Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*)

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Abstract

Disturbances of lipid metabolism are a major problem in livestock fish and the present study analysed the different tissue expression pattern and regulation of 40 lipid-relevant genes in gilthead sea bream. Nineteen sequences, including fatty acid elongases (4), phospholipases (7), acylglycerol lipases (8) and lipase-maturating enzymes (1), were new for gilthead sea bream (GenBank, JX975700-JX975718). Up to six different lipase-related enzymes were highly expressed in adipose tissue and liver, which also showed a high expression level of Δ6 and Δ9 desaturases. In the brain, the greatest gene expression level was achieved by the very long chain fatty acid elongation 1, along with relatively high levels of Δ9 desaturases and the phospholipase retinoic acid receptor responder. These two enzymes were also expressed at a high level in white skeletal muscle, which also shared a high expression of lipid oxidative enzymes. An overall down-regulation trend was observed in liver and adipose tissue in response to fasting following the depletion of lipid stores. The white skeletal muscle of fasted fish showed a strong down-regulation of Δ9 desaturases in conjunction with a consistent up-regulation of the "lipolytic machinery" including key enzymes of tissue fatty acid uptake and mitochondrial fatty acid transport and oxidation. In contrast, the gene expression profile of the brain remained almost unaltered in fasted fish, which highlights the different tissue plasticity of lipid-related genes. Taken together, these findings provide new fish genomic resources and contribute to define the most informative set of lipid-relevant genes for a given tissue and physiological condition in gilthead sea bream.

Keywords: fish; lipid metabolism; fasting; lipogenesis; lipolysis; biomarkers.

1. Introduction

Whereas global fisheries are in decline, the aquaculture industry is continuously growing and several efforts have been directed towards the reduction of wild-fishery-derived raw materials in the feeds of farmed fish. Much attention has been focused on plant ingredients and there is now accumulating evidence for a successful and combined replacement of fish meal (FM) and fish oil (FO) in fish feeds (Benedito-Palos et al., 2009; Nasopoulou and Zabetakis, 2012; Tacon and Metian, 2008). Hence, the current targets for FM and FO give Fish-In Fish-Out (FIFO) coefficients lower than 1.5 for most fish production systems, including those of salmonids and typically marine fish (Kaushik and Troell, 2010; Shepherd and Jackson, 2013). However, concerted efforts are still needed to undertake an in-depth assessment of the consequences of feeding very low FM and FO diets (less than 10% inclusion levels for marine ingredients), and additional knowledge is welcome to fill the gaps in nutritionally-mediated disorders arising from reduced growth and survival, impaired reproductive function, fin erosion and lordosis, stressful behaviour, heart failure, liver steatosis and the intense accumulation of lipid droplets in the hindgut (Francis et al., 2001; Glencross, 2009; Montero and Izquierdo, 2011). Most of these symptoms of nutrient deficiencies disappear with the fortification of fish feeds with essential fatty acids and phospholipids (Benedito-Palos et al., 2008; Olsen et al., 2003), although specific requirements for the latter have not yet been reported for juvenile fish (Tocher et al., 2008).Therefore, there is a strong need for integrative tools that are capable of describing and predicting any metabolic disturbance of lipid metabolism in fish.

Genome-wide expression analyses demonstrate that lipid accumulation and fuel partitioning are highly regulated in salmonids by changes in the expression and activity of fatty acid elongases, desaturases and key enzymes involved in cholesterol and lipoprotein metabolism (Morais et al., 2012; 2011; Panserat et al., 2009). Likewise, the expression and activity of lipoprotein lipases and Δ9 fatty acid desaturases are highly regulated by hormones and dietary nutrients in gilthead sea bream (Albalat et al., 2007; Benedito-Palos et al., 2013; Saera-Vila et al., 2005). Attempts to identify hormonally- and nutritionally-regulated transcription factors of lipolysis, lipogenesis and adipogenesis (e.g., peroxisome proliferator-activated receptors, tumour necrosis factor α, liver x receptor) have also been performed in a wide range of fish species, including

gilthead sea bream (Benedito-Palos et al., 2013; Cruz-García et al., 2009a; 2009b; Saera-Vila et al., 2007), Atlantic salmon (Martínez-Rubio et al., 2013), rainbow trout (Cruz-García et al., 2009a; Cruz-García et al., 2011) and flounder fish (Cho et al., 2012). However, for a given tissue and physiological condition, the transcriptional plasticity of most lipid-related genes remains uncertain. Accordingly, the aim of the present study was to phenotype at the transcriptional level the tissue-specific regulation of lipid metabolism in nutritionally challenged fish, focusing on a panel of 40 genes which were selected as markers of fatty acid, phospholipid (PL), acylglycerol and lipoprotein metabolism. The final aim is to define a data set of highly informative molecular markers of nutritional condition for any given tissue. To pursue this issue, we took advantage of the recently updated transcriptomic database of gilthead sea bream, hosted at www.nutrigroup-iats.org/seabreamdb, that contains assembled nucleotide data from public and private repositories, ESTs collection and high-throughput sequence libraries, which has been annotated by homology comparison to several protein and nucleotide databases (Calduch-Giner et al., 2013).

2. Materials and methods

2.1. Animal care and experimental setup

Juvenile gilthead sea bream (*Sparus aurata* L.) of Atlantic origin (Ferme Marine de Douhet, Ile d'Oléron, France) were cultured in the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS). Photoperiod and water temperature followed natural changes at IATS latitude (40º5N; 0º10E), and fish were fed to visual satiety with a commercial diet (Efico YM 564, Biomar, Aarhus, Denmark) for 74 days, from 21st July to 3th October. Following this period, fish of 80-90 g body mass were randomly distributed in duplicate tanks of 500 L in groups of 30 fish each. One group of fish continued to be fed as usual (CTRL group) while the other group was fasted for 10 days prior to the tissue sampling (fasted group). At harvest, eight randomly selected fish per experimental group were anaesthetised following overnight fasting with 3-aminobenzoic acid ethyl ester (MS-222, 100 mg/ml). Mesenteric adipose tissue, liver, brain and a representative portion of the skeletal muscle (dorsal white muscle) were

106 rapidly excised under RNAse-free conditions, frozen in liquid nitrogen and stored at -
107 80ºC until gene expression analyses were performed.
108 All procedures were carried out according to the national (IATS-CSIC Review
109 Board) and present EU legislation on the handling of experimental animals.
110
111 *2.2. Gene sequence analysis*
112
113 The nutrigroup-iats.org/seabreamdb database contains 594 non-redundant
114 sequences with the Gene Ontology term "lipid metabolic process" (GO:0006629). This
115 allowed the unequivocal identification (E-value < 5e-66) of 19 new gilthead sea bream
116 sequences uploaded to GenBank with accession numbers JX975700-JX975718 (Table
117 1). The list included four fatty acid elongases (elongation of very long chain fatty acids
118 1, 4, 6 and 7: *ELOVL1b, ELOVL4, ELOVL6, ELOVL7*), seven phospholipases (group
119 XIIA secretory phospholipase A2, *PLA2G12A*; group XIIB secretory phospholipase A2,
120 *PLA2G12B*; group XV phospholipase A2, *PLA2G15*; retinoic acid receptor responder
121 protein 3, *RARRES3*; 85kDa calcium-independent phospholipase A2, *PLA2G6*;
122 cytosolic phospholipase A2, *cyt-PLA2*; intestinal phospholipaseA2, *int-PLA2*) and eight
123 lipase-related enzymes (lipase member H, *LIPH*; adipose triglyceride lipase, *ATGL*;
124 sn1-specific diacylglycerol lipase β, *DAGLB*; monoacylglycerol lipase abhydrolase
125 domain-containing protein 6a and 12b, *ABHD6a*, *ABHD12b*; carboxyl ester lipase,
126 *CEL*; colipase-dependent pancreatic lipase, *cd-PL*; lipase maturation factor 1, *LMF1*).
127 Fourteen out of the 19 comprised complete codifying sequences with open reading
128 frames of 446–1733 nucleotides in length and a variable number of reads (8-693)
129 composing the assembled sequences.
130
131 *2.3. Gene expression analysis*
132
133 Total RNA from tissues was extracted using a MagMAX^{TM}-96 total RNA
134 isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was 50–100 µg with
135 UV absorbance measures ($A_{260/280}$) of 1.9–2.1 and RIN (RNA integrity number) values
136 of 8-10 as measured on an Agilent 2100 Bioanalyser, which is indicative of clean and
137 intact RNA. Reverse transcription (RT) of 500 ng of total RNA was performed with
138 random decamers using the High-Capacity cDNA Archive Kit (Applied Biosystems,
139 Foster City, CA, USA) according to the manufacturer's instructions. Negative control

reactions were run without reverse transcriptase. Quantitative real-time PCR (qPCR) was performed using an Eppendorf Mastercycler Ep Realplex real-time PCR system (Eppendorf, Wesseling-Berzdorf, Germany).

The 96-well PCR array layout was designed for the simultaneous profiling of a panel of 40 genes in duplicate format (Supplemental Table 1). The set of genes included five fatty elongases (*ELOVL1b*, *ELOVL4*, *ELOVL5*, *ELOVL6*, *ELOVL7*), three fatty acid desaturases (fatty acid desaturase 2, *FADS2*; stearoyl-CoA desaturase 1, *SCD1a* and *SCD1b*), ten PL synthesis and remodelling enzymes (lysophosphatidylcholine acyltransferase 1-3, *LPCAT1*, *LPCAT2*, *LPCAT3*; phosphatidylethanolamine N-methyltransferase, *PEMT*; *PLA2G12A*; *PLA2G12B*; *PLA2G15*; *RARRES3*; *PLA2G6*; *cyt-PLA2*; *int-PLA2*), thirteen lipase-related genes (hepatic lipase, *HL*; lipoprotein lipase, *LPL*; lipoprotein lipase-like, *LPL-like*; *LMF1*; *LIPH*; lysosomal acid lipase, *LIPA*; hormone sensitive lipase, *HSL*; *ATGL*; *DAGLB*; *ABHD6a*; *ABHD12b*; *CEL*; *cd-PL*; *int-PLA2*), four enzymes of fatty acid β-oxidation (carnitine palmitoyltransferases, *CPT1A* and *CPT1B*; enoyl-CoA hydratase, *ECH*; hydroxyacyl-CoA dehydrogenase, *HADH*), and four transcription factors (liver X receptor α, *LXRα*; peroxisome proliferator-activated receptors, *PPARα*, *PPARβ*, *PPARγ*). Housekeeping genes and controls of general PCR performance were included on each array, with all of the pipetting operations performed using the EpMotion 5070 Liquid Handling Robot (Eppendorf). Briefly, RT reactions were diluted to convenient concentrations and the equivalent of 660 pg of total input RNA was used in a 25 µL volume for each PCR reaction. PCR-wells contained a 2x SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA), and specific primers at a final concentration of 0.9 µM were used to obtain amplicons of 50–150 bp in length (Supplemental Table 2).

The program used for PCR amplification included an initial denaturation step at 95ºC for 3 min, followed by 40 cycles of denaturation for 15 s at 95ºC and annealing/extension for 60 s at 60ºC. The efficiency of PCR reactions was always higher than 90%, and negative controls without sample templates were routinely used for each primer set. The specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5ºC/10 s over a temperature range of 55–95ºC), linearity of serial dilutions of RT reactions, and electrophoresis and sequencing of PCR amplified products. Fluorescence data acquired during the PCR extension phase were normalised using the delta-delta Ct method (Livak and Schmittgen, 2001). *β-actin*, elongation factor 1, α-tubulin and 18S rRNA were initially tested for gene expression stability

using GeNorm software, and the most stable gene was found to be *β-actin* (M score = 0.21); therefore, this gene was used as a housekeeping gene in the normalisation procedure for routine assays. Fold-change calculations for each gene in a given tissue were in reference to the expression ratio between fasted and CTRL fish (values > 1 indicate fasting up-regulated genes; values < 1 indicate fating down-regulated genes). For the clarity of results in a scalable manner, all data values were in reference to the expression level of *LXRα* in CTRL fish with an arbitrarily assigned value of 1 in multi-gene analysis comparisons.

2.4. Statistical analysis

Changes in gene expression for a given tissue and nutritional condition were analysed by one-way analysis of variance followed by the Student-Newman-Keuls tests at a significance level of 5%. The fasting-mediated effects on growth performance and gene expression were analysed by Student t-test at a significance level of 5%. All analyses were performed using the SPSS package version 20.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Growth performance

Continuously fed fish (CTRL) grew efficiently with an 18-20% increase in body weight and a feed efficiency (wet weight gain/dry feed intake) of 1.04 over the course of the 10 day experimental period. Fasted fish shared a 6-8% loss in body weight mass. In tissue-sampled fish, viscera weight and liver weight of CTRL fish were significantly higher than those of fasted fish; the resulting viscerosomatic and hepatosomatic indexes (100 x tissue weight/fish weight) varied from 8.5% to 5.4% and from 2.1% to 0.6%, respectively (Table 2).

3.2. Gene expression profiling

All data for tissue gene expression are shown in supplemental Table 3. Note that *int-PLA2* was not expressed in any of the analysed tissues, and was used as a negative control of PCR reactions. *ELOV7* and *CPT1B* were not found at detectable levels in the liver tissue, whereas the expression of *HL* was only detectable in that tissue. *LIPH* and *cd-PL* were not expressed at detectable levels in the white skeletal muscle, while *LPL*, *CEL* and *cd-PL* remained below detectable levels in the brain. The remaining genes (30) were found at detectable levels in all four analysed tissues, with a gene expression profile that reflected the lipid storage capacity and biosynthetic and lipid oxidative capabilities of each tissue in a given nutritional condition. Accordingly, the tissue-specific molecular signature of lipases, desaturases, elongases, phospholipid-related enzymes and lipid oxidative enzymes changed gradually from adipose tissue, liver, brain and white skeletal muscle (Figure 1). Hence, regarding the top 10 genes with the highest expression, the mesenteric adipose tissue of CTRL fish was characterised by a high expression level of acylglycerol lipases (*CEL* > *LPL*, *cd-PL*, *HSL*, *ATGL*) and Δ9 desaturases (*SCD1b*), and to a lesser extent, of *ELOVL1b*, *PPARγ* and *HADH*. In the liver tissue, a high level of expression was also found for acylglycerol lipases (*CEL* > *HL*, *cd-PL*), but the relative weight of elongases (*ELOVL1b*, *ELOVL5*, *ELOVL6*), Δ6 and Δ9 desaturases (*FADS2*, *SCD1b* > *SCD1a*) and the phospholipase *PLA2G12B* was more important than in adipose tissue. This was more evident in brain, where the greatest expression level was achieved by elongases (*ELOVL1b* > *ELOVL5*, *ELOVL* 6) and secondly by Δ9 desaturases (*SCD1b* > *SCD1a*) and the phospholipase *RARRES3*. In contrast, the lipid transcriptome of white skeletal muscle was mostly characterised by the high expression level of fatty acid oxidative markers (*HADH* > *ECH*, *CPTIA*).

To compare the tissue gene expression pattern of CTRL and fasted fish, the relative expression level of all genes in the PCR array were plotted against each other in a scatter plot with a fold-change cut-off of 1.25 and 0.8 for differentially expressed genes at a significance level of 5%. The adipose tissue showed an overall down-regulated response that was statistically significant for *PPARγ*, *ELOVL5*, Δ9 desaturases (*SCD1a* and *SCD1b*) and several lipases (*CEL*, *LPL*, *LIPA*) with fold-changes varying between 0.04 and 0.6 (Figure 2A). The magnitude and intensity of change was higher in the liver tissue with a strong down-regulation of elongases (*ELOVL4-6*), desaturases (*SCD1a*, *SCD1b*, *FADS2*), phospholipid-related enzymes (*PEMT*, *LPCAT2*, *LPCAT3*, *RARRES3*, *PLA2G12B*, *PLA2G6*), acylglycerol lipases (*cd-PL*, *CEL*, *HL*), *LMF1*, *ECH* and transcription factors (*PPARγ* and *LXRα*), which in turn was accompanied by the

up-regulation of *ATGL*, *LPL* and *LPL-like* as well as *HADH* and *CPT1A* (Figure 2B). The genes for *LPL-like*, *LMF1*, the phospholipase *PLA2g15* and markers of fatty acid-β oxidation (*HADH*, *CPT1A*, *CPT1B*) were also up-regulated in the skeletal muscle of fasted fish, in association with a strong down-regulation of Δ9 desaturases (*SCD1a* and *SCD1b*) (Figure 3A). All of this is in contrast with the observations made in the brain tissue, where the analysed lipid markers remained almost unaltered by fasting (Figure 3B). For a corollary overview of fold-changes in gene expression between CTRL and fasted fish for a given tissue and differentially expressed gene, see Figure 4.

4. Discussion

Lipid content and fatty acid composition are important traits for meat fish quality, and research efforts have been focused on developing breeding programs to select lean fish with a high content of n-3 long chain polyunsaturated fatty acids (LC-PUFA) (Bell et al., 2010; Lefevre et al., 2007). This strengthens the importance of web tools (www.nutrigtoup-iats.org/aquafatdb) for predictive modelling the fillet fatty acid composition on the basis of different lipid deposition rates and diet composition as independent variables (Ballester-Lozano et al., 2011; 2014). However, there are important gaps in fish lipid metabolism and the rationale of the present study is to upload new gilthead sea bream nucleotide sequences (up to 19) to public databases, also addressing the tissue-specific co-regulation of a panel of 40 lipid-related genes, selected as markers of fatty acid, triacylglycerol, PL and lipoprotein metabolism. Liver, skeletal muscle, adipose tissue and brain were chosen as target tissues on the basis of their different lipid metabolic capabilities, with the brain being the most refractory tissue to changes in nutrient availability. This concurs with the great robustness of the brain fatty acid profile of gilthead sea bream when fish are challenged with dietary vegetal oils (Benedito-Palos et al., 2010). In contrast, liver and secondly adipose tissue and skeletal muscle showed pronounced changes in their lipid gene expression profile in response to nutrient deprivation and the depletion of body fat stores, affecting fatty acid elongases, desaturases, acyltransferases, methyltransferases, phospholipases and several enzymes of the lipase gene superfamily, as described below.

4.1. Fatty acid bioconversion

To date, seven ELOVLs (*ELOVL1–7*) have been identified in mammals (Jakobsson et al., 2006). Among them *ELOVL1*, *ELOVL3*, *ELOVL6* and *ELOVL7* show a substrate preference for saturated and monounsaturated fatty acids, whereas *ELOVL2*, *ELOVL4* and *ELOVL5* are more selective for polyunsaturated fatty acids (PUFA). *ELOVL5* has been cloned and functionally characterised in several fish species, including freshwater fish (Agaba et al., 2004), salmonids (Morais et al., 2009) and marine fish (Gregory et al., 2010; Mohd-Yusof et al., 2010; Monroig et al., 2013; Morais et al., 2012), whereas *ELOVL2* remains restricted to freshwater fish and salmonids (Monroig et al., 2009; Morais et al., 2009; Tan et al., 2010). More recently, *ELOVL4* has also been characterised in zebrafish (Monroig et al., 2010), Atlantic salmon (Carmona-Antonanzas et al., 2011), cobia (Monroig et al., 2011) and rabbitfish (Monroig et al., 2012), but to the best of our knowledge, only *ELOVL5* has been characterised in gilthead sea bream (Agaba et al., 2005). Therefore, the uploading of four new sequences to public repositories, unequivocally annotated as *ELOVL1b*, *ELOVL4*, *ELOVL6* and *ELOVL7*, is of relevance for gilthead sea bream, which is now one the few fish species with an almost complete dataset of actively transcribed ELOVLs. In that sense, the lack of *ELOVL2* is noteworthy, and is consistent with the blockage of the last elongation steps of the LC-PUFA biosynthetic pathways in marine fish (Monroig et al., 2009; Morais et al., 2009). Likewise, *ELOVL3* is related to the synthesis of saturated and monounsaturated fatty acids of very long chains, and to the best of our knowledge, an orthologous gene has not yet been reported in gilthead sea bream or any other fish. A number of authors have highlighted some ELOVL functional redundancy, and suggest that *ELOVL4* might serve to compensate, at least in part, for the lack of *ELOVL2* (Monroig et al., 2012; 2011), although our results only supported a modest expression of *ELOVL4* in liver. A tissue-specific expression pattern was found for *ELOVL7* with no detectable levels in the liver and a moderately high expression in the brain, although the physiological significance of this finding remains to be established. From our results, it is also clear that the hepatic expression of *ELOVL4*, *ELOVL5* and *ELOVL6* is highly regulated at the transcriptional level by the nutritional condition, with a pronounced down-regulation in fasted fish. In contrast, *ELOVL1b* remained almost unaltered in all of the analysed tissues, which agrees with the proposed role as a “housekeeping elongase” in mammals (Guillou et al., 2010).

A general statement is that enzymes with Δ5 desaturase activity are not found in the genome of marine fish and the characterised *FADS2* only has Δ6 desaturase activity

(Seiliez et al., 2003; Zheng et al., 2004a; 2009). Experimental evidence also indicates that the expression of FADS2 is subjected to nutritional regulation in different fish species (reviewed by Vagner and Santigosa, 2011) with an enhanced expression with the replacement of FO with vegetable oils in either salmonids (Zheng et al., 2005; 2004b) or marine fish (González-Rovira et al., 2009; Izquierdo et al., 2008). The same is known to occur in Atlantic salmon fed low energy diets (Martínez-Rubio et al., 2013), whereas low expression levels have been reported in Atlantic cod (Tocher et al., 2006) and European sea bass (González-Rovira et al., 2009) regardless of the dietary composition. However, at least in gilthead sea bream, this metabolic feature is changing with the advent of new fish feed formulations, and our results demonstrate high expression of *FADS2* in the liver of fish fed continuously when comparisons are made with other lipid-related genes. Furthermore, the expression of *FADS2* was markedly down-regulated by fasting (11-fold decrease), which might primarily reflect a low FA bioconversion with a reduced conversion of 18:3n-3 to 18:4n-3 and 18:2n-6 to 18:3n-6. Measurements of enzymes activities are needed to confirm the high activity of *FASD2* in currently marine farmed fish, as this would implicate *FADS2* as a non-limiting enzyme in the LC-PUFA biosynthetic pathway of marine fish and gilthead sea bream in particular.

In contrast to Δ5 and Δ6 desaturases, SCD1 enzymes with Δ9 desaturase activity are mostly ubiquitous in all living organisms with palmitoleic acid and oleic acid being the major products of SCD activity. In teleost fish, two SCD isoforms have been found as duplicated genes of SCD1-type (Castro et al., 2011), but the *SCD1b* variant is clearly expressed at a higher level in the skeletal muscle of gilthead sea bream (Benedito-Palos et al., 2013). In the present study, the same tendency appears to occur in liver, mesenteric adipose tissue and brain; however, regardless of this, *SCD1a* and *SCD1b* transcripts were markedly down-regulated by fasting in skeletal muscle (10- and 5-fold lower expression, respectively), adipose tissue (4- and 25-fold lower expression, respectively) and liver (33- and 100-fold lower expression, respectively), which confirms and extends the idea that SCD1 enzymes are strong markers of lipogenesis in a wide range of tissues and species, including fish and mammals (Ntambi et al., 2002; Sampath et al., 2007).

4.2. Phospholipids synthesis

Phospholipids are first synthesised in the *de novo* Kennedy pathway (Kennedy and Weiss, 1956), but their FA composition at the sn-2 position is altered in the remodelling Land's cycle (Lands, 1958) through the concerted action of acyltransferases and phospholipase A2 enzymes. Other important regulatory steps of PL synthesis are the transmethylation reactions catalysed by the *PEMT* enzyme to convert phosphatidylethanolamine to phosphatidylcholine. Therefore, the down-regulated expression of *PEMT* and *LPCAT1-3* in the liver tissue of fasted fish is indicative that PL synthesis and remodelling are highly regulated at the transcriptional level in this tissue by the nutritional condition. Conversely, none of these enzymes were transcriptionally regulated by fasting in muscle, adipose tissue or brain, as previously reported for the skeletal muscle of fish fed the maintenance ration (Benedito-Palos et al., 2013). Regarding the phospholipase superfamily, we also found down-regulated expression of *PLA2G12B*, *RARRES3* and *PLA2G6* in the liver tissue of fasted fish. Of note, the opposite regulation was found for *PLA2G15* in skeletal muscle, although it remains unclear whether *PLA2G15* might drive the increased retention of arachidonic acid and docosahexaenoic acid in the muscle PLs of fasted fish (Benedito-Palos et al., 2013).

4.3. Tissue fatty acid uptake

Most of the metabolic re-arrangements that occur during fasting to maintain energy homeostasis also involve several vascular enzymes of the lipase superfamily, which act as key-limiting enzymes of tissue fatty acid uptake. In that sense, it is noteworthy that the overall literature strongly supports a distinct substrate specificity of *LPL* and *HL* to accommodate to the full spectrum of circulating lipoproteins (reviewed by Wong and Schotz, 2002). Hence, *LPL* catalyses the hydrolysis of triglycerides transported in the bloodstream as chylomicrons and very-low-density lipoproteins (VLDL) (Mead et al., 2002), whereas *HL* preferentially uses intermediate-density lipoproteins (IDL) as substrates (Berg, 2001). Importantly, the expression of *HL* is restricted to liver, whereas *LPL* is more ubiquitous with an enhanced expression and activity in adipogenic tissues, which follows the changes in lipolysis and adipose tissue mass in gilthead sea bream arising from a wide range of environmental and nutritional challenges (Albalat et al., 2007; Pérez-Sánchez et al., 2013; Saera-Vila et al., 2005; 2007). Furthermore, the regulation of *LPL* is very often opposite in liver and adipose tissue in order to drive the flux of lipids from adipose tissue towards the liver and vice

versa. This was also found herein in response to fasting, although, paradoxically, the trend for *LPL* and *HL* expression is not the same in the liver tissue. Furthermore, in previous studies of stress kinetics (Calduch-Giner et al., 2010; Pérez-Sánchez et al., 2013), the magnitude of change in *HL* expression is lower than that of *LPL*. This agrees with the observation that the hepatic expression of *LPL* is up-regulated by short-term fasting in red sea bream (Oku et al., 2006), whereas the expression of *HL* remains almost unaltered.

There is also experimental evidence for a second isoform of LPL, exclusive of fish lineage and annotated by us as *LPL-like* (Benedito-Palos et al., 2013). Importantly, this isoform is preferentially expressed in the skeletal muscle of gilthead sea bream, and a different tissue distribution has also been reported in red sea bream (Oku et al., 2006) and torafugu (Kaneko et al., 2013). This is also substantiated in the present study where the expression of *LPL-like* was up-regulated in a consistent manner by feed restriction in the skeletal muscle, making this *LPL* isoform a candidate gene in selective breeding programmes for lean muscle fish phenotypes. The mechanisms underlying the fine regulation of *LPL-like* are far from being established, although it is noteworthy that changes in *LPL-like* and *LMF1* transcript levels are highly correlated in the skeletal muscle of gilthead sea bream. Since *LMF1* is an endoplasmic reticulum chaperone with a critical role in mammals with regard to the post-translational activation of *LPL*, *HL* and endothelial lipase (Ben-Zeev et al., 2011; Doolittle et al., 2010), a functional association between *LPL-like* and *LMF1* is also suspected in fish.

4.4. Lipolysis and β-oxidation

The mobilisation of metabolic fuels from fat depots is also a complex process where *ATGL* and *HSL* act sequentially for the proper hydrolysis of tri- and diglycerides, respectively (Zimmermann et al., 2009). Together with the last step performed by monoglyceride lipases, this set of enzymes constitute the basic "lipolytic machinery" with high expression reported in adipose tissue of mammals (Jenkins et al., 2004; Kershaw et al., 2006; Lass et al., 2011) and fish, as has been evidenced in the present study. To our knowledge, this is the first report in fish analysing the gene expression profile of an almost complete set of acylglycerol lipases. Of note, the expression of *ATGL* seems to be particularly regulated at the transcriptional level, and its expression was significantly up-regulated in the liver of fasted fish following the increased gene

410 expression of oxidative enzymes involved in fatty acid β-oxidation (*HADH*, *CPT1A*).
411 Likewise, *ATGL* expression is up-regulated by fasting in several experimental models of
412 mammals (Bertile and Raclot, 2011; Kershaw et al., 2006), and a down-regulation has
413 been recently reported in another fish species (large yellow croaker) with the use of
414 enriched-lipid diets (Wang et al., 2013). It must be taken into account that *ATGL* shares
415 a high sequence homology with adiponutrin, a nutritionally regulated obesity-protein,
416 which is dramatically down-regulated during fasting and restored by re-feeding, both in
417 mice and rats (Bertile and Raclot, 2011; Kershaw et al., 2006). Therefore, further
418 studies are needed to determine if these two close-related lipolytic enzymes with an
419 opposite regulation are conserved in the fish lineage.
420 Other enzymes with a theoretical TAG lipase activity are *LIPA*, *LIPH* and *cd-*
421 *PL*. Interestingly, *LIPA* is involved in the intracellular hydrolysis of cholesteryl esters
422 and TAGs which have been internalised via receptor-mediated endocytosis of
423 lipoprotein particles (Anderson and Sando, 1991). In the present study, a relatively high
424 expression of *LIPA* was found in all of the analysed tissues, with its reduced expression
425 becoming noticeable with the loss of adipose tissue mass in fasted fish. Likewise, the
426 expression of *cd-PL*, closely related to pancreatic lipases (Oku et al., 2006), was mostly
427 restricted to liver and adipose tissue, with reduced expression in the liver of fasted fish.
428 Conversely, *LIPH* was expressed at a relatively low level in all of the analysed tissues,
429 which is probably indicative that this enzyme does not play a vital role and simply
430 supports the action of other lipases, as previously suggested in laying hens (Sato et al.,
431 2010).
432 *CEL*, also known as bile salt-activated lipase, is another lipolytic enzyme with a
433 role not only in the digestion and absorption of lipids, but also in the metabolism of
434 lipoproteins (Hui and Howles, 2002). In that sense, a clearly down-regulated response
435 was found herein in response to fasting in liver and adipose tissue. However, the
436 noteworthy expression of *CEL* in these two tissues is surprising, because this enzyme is
437 primarily synthesised in the pancreas and lactating mammary gland in mammals.
438 Indeed, low expression levels of *CEL* have been found in the adipose tissue of mouse
439 (Holmes and Cox, 2011) and laying hens (Sato et al., 2010). Nevertheless, the ancestor
440 of *CEL* appeared early in vertebrate evolution (Holmes and Cox, 2011), and this
441 enzyme might have acquired new functions in the fish lineage that were not necessarily
442 represented in tetrapods, although their precise physiological significance remains
443 unclear.

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445

446 *4.5. Transcription factors*

447

448 *PPARs* and *LXRs* belong to the nuclear receptor superfamily and play a key role
449 in lipid metabolism, acting as specific sensors of fatty acids and cholesterol. In that
450 sense, both in this and previous studies in gilthead sea bream (Cruz-García et al., 2009b;
451 Diez et al., 2007; Leaver et al., 2005) and salmonids (Jordal et al., 2007; Kennedy et al.,
452 2006), *PPARγ* has been shown to be the most highly expressed PPAR isoform in
453 adipogenic tissues with a pronounced down-regulation by fasting in liver and adipose
454 tissue. This response was concurrent with the down-regulated expression of *LXRα* in
455 liver, although it is noteworthy that target genes for lipolytic cytokines, and tumour
456 necrosis factor α in particular, are different in fish with lean and fat phenotypes (Cruz-
457 García et al., 2009a). In that sense, it is important to note that the expression of the
458 lipolytic *PPARα* is increased in fish by fasting and tissue oxidative capacity (Leaver et
459 al., 2005; 2008), which is viewed as part of the complex trade-off between lipolytic and
460 lipogenic signals. However, in the present study, the fasting-mediated effects on *PPARα*
461 expression were mostly reduced to a non-statistically significant increase at the liver
462 level, which suggests that other factors are the upstream regulators of the increased
463 oxidative capacity of skeletal muscle arising from a consistent up-regulation of
464 mitochondrial fatty acid transporters (*CPT1A*, *CPT1B*) and enzymes of β-oxidation
465 (*HADH*).

466

467 *4.6. Conclusions*

468

469 In summary, the present study analyses how 40 genes selected as markers of
470 lipid and lipoprotein metabolism are regulated in concert in fish challenged by fasting.
471 The results clearly show a tissue-specific regulation according to the different metabolic
472 capabilities of each tissue and vital functions for life, which makes the brain highly
473 refractory at the transcriptional level to changes in nutrient and energy availability. In
474 contrast, the liver is clearly the most reactive tissue, with changes in gene expression
475 affecting not only *ELOVL4-6* and Δ6-Δ9 desaturases, but also to a high extent to the
476 "lipolytic machinery", including *ATGL*, *HADH* and *CPT1A* enzymes. The two isoforms
477 of LPL have also been confirmed as key enzymes of lipid metabolism in fish, with a
478 differential and tissue-specific gene expression pattern in adipogenic and muscle tissues.
479 This kind of differential tissue regulation has also been reported for most of the genes

480 included in the array, which is of interest when defining the set of the most informative
481 lipid markers for a given tissue and experimental condition. Secondly, this knowledge
482 will contribute to define in future studies how the different tissue-specific gene
483 expression patterns are segregated with the selection pressure for fast growth and
484 alternative diets on intensive fish farming.
485
486
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488
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495

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771

772 **Figure legends**

773

774 Figure 1. Gene expression values for the top 10 genes with the highest expression in
775 different tissues (A, mesenteric adipose tissue; B, liver; C, brain; D, skeletal muscle) of
776 gilthead sea bream of the control group. Different colours indicate the biological
777 function of the genes. Data are the mean of 8 fish ± SEM. β-actin was used as a
778 housekeeping gene. Within a tissue, different superscript letters indicate significant
779 differences (Student-Newman Keuls test, $P<0.05$).

780

781

782 Figure 2. Scatterplots for gene expression values in gilthead sea bream of the control
783 and fasted groups plotted against each other in mesenteric adipose tissue (A) and liver
784 (B). Data are the mean of 8 fish. Standard error is not plotted to simplify the graphical
785 representation. β-actin was used as a housekeeping gene; regardless of dietary group, all
786 data values referred to the expression level of *LXRα* in control fish. Red text denotes up-
787 regulated genes ($P<0.05$) in fasted fish; green text denotes down-regulated genes
788 ($P<0.05$) in fasted fish; black text indicates non-differentially regulated genes (for
789 simplicity, only the most representative are indicated).

790

791 Figure 3. Scatterplots for gene expression values in gilthead sea bream of the control
792 and fasted groups plotted against each other in skeletal muscle (A) and brain (B). Data
793 are the mean of 8 fish. Standard error is not plotted to simplify the graphical
794 representation. β-actin was used as a housekeeping gene; regardless of dietary group, all
795 data values referred to the expression level of *LXRα* in control fish. Red text denotes up-
796 regulated genes ($P<0.05$) in fasted fish; green text denotes down-regulated genes
797 ($P<0.05$) in fasted fish; black text indicates non-differentially regulated genes (for
798 simplicity, only the most representative are indicated).

799

800 Figure 4. Graphical representation of fold-changes of differentially expressed genes
801 (fasted *versus* control fish) for liver (A), mesenteric adipose tissue (B) and skeletal
802 muscle (C) of gilthead sea bream. Different colours indicate the biological function of
803 the genes...

804

Table 1. Characteristics of new assembled sequences according to BLAST searches.

GenBank	Contig	F[a]	size (nt)	Annotation[b]	Best match[c]	E[d]	CDS[e]
JX975700	C2_316	693	1529	*ELOVL1b*	XP_003439861	0.0	124-1089
JX975701	C2_13708	30	1315	*ELOVL4*	XP_003440051	1e-158	229-1020
JX975702	C2_43870	8	1290	*ELOVL6*	XP_003443447	2e-163	165-971
JX975703	C2_63003	8	580	*ELOVL7*	XP_003446185	1e-118	31->580
JX975704	C2_6651	86	1316	*PLA2G12A*	XP_003452268	7e-125	185-766
JX975705	C2_8678	62	1070	*PLA2G12B*	ACQ58624	7e-123	26-664
JX975706	C2_4128	172	3424	*PLA2G15*	XP_003439481	0.0	221-1486
JX975707	C2_6806	137	1070	*RARRES3*	AC009640	3e-71	159-674
JX975708	C2_6027	63	1647	*PLA2G6*	CAK05383	0.0	<1-1066
JX975709	C2_7071	70	2777	*cyt-PLA2*	XP_003445168	0.0	456-2669
JX975710	C2_1462	348	1229	*int-PLA2*	BAB20241	5e-66	187-633
JX975711	C2_10823	60	2384	*ATGL*	ADY89608	0.0	<1-1284
JX975712	C2_4226	149	2204	*ABHD12b*	XP_003441119	0.0	144-1196
JX975713	C2_14725	37	1587	*ABHD6a*	XP_003447746	0.0	92-1099
JX975714	C2_73027	201	1807	*CEL*	CAF94246	0.0	23-1687
JX975715	C2_33966	25	1074	*LIPH*	XP_003446885	0.0	<1->1074
JX975716	C2_12991	71	1866	*DAGLB*	XP_003438553	0.0	<1-1485
JX975717	C2_18392	20	1487	*cd-PL*	BAF31237	0.0	24-1433
JX975718	C2_7945	75	2083	*LMF1*	CBN81183	0.0	31-1764

[a]Number of sequences.

[b]Gene identity determined through BLAST searches. *ELOVL1b*, elongation of very long chain fatty acids 1b; *ELOVL4*, elongation of very long chain fatty acids 4; *ELOVL6*, elongation of very long chain fatty acids 6; *ELOVL7*, elongation of very long chain fatty acids 7; *PLA2G12A*, group XIIA secretory phospholipase A2; *PLA2G12B*, group XIIB secretory phospholipase A2; *PLA2G15*, group XV phospholipase A2; *RARRES3*, retinoic acid receptor responder protein 3; *PLA2G6*, 85kDa calcium-independent phospholipase A2; *cyt-PLA2*, cytosolic phospholipase A2; *int-PLA2*, intestinal phospholipase A2; *ATGL*, adipose triglyceride lipase; *ABHD12b*, monoacylglycerol lipase abhydrolase domain-containing protein 12; *ABHD6a*, monoacylglycerol lipase abhydrolase domain-containing protein 6a; *CEL*, carboxyl ester lipase; *LIPH*, lipase member H; *DAGLB*, sn1-specific diacylglycerol lipase β; *cd-PL*, colipase-dependent pancreatic lipase; *LMF1*, lipase maturation factor 1.

[c]Best BLAST-X protein sequence match.

[d]Expectation value.

[e]Codifying sequence.

Table 2. Growth and biometric parameters of feed (CTRL group) and fasted gilthead sea bream. Each value is the mean ± SEM of the 8 sampled fish for transcriptional analysis.

	CTRL	Fasted	*P* [a]
Final body weight (g)	109.48 ± 3.42	79.93 ± 1.82	*<0.001*
Viscera (g)	9.35 ± 0.49	4.34 ± 0.23	*<0.001*
Liver (g)	2.31 ± 0.13	0.52 ± 0.03	*<0.001*
VSI (%) [b]	8.52 ± 0.23	5.41 ± 0.19	*<0.001*
HSI (%) [c]	2.10 ± 0.06	0.64 ± 0.02	*<0.001*
DM intake (g/fish)	17.25	-	

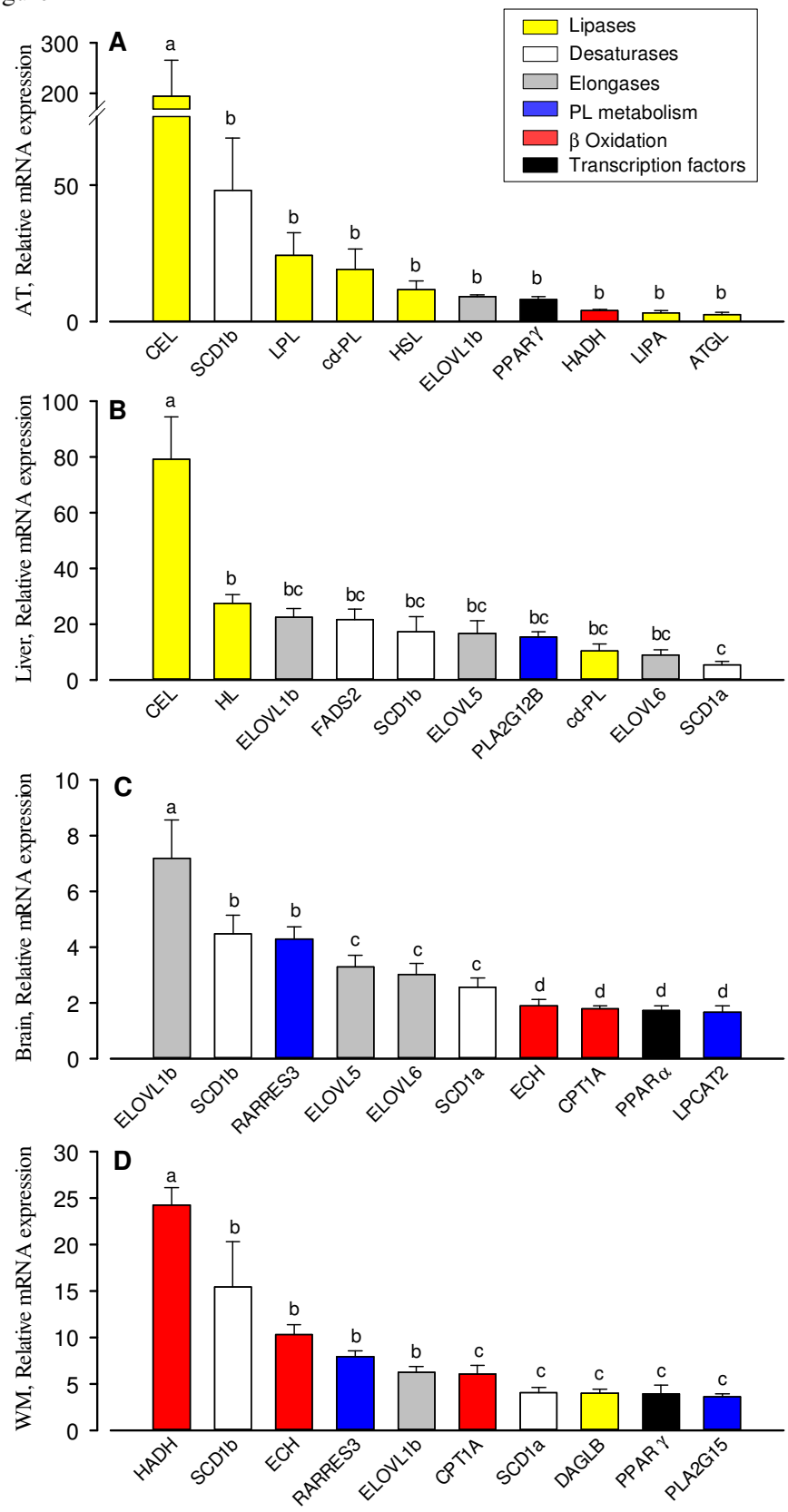
Initial average weight for the entire population was 86 ± 0.08 g.

[a]P values result from Student-t test.

[b]Viscerosomatix index = (100 × viscera wt.) / fish wt.

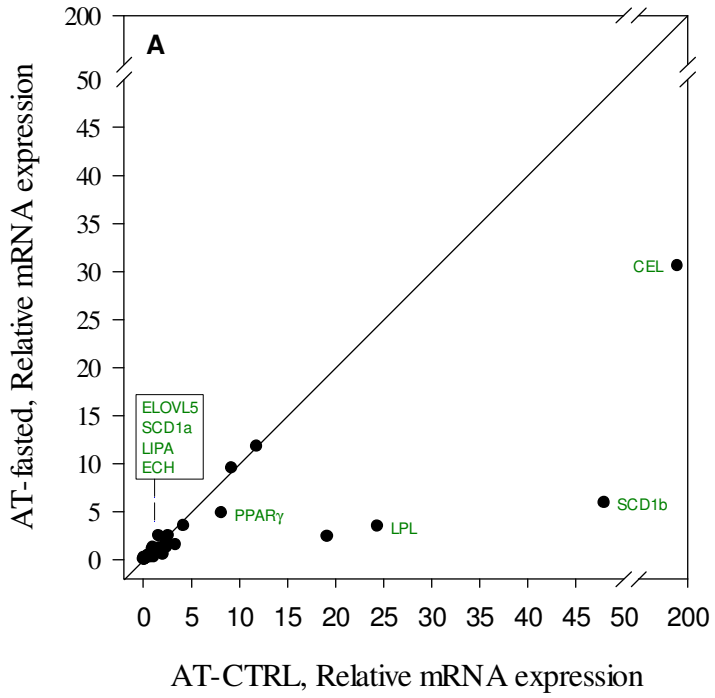
[c]Hepatosomatic index = (100 × liver wt.) / fish wt.

832 Figure 1



833
834

835 Figure 2



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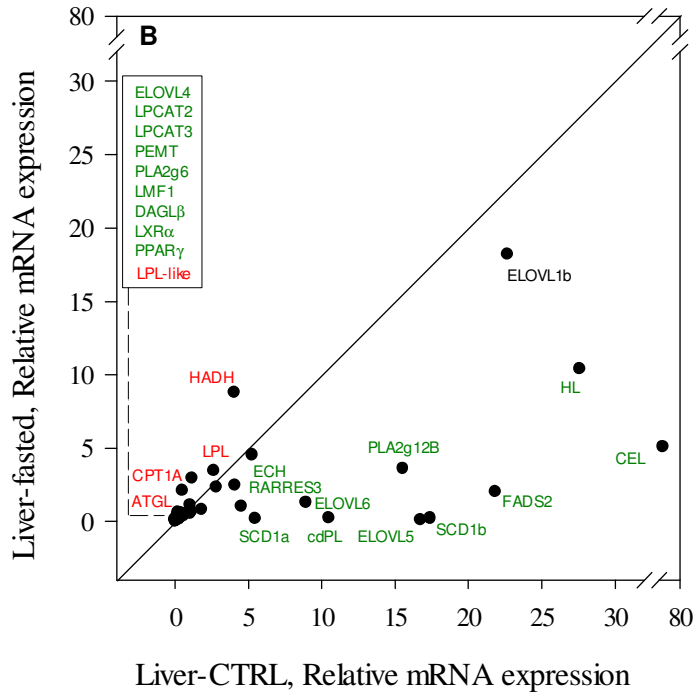

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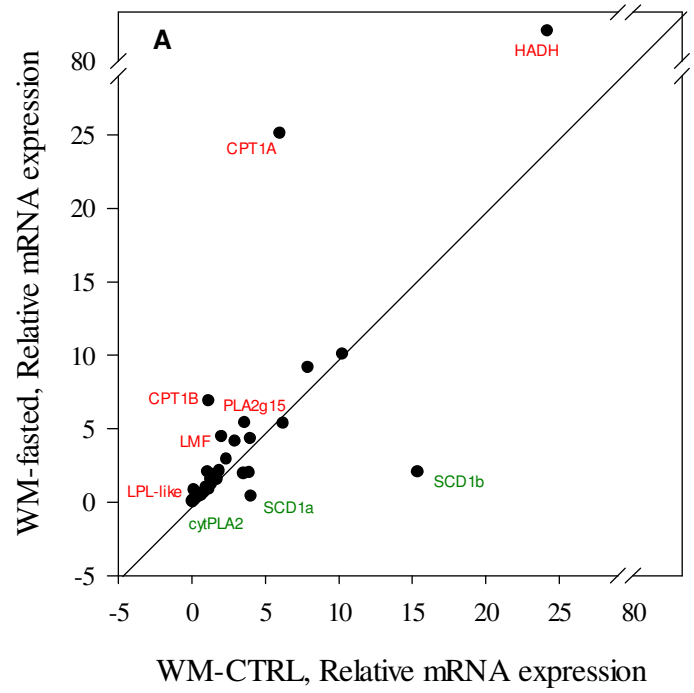
839 Figure 3



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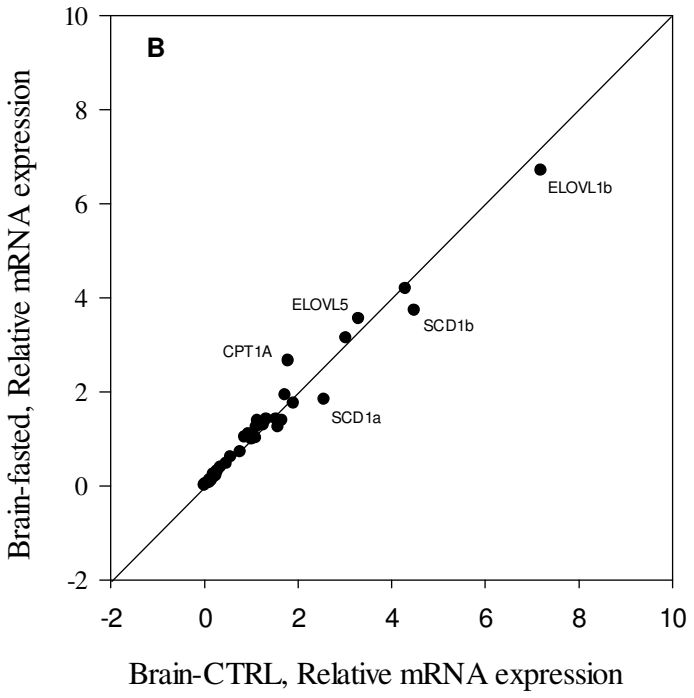

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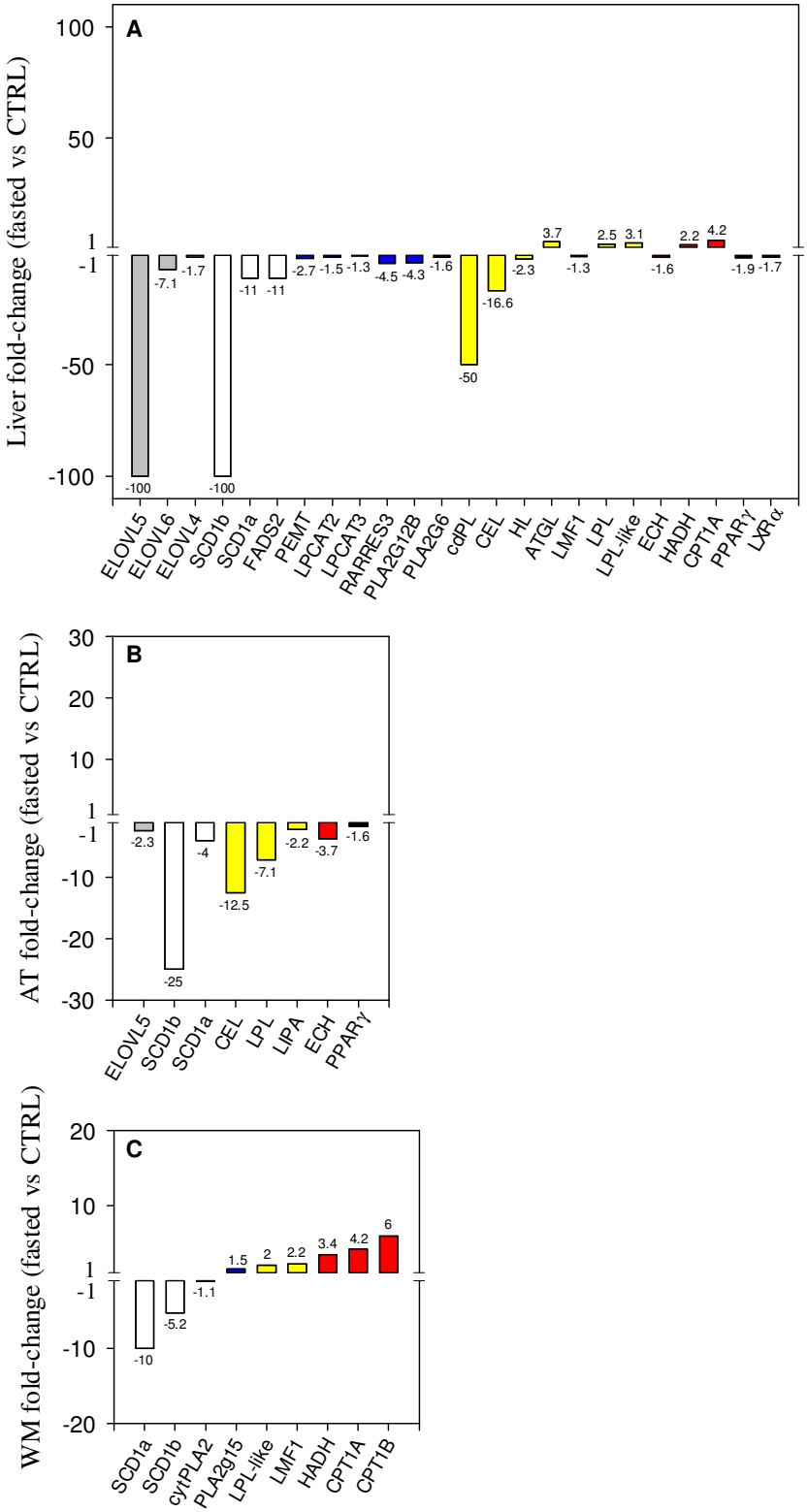
843 Figure 4



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