of n-3 and n-9 fatty acids. Biochim. Biophys. Acta 1781, 326-335.
587 Torstensen, B.E., Espe, M., Sanden, M., Stubhaug, I., Waagbø, R., Hemre, G.I., Fontanillas, R.,
588 Nordgarden, U., Hevrøy, E.M., Olsvik, P., Berntssen, M.H.G., 2008. Novel production of Atlantic
589 salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant
590 meal and vegetable oil blends. Aquaculture 285, 193-200.
591 Turchini, G.M., Torstensen, B.E., Ng, W.-K., 2009. Fish oil replacement in finfish nutrition. Reviews in
592 Aquaculture 1, 10-57.
593 Watanabe, T., 2002. Strategies for further development of aquatic feeds. Fish. Sci. 68, 242-252.
594
595 **Figure legends**
596
597 **Figure 1. Adipocyte cell size distribution of gilthead sea bream fed with FO, 66VO and**

598 **66VO/FO diets.** Data are shown as mean ± S.E.M. (n=3). Results were analysed by one-way
599 analysis of variance (ANOVA), followed by Tukey's test. Values that do not share a common
600 letter are significantly different (P<0.05).
601
602 **Figure 2. Hormone-sensitive lipase (HSL) activity in adipose tissue of gilthead sea bream**
603 **fed with FO, 66VO and 66VO/FO diets.** Data are shown as mean ± S.E.M. (n=6). Results
604 were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test. Values
605 that do not share a common letter are significantly different (P<0.05).
606
607 **Figure 3. Basal lipolysis levels and effects of incubation with TNFα, linoleic acid (LA), *cis*-9**
608 ***trans*-11 conjugated linoleic acid (c9,t11-CLA) and *trans*-10 *cis*-12 conjugated linoleic acid**
609 **(t10,c12-CLA) on lipolysis of isolated adipocytes of gilthead sea bream fed with FO, 66VO**
610 **and 66VO/FO diets.** Comparison of the basal lipolysis levels from the adipocytes of FO, 66VO
611 and 66VO/FO groups (A). The isolated adipocytes from each experimental group were also
612 incubated with the different treatments and the lipolysis as glycerol released into the medium
613 was measured; FO (B), 66VO (C) and 66VO/FO (D) groups. The treatment vehicle, ethanol
614 (EtOH for fatty acids), was also tested. Data are shown as mean ± S.E.M. (n=3). Results were
615 analysed by one-way analysis of variance (ANOVA), followed by Tukey's test. Values that do
616 not share a common letter are significantly different (P<0.05). Comparisons between each
617 separate treatment were analysed by one-way analysis of variance (ANOVA), followed by
618 Tukey's test. The treatments (TNFα, LA, c9,t11-CLA, t10,c12-CLA) that do not share a
619 common number are significantly different from the other dietary groups (P<0.05).
620
621 **Figure 4. LPL, HSL and LXR gene expression profile on isolated adipocytes of gilthead sea**
622 **bream fed with FO, 66VO and 66VO/FO diets and effects of TNFα, linoleic acid (LA), *cis*-9**
623 ***trans*-11 conjugated linoleic acid (c9,t11-CLA) and *trans*-10 *cis*-12 conjugated linoleic acid**
624 **(t10,c12-CLA) on LPL, HSL and LPL expression on isolated adipocytes of gilthead sea**
625 **bream fed with FO, 66VO and 66VO/FO diets.** Gene expression of LPL (A), HSL (B) and
626 LXR (C) of gilthead sea bream fed with FO, 66VO and 66VO/FO diets. Effects of TNFα; LA;
627 c9,t11-CLA; and t10,c12-CLA on LPL, HSL and LXR of isolated adipocytes of gilthead sea
628 bream fed with FO (D), 66VO (E) and 66VO/FO (F) diets. In the panels D, E and F, the data are
629 compared to the control levels of each gene and also between the different gene expression
630 levels of the three genes analysed. The treatment vehicle, ethanol (EtOH for fatty acids), was
631 also tested. The gene expression data are presented as relative units (RU) using β-actin as a
632 housekeeping gene (delta-delta method). Data are shown as mean ± S.E.M. (n=3). Results for
633 each gene were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test.
634 Values that do not share a common letter (capital letters for LPL, Greek letters for HSL and
635 lower case letters for LXR) are significantly different (P<0.05). The effect of the treatments on
636 LPL, HSL and LXR was also analysed between the three diets (G). Data are shown as mean ±

S.E.M. (n=3). Data are shown as mean ± S.E.M. (n=3). Results were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test. Values that do not share a common letter are significantly different ($P<0.05$).

Figure 5. Basal levels of glucose uptake (2-deoxyglucose, 2-DG) on isolated adipocytes from FO, 66VO and 66VO/FO (A) and effect of insulin (Ins) and TNFα on the glucose uptake; FO (B), 66VO (C) and 66VO/FO (D) groups. Data are shown as mean ± S.E.M. (n=3). Results were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test. Values that do not share a common letter are significantly different ($P<0.05$).

646

647 **Table 1.** Ingredients and chemical composition of experimental diets

648

649

650 [a]

Ingredient (g/Kg)	FO	66VO
Fish meal (CP 70%)[a]	15	15
CPSP 90[b]	5	5
Corn gluten meal	40	40
soybean meal	14.3	14.3
Extruded wheat	4	4
Fish oil[c]	15.1	5.1
Rapeseed oil	0	1.7
Linseed oil	0	5.8
Palm oil	0	2.5
Soya lecithin	1	1
Blinder (sodium alginate)	1	1
Mineral premix[d]	1	1
Vitamin premix[e]	1	1
$CaHPO_4.2H_2O$ (18%P)	2	2
L-lysine	0.55	0.55
Proximate composition		
Dry matter (DM, %)	93.4	92.77
Crude protein (%DM)	53.2	52.62
Crude fat (%DM)	21.09	20.99
Ash (%DM)	6.52	6.57

680 Fish meal (Scandinavian LT).

681 [b] Fish soluble protein concentrate (Sopropêche, France).

682 [c] Fish oil (Sopropêche, France).

683 [d] Supplied the following (mg/kg diet, except as noted): calcium carbonate (40% Ca)
684 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric
685 citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen
686 phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese
687 sulphate 30, sodium selenite 0.3.

688 [e] Supplied the following (mg/kg diet): retinyl acetate 2.58, DL-cholecalciferol
689 0.037, DL-α tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5,
690 riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium
691 pantothenate 2.5, vitamin B12 0.025, ascorbic acid 250, inositol 500, biotin 1.25
692 and choline chloride 500.

693

694

694
695
696 **Table 2**
697
698 Gilthead sea bream primer sequences used for real-time PCR
699
700

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
LXR	**-** **FJ502320**	F	GCA CTT CGC CTC CAG GAC AAG	476-496
		R	CAG TCT TCA CAC AGC CAC ATC AGG	582-559
β-actin	**-** **X89920**	F	TCC TGC GGA ATC CAT GAG A	811-829
		R	GAC GTC GCA CTT CAT GAT GCT	861-841

701

Table 3

Biometric and biochemical plasma parameters of fish sampled at the end of the feeding trial.

	FO	66VO	66VO/FO
Body mass (g)	528 ± 14,2[a]	552 ± 13,35[a]	563 ± 10,83[a]
Adipose tissue (g)	6.44 ± 0.32	6.71 ± 0.73	7.04 ± 0.75
Liver (g)	6.27 ± 0.24[a]	8.38 ± 0.44[b]	7.74 ± 0.28[b]
MFI (%)[1]	1.23 ± 0.07	1.14 ± 0.11	1.24 ± 0.12
HSI (%)[2]	1.18 ± 0.03[a]	1.4 ± 0.07[b]	1.37 ± 0.04[b]
Plasma parameters			
Glucose (mM)	4.23±0.36	4.43±0.40	4.45±0.40
NEFAs (mEq/L)[3]	0.20±0.01[ab]	0.21±0.02[a]	0.16±0.01[b]
Triglycerides (mM)	8.85±0.56	8.86±1.16	7.85±0.50

Values are the mean ± S.E.M. (n=18). Differences between groups were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test.

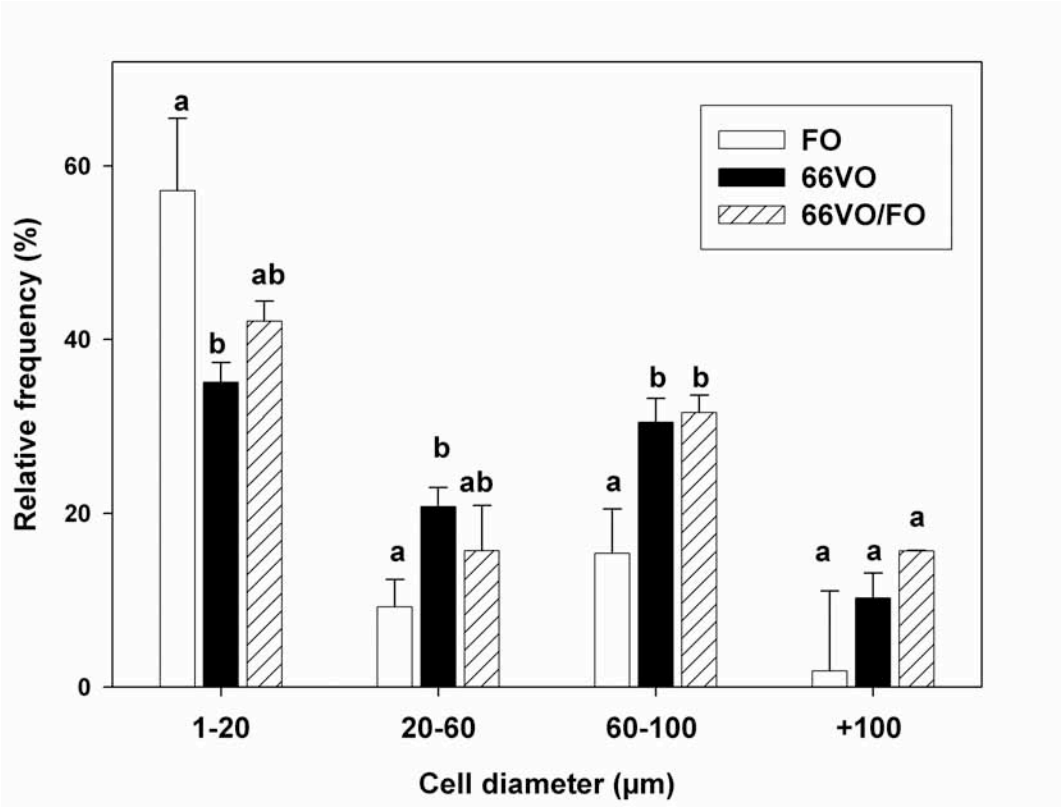
Values that do not share a common letter are significantly different ($P<0.05$)

[1]Mesenteric fat index (MFI)=(mesenteric fat mass/fish mass) ×100.

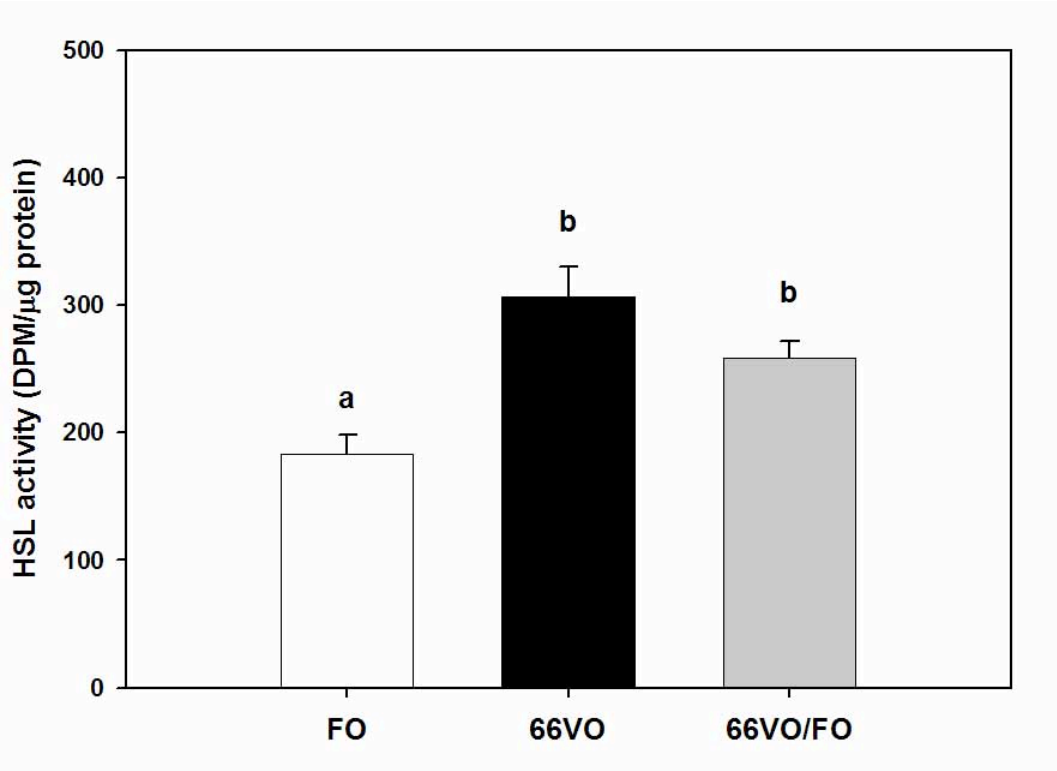
[2]Hepatosomatic index (HSI)=(liver mass/fish mass) ×100.

[3]NEFAs: non-esterified fatty acids

Fig 1

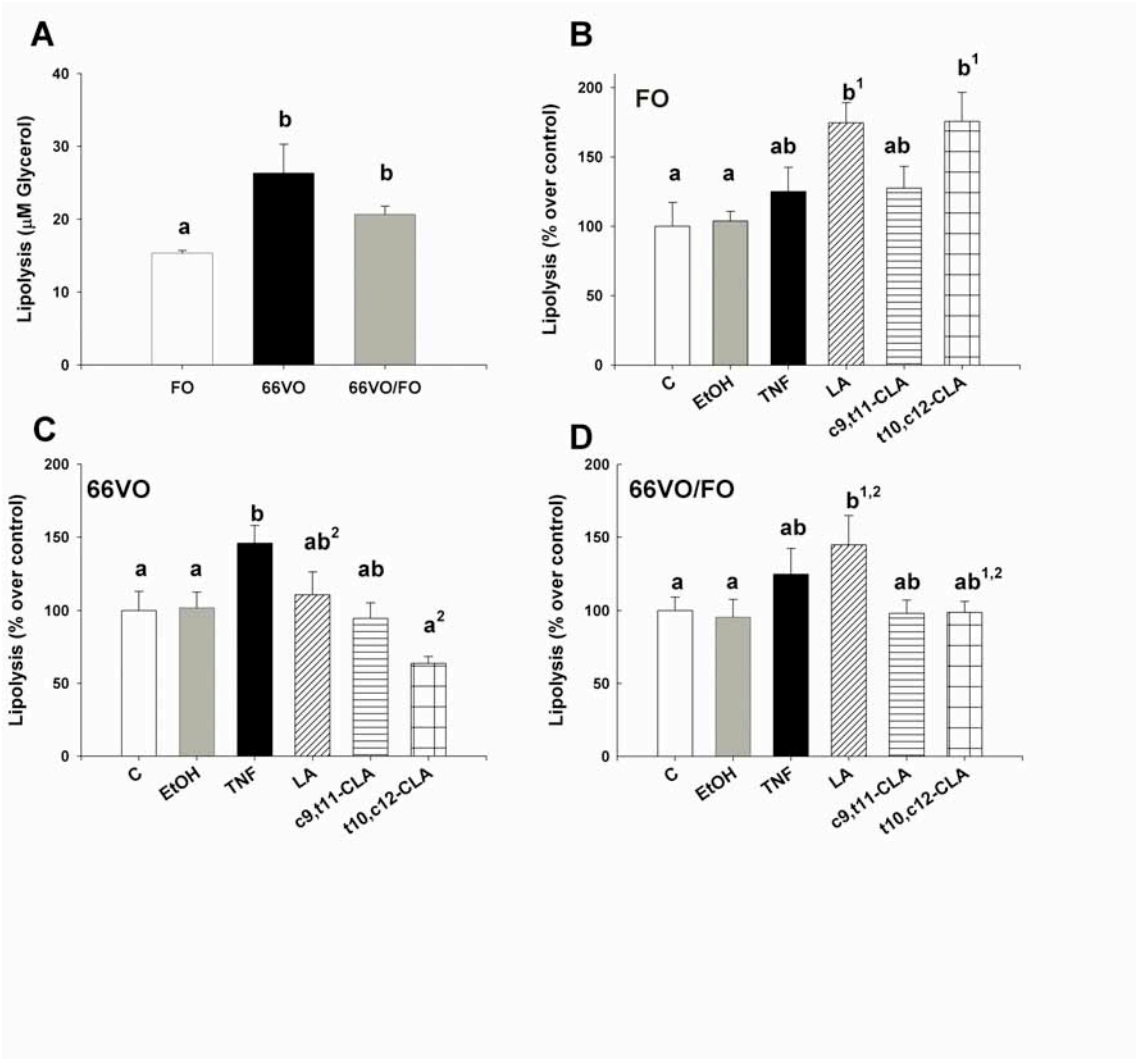

FO
66VO
66VO/FO
Relative frequency (%)
0
20
40
60
a
b
ab
a
b
ab
a
b
b
a
a
a
1-20
20-60
60-100
+100
Cell diameter (µm)

730 **Fig 2**
731
732
733

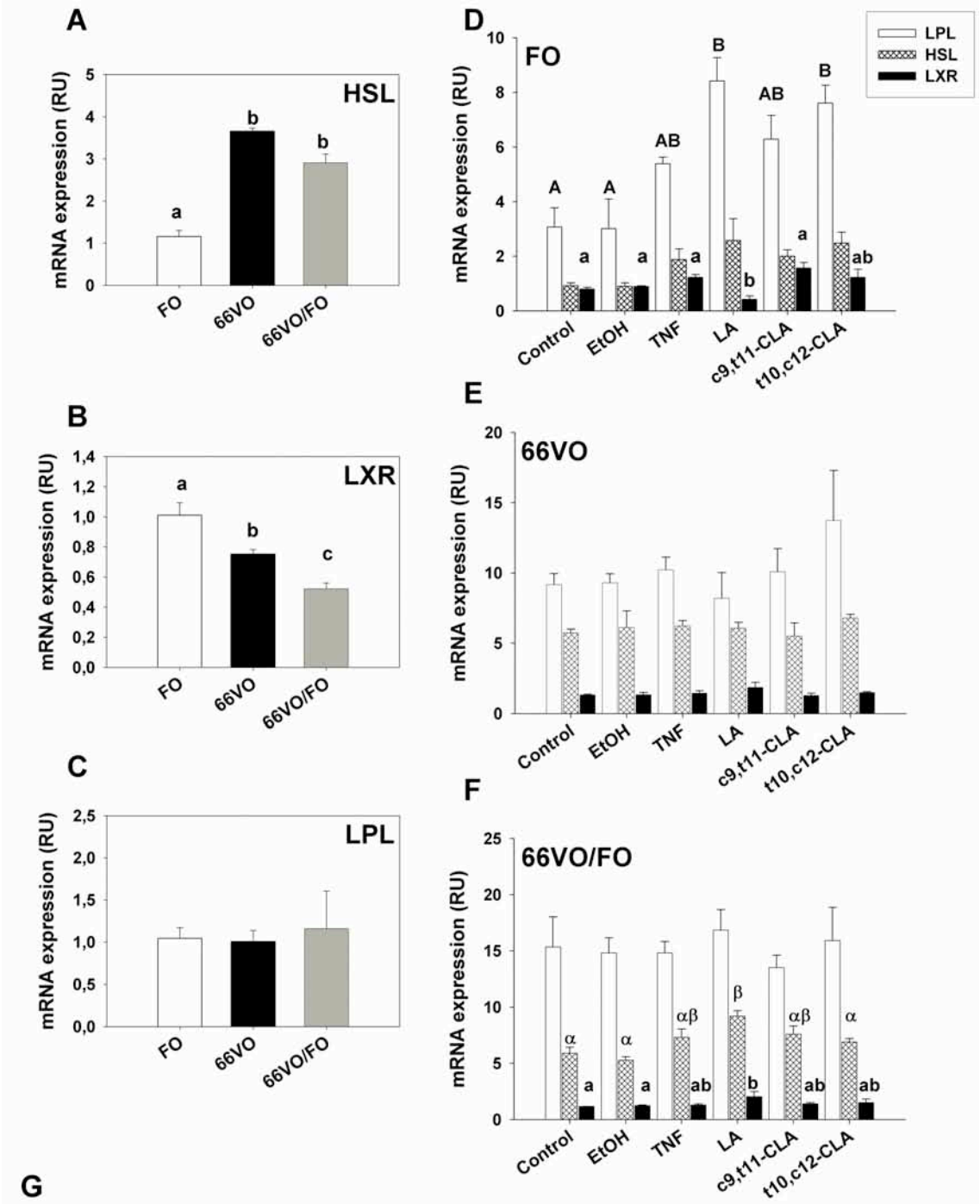


734
735

Fig 3


A
Lipolysis (μM Glycerol)
40
30
20
10
0
a
b
b
FO
66VO
66VO/FO
B
FO
Lipolysis (% over control)
200
150
100
50
0
a
a
ab
b1
ab
b1
C
EtOH
TNF
LA
c9,t11-CLA
t10,c12-CLA
C
66VO
a
a
b
ab2
ab
a2
D
66VO/FO
ab
b1,2
ab
ab1,2

737 **Fig 4**
738



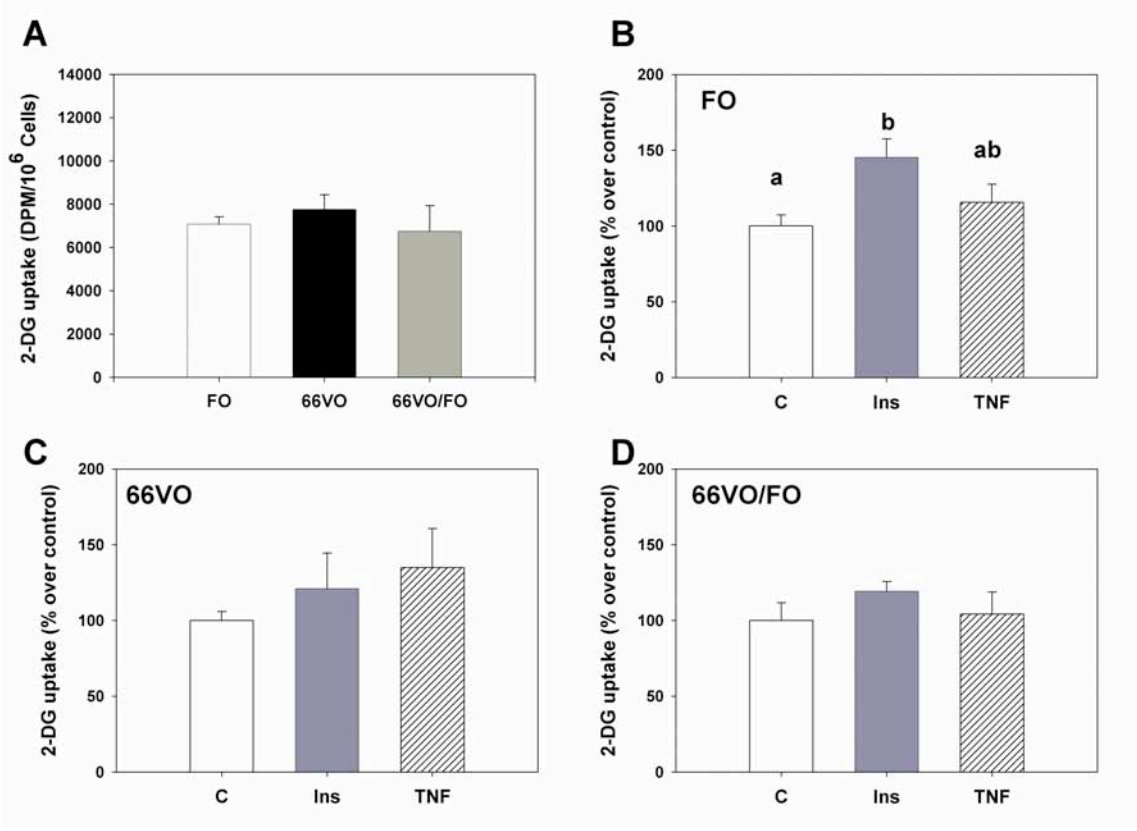
G

Treatments vs diets

	LPL			HSL			LXR		
	FO	66VO	66VO/FO	FO	66VO	66VO/FO	FO	66VO	66VO/FO
TNFα	1.78±0.29^{a}	1.15±0.09^{ab}	0.94±0.08^{b}	1.09±0.31	0.92±0.06	1.22±0.12	1.22±0.10	1.11±0.15	1.16±0.11
LA	2.83±0.28^{a}	0.92±0.21^{b}	0.97±0.13^{b}	1.64±0.51	0.81±0.10	1.54±0.08	0.42±0.13^{a}	1.54±0.30^{ab}	2.05±0.45^{b}
c9,t11-CLA	2.12±0.29^{a}	1.13±0.19^{b}	0.91±0.07^{b}	0.98±0.21	0.82±0.14	1.27±0.12	1.57±0.21	1.05±0.16	1.42±0.10
t10,c12-CLA	2.56±0.22^{a}	1.54±0.40^{ab}	0.98±0.13^{b}	1.58±0.25	0.93±0.09	1.15±0.05	1.23±0.29	1.23±0.06	1.51±0.32

739
740

Fig 5


A
2-DG uptake (DPM/10^6 Cells)
14000
12000
10000
8000
6000
4000
2000
0
FO
66VO
66VO/FO
B
FO
2-DG uptake (% over control)
200
150
100
50
0
a
b
ab
C
Ins
TNF
C
66VO
2-DG uptake (% over control)
200
150
100
50
0
C
Ins
TNF
D
66VO/FO
2-DG uptake (% over control)
200
150
100
50
0
C
Ins
TNF