J Appl Genet 51(4), 2010,pp. 479–487

Original article

Genes associated with long-chain omega-3 fatty acids in bovine skeletal muscle

R. Perez, J. Cañón, S. Dunner

Department of Animal Production, Veterinary Faculty, Complutense University of Madrid, Spain

Abstract. Long-chain omega-3 fatty acids (n-3 FAs) influence meat tenderness, juiciness, and flavor, and are beneficial to human health. The percentage of long-chain n-3 FAs in total FAs is termed the omega-3 index (O3I). It is thus of great interest to favor rising this index in bovine skeletal muscle, to obtain healthier, tastier, and more nutritive meat. This study was aimed to detect transcriptomic variations related to O3I in muscles in 15-month-old males of 4 Spanish cattle breeds raised under the same conditions. Through the analysis of extreme O3I phenotypes, 3 genes of interest (*AANAT, UCP2* and *AHA1*) were identified. *AANAT* and *UCP2* were strongly up-regulated, while *AHA1* was repressed in animals with a high O3I. Moreover, gene expression differed between *GDF8*-null animal muscles (tested for nt821del11 and Q204X mutations) and the wild-type muscles for genes *GDH1, IGF2R, FADS1, ASPH*, and *AIM1*, all showing down-regulation in Asturiana de los Valles calves with muscle hypertrophy (*GDF8*-null). This shows that in *GDF8*-null animals other pathways are used for FA synthesis.

Keywords: differential display, lipid metabolism, long-chain fatty acids, meat quality, omega-3 fatty acids.

Introduction

Fatty acid (FA) composition is known to affect the technological quality of fresh meat and the sensory quality of meat products, as well as to influence human health. Although meat quality assessment depends on socio-cultural preferences of the consumers, fat plays a crucial role in the eating quality of meat, as it influences meat tenderness, juiciness, and flavor (Wood et al. 1999). Increased intramuscular fat, at least in loin meat, is also associated with an improvement in consumer perception of texture and taste (Fernandez et al. 1999). In particular, the proportion of polyunsaturated FAs (PUFAs) in animal products is considered by the consumer as important for the dietetic value of the meat. Particularly relevant is the percentage of long-chain omega-3 FAs in total FAs, which is termed the omega-3 index (O3I).

The relationships between lipids and diseases have been studied extensively, e.g. dietary fat has been hypothesized to increase the risk of various cancers, although no clear association has been demonstrated (Lin et al. 2004). An increase in O3I in the diet has been shown to prevent and treat several cardiovascular and associated diseases (e.g. von Schacky 2000), and to modulate inflammatory responses positively (see Calder 2006). As a consequence, recommendations have recently emphasized the importance of fat quality (rather than fat quantity) and of an increase in n-3 PUFAs at the expense of n-6 PUFAs (Laaksonen et al. 2005; Ohlund et al. 2007).

The proportions of nutrients and the composition of FAs in meat are influenced by numerous factors, including diet, age, gender, breed, ambient temperature, and hormones (Nürnberg et al. 1998). Genetic factors are also known to affect the biological characteristics of muscles, e.g. fiber type, collagen, intramuscular adipose tissue, and protease activities (De Smet et al. 2004). Considering the importance of lipids and FAs for both

Received: November 19, 2009. Revised: March 4, 2010. Accepted: May 20, 2010.
Correspondence: S. Dunner, Department of Animal Production, Veterinary Faculty, Complutense University of Madrid, Spain; e-mail: dunner@vet.ucm.es

carcass value and meat quality, two major methods have been used to control lipid content and composition: feeding and selection. As regards the first one, some trials have been proposed to change the fat ratio in muscle (Mir et al. 2004, Dannenberger et al. 2007) by diet supplementation with fish oil (Scollan et al. 2003), pasture grass (Enser et al. 1998) or linseed (Raes et al.; 2004), but the results were inconsistent (see Webb and O'Neill 2008). The second approach to increase beneficial FAs in bovine muscle for human consumption is to identify the genes associated with the amount of these FAs (namely O3I) and the mechanisms that allow them to be produced in individuals. The identification of causal genes associated with adiposity and their expression profiles in skeletal muscle would provide a better understanding of lipid metabolism processes and their influence on meat quality traits for genetic selection, specifically the selection of individuals bearing polymorphisms in genes producing higher levels of long-chain n-3 FAs and, as a result, healthier and tastier meat.

The purpose of this study was to utilize the natural variation in lipid profiles found in four Spanish cattle breeds, including *GDF8*-null animals characterized by muscular hypertrophy, to identify genetic factors affecting O3I through the use of differential display (DD-PCR) and validation by quantitative real-time PCR (Q-PCR).

Materials and methods

Animals, muscle samples, and phenotypes

The study population consisted of 99 animals of 4 Spanish bovine breeds (25 Asturiana de los Valles, 24 Asturiana de la Montaña, 25 Avileña, and 25 Pirenaica). All individuals were male calves reared from weaning to approximately 15 months old (70% mature weight) in the same conditions and fed a complete pelleted diet (composed of barley and soya with appropriate minerals and vitamins and energy of approximately 12.5 kJ/kg dry matter) and straw provided *ad libitum*. Individuals were blood sampled in Magic Buffer (Biogen Diagnostica, Spain). Shortly after slaughter a small sample was excised from the *longissimus dorsi* muscle at the 6th vertebra of each animal and immediately frozen in liquid nitrogen and stored at –80ºC until analyzed.

The FA composition of neutral lipids and phospholipids in the muscle was assessed as described by Scollan et al. (2001). The trait of interest in this study was the omega-3 index (O3I), calculated from the formula:

O3I = (%EPA + %DPA + %DHA) / total FA

All samples were also genotyped through a Capillary Primer-Extension Assay for the disruptive mutations nt821del11 and Q204X at the *GDF8* locus (to determine the presence/absence of functional myostatin protein and hence of muscle hypertrophy).

RNA source, total RNA extraction

A 25 mg sample of longissimus dorsi was homogenized and RNA was extracted using commercial spin-columns (RNeasy® Fibrous Tissue Mini Kit, QIAGEN), yielding around 10-20 μg of total RNA protected against RNase degradation with RNAsecureTM Reagent 1X (Ambion).

The quality and concentration of total RNA representing each sample was assessed by conventional agarose electrophoresis and through absorbance measurements (260/280 ratio < 1.8). Intact 28S and 18S rRNA subunits were observed on 1% agarose gel, indicating minimal degradation of the RNA.

Differential display (DD-PCR)

Out of the 83 wild-type *GDF8* samples, we selected 12 samples showing extreme levels of O3I: 2 Avileña, 2 Pirenaica, and 2 Asturiana de la Montaña samples with the lowest O3I, and the same numbers of samples with the highest O3I from these breeds.

Amplification reactions were performed using 300 ng of total mRNA, with one-step Q-PCR (Superscript™ III One-Step RT-PCR with Platinum® Taq from Invitrogen) and the corresponding combination of polyT primers (ending with bases A or C or G), containing viral T7 tail primers [T7(dT12)], and 20 reverse primers formed by an arbitrary random sequence with a viral M13 tail [M13-ARP] (the viral tails provide a method for further re-amplification). The reactions were electrophoresed in denaturing polyacrylamide gels [6% (19:1) acrylamide:bis-acrylamide, 7M urea, 0.5× TBE] for 4 h at 60W, and stained with silver nitrate as described by Bassam et al. (1991). DD-PCR bands were excised from the acrylamide gel, re-amplified by PCR with AmpliTaq Gold (Applied Biosystems) with the T7/M13 primer pairs, and sequenced on an ABI Prism 3130 (Applied Biosystems) automated sequencer. Nucleotide sequences obtained were aligned by BLAST

(Altschul et al. 1990; Wheeler and Bhagwat 2007) against public databases, and corresponding transcripts with at least 90% homology were identified.

Primer pair design

Known sequences were used to design primer pairs for each gene identified (Table 1) using primer 3 (Rozen and Skaletsky 2000), preventing possible secondary structures by screening with QIAGEN Oligo Toolkit (http://www.operon.com/) and Dinamelt server (Markham and Zuker 2005), and ensuring specificity of the sequence by BLAST (Altschul et al. 1990; Wheeler and Bhagwat 2007).

Quantitative real-time PCR (Q-PCR)

A larger set of individuals was selected out of the initial 99 individuals to contrast gene expression in high vs low O3I muscles and in *GDF8*-null (*mh/mh*) and wild-type homozygous (+/+) muscles. Here individuals from Asturiana de los Valles (some showing muscular hypertrophy) were also used. The samples included 21 +/+ muscles (average O3I ranging between 0.3 and 1.2) (7 Avileña, 7 Pirenaica and 7 Asturiana de la Montaña) and 10 *mh/mh* muscles (average O3I ranging between 0.7 and 1.6) (7 Asturiana de los Valles). The genes were quantified and transcriptome variations validated by Q-PCR. Total RNA (0.3 μg) was used to produce a retro-transcription reaction using the iScript™ cDNA Synthesis Kit (Bio-Rad), following the manufacturer's recommendations. To perform gene validation, reactions from all individuals were pooled and serially diluted to plot the standard curves. All the aliquots were stored at -80ºC until used. Reactions were performed in an iCycler IQ Real-Time PCR Detection System (Bio-Rad) and a mix was prepared in a 15–μL reaction volume using Dynamo™ HS SYBR® Green qPCR Kit (Finnzymes), 0.4 mM of each primer and 2.7 μL of 1/10 diluted cDNA (except for *AANAT*, which was diluted to ¼), regardless of the initial concentration. The selected reference genes for data normalization were *EEF1A2* and *SF3A1*, whose stability in muscle tissue has been shown elsewhere (Perez et al. 2008). Standard curves and no template controls were produced in triplicate for each gene, together with the sample assays. The following experimental protocol was used: quantification program consisting of 45 cycles of 95ºC for 25 s, annealing temperature (Table 1) for 10 s, and 72ºC for 15 s, ending with a melting program of 68-95ºC with a heating rate of 0.01ºC/s and continuous fluorescence measurement.

Statistical analysis

The results were exported from the iCycler IQ Real-Time PCR Detection System into Microsoft Excel files and analyzed using qBase Excel application (Vandesompele et al. 2002), which allows inter-run calibration, normalization, and calcula-

Table 1. Primer pairs designed for real-time PCR (Q-PCR). T_a = empirical annealing temperature.

Gene symbol	Q-PCR primer pairs		T_a	Amplicon size (bp)
	forward primer	reverse primer		
AANAT	CCTGAAACCCTCGCCTCT	CCCTCTCGCTCAATCTCAAAC	59	141
AHA1	TGAAGGAAGAAGGGGTGAAA	TGGGTTTTTGAAGGAGCAGA	57	181
AIM1	GCTCCAGAAGATAGAATTGAGTCAC	TTCATCCTAAAGGGAGGCACT	59	110
ASPH locus	AACCTGCTCCAAAGCCAAC	TGCTGGTCCTTCTGGTTCTT	59	169
CAMK2A	ATGCGGGTCCAAAAAGAAC	CAAGGGAAGAGGGAAAGAGAA	57	100
DAG1	CGGCATCTCCAGTTTGTCC	ATCGTCCTCGCTGCTCTTCT	59	105
FADS1	TTGCTGCCTGTCTACTTCCA	AGCCTTTCAGTCCCAACA	59	136
GDH1	AGTTTCTTCCGCCTGCCTGT	AAGTGACCGACTGGTCTTGG	59	104
IGF2R	GTGAGAACCAACGGGGACAG	TTCCGACTTTGCGACTGAA	59	136
KIF3C	CCCTCTGTCTCCTTGGCTTA	TCATTCCTTTATTATCGACTGTGTT	59	127
MTCO3	ATTGGCGGAAGAAGCAGA	GACGGAGTTTACGGCTCAA	59	100
ND5	TCTGCAATAGAAGGCCCAAC	CTGTGAGGGGATAGAAACGG	59	100
THRAP3	TGCTACTTTCCCCTTACTTTGC	TTGCAGGAGGAGTTGAAACA	59	120
TNNT1	GGAAGCGCATGGAAAAGGAC	CCTGACGCTCTCGCTCCTTC	59	174
UCP2	CCTGCTTCCTTTTCCTTCCT	CCTGGACTGTGAGAGGCTAC	59	136
Reference genes				
SF3A1	GCGGGAGGAAGAAGTAGGAG	TCAGCAAGAGGGACACAAA	57	125
EEF1A2	GCAGCCATTGTGGAGATG	ACTTGCCCGCCTTCTGTG	59	196

tion of relative expression levels for each gene and each sample. Association between gene expression variation and the O3I phenotype measured was tested through the non-parametric Kruskal-Wallis test using the SAS system package (v.9) NPAR1WAY procedure. Muscular hypertrophy genotypes were also analyzed for association of the quantified genes and this genotype.

Results

Variation in O3I

The O3I of *longissimus dorsi* from the 4 bovine Spanish breeds ranged from 0.2% to 1.6% in *GDF8* wild-type muscles, and from 0.4% to 1.7% in hypertrophic muscles. Average O3I was 0.8% and its coefficient of variation (CV) was 39.5%. This phenotypic character thus presents a high coefficient of variation. Furthermore, 16 individuals from the study population were *GDF8*-null animals and their average O3I in muscles was 1.2%, while CV was 31.6%. Their O3I was significantly higher ($P < 0.001$, data not shown) than in wild-type animals. All calves were reared on the same diet and under the same production system, suggesting that genetic differences in the populations underlie the lipid profile variations.

Differential display (DD-PCR)

Expression profiles were generated by DD-PCR and compared between extreme phenotypes for O3I. Both presence/absence and intensity variations were observed between the groups of high versus low O3I muscles. From the polyacrylamide gels, 43 differentially expressed bands were excised, but only 24 bands were successfully re-amplified and sequenced, and corresponding genes were identified, with at least 95% identity with annotated sequences in public databases. These genes belong to various functional classes (Table 2), such as energy metabolism (e.g. *CYB*, *MTCO*, *ND*, *GDH1*) or sarcomeric structure (e.g. *ACTA*, *TNNT*, *TTN*), as well as genes involved in hormone signaling (*THRAP3*), FA metabolism (*FADS1*) and intracellular trafficking (*KIF3C*). The remaining excised bands (21) were unsuccessfully amplified or did not yield any clear sequence, thus not allowing identification of the gene involved.

Quantitative real-time PCR (Q-PCR)

Not all genes identified by DD-PCR were chosen for quantification by Q-PCR. In an attempt to validate the transcriptomic variations found by DD-PCR, we chose at least one gene from each functional group as the selection criteria, with the exception of the ubiquitinization system. Gene expression levels were measured in a larger sample of extreme O3I phenotypes ($N = 31$), which included both *GDF8*-null (*mh/mh*) and wild-type (+/+) individuals.

The Q-PCR results were calibrated and normalized, using 2 housekeeping genes (*SF3A1* and *EEF1A2*) and qBase Excel application for automated analysis. The data obtained did not fit a normal distribution nor presented homoscedasticity of variances, and thus were statistically analyzed using the non-parametric Kruskal Wallis test, as recommended by other authors (Thellin et al. 1999; Olsvik et al. 2005). Two different sources of variation were checked (Table 3): low vs high O3I and wild-type homozygous vs null *GDF8*.

Our results show differentially expressed genes both for O3I and for the *GDF8* genotype. In the first case (O3I), genes *AANAT* (arylalkylamine N-acetyltransferase) and *UCP2* (uncoupling protein 2) have a 2-fold significant expression increase ($P < 0.01$ and $P < 0.05$, respectively) in muscles with high O3I. By contrast, gene *AHA1* shows a suggestive down-regulation in these samples ($P < 0.07$). In the *GDF8* genotype, genes like *AIM1* (absent in melanoma 1), *ASPH* (aspartyl/asparaginyl β-hydroxylase), *GDH1* (glutamate dehydrogenase 1) and *IGF2R* (insulin-like growth factor 2 receptor) are significantly down-regulated (see Table 3) in *longissimus dorsi* from hypertrophic animals. Also, there is a tendency for gene *FADS1* (fatty acid desaturase 1) to have a lower expression. There is an interaction between sources of variation for gene *DAG1* (dystroglican 1).

Discussion

Outcome of DD-PCR

The trait measured here (O3I) is computed as the percentage of the sum of long-chain n-3 FAs [(20:5, n-3; EPA) + (22:5, n-3; DPA) + (22:6, n-3; DHA)] in total FAs measured in a sample of frozen meat excised from *longissimus dorsi.* This trait has been chosen among other FA measures due to its high coefficient of variation and the fact that it showed no breed effect. Expression differences of genes underlying this trait were mainly detected through intensity differences between DD-PCR bands, rather than through band

Table 2. Genes identified by differential display (DD-PCR) and real-time PCR (Q-PCR). The last column indicates genes quantified by Q-PCR to validate differences in expression.

Gene symbol	Full gene name	GenBank access. number	Function	Q-PCR
Energy metabolism				
CYB	Cytochrome b	AY676873.1	Mitochondrial electron transport chain (complex III)	
MTCO1	Cytochrome c oxidase subunit 1	AY676873.1	Mitochondrial electron transport chain (complex IV)	
MTCO3	Cytochrome c oxidase subunit 3	AY676873.1		X
ND1	NADH dehydrogenase subunit 1	AY676873.1	Mitochondrial electron transport chain (complex I)	
ND5	NADH dehydrogenase subunit 5	AY676873.1		X
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	NM_176655.2		
GDH1	Glutamate dehydrogenase 1, mitochondrial precursor	NM_182652.1	Oxidative deamination of L-glutamate to α-ketoglutarate. Provides TCAIs	X
Sarcomeric system, structural				
ACTA1	Actin, alpha 1	NM_174225.1	Constituent of thin filaments	
MYH1	Myosin heavy chain 1	NM_174117.1	Constituent of thick filaments, predominant in type IIb fibers	
TNNT1	Troponin T1	NM_174474.1	Constituent of thin filaments, predominant in type I fibers	X
TTN	Titin	XR_027685.2	Constituent of elastic filaments	
Ubiquitinization system				
CUEDC1	CUE domain-containing, protein 1	XM_876128.1	Coupling of ubiquitine conjugation to endoplasmic reticulum degradation	
UBE4A	Ubiquitinization factor E4A	XM_867226.1	Participates in ubiquitinization multimeric complex	
Other functions				
AANAT	Arylalkylamine N-Acetyltransferase	XM_870926.3	Limiting step in the synthesis of melatonin from serotonin	X
AHA1	Activator of heat shock 90kDa protein ATPase, homolog 1	NM_001034666.1	Co-chaperone activator of Hsp90 chaperone activity	X
AIM1	Absent in melanoma 1	XM_594681.2	Deleted in melanoma cells	X
CAMK2A	Calcium/calmodulin-dependent protein kinase type II alpha chain	XM_874423.1	Ca2+/calmodulin dependent serine/threonine protein kinase, involved in intracellular signaling cascades	X
DAG1	Dystroglycan 1	AB009079.1	Dystrophin-associated glycoprotein, intermediary between sarcoplasm and extracellular matrix	X
FADS1	Fatty Acid Desaturase 1	XM_612398.4	Introduce unsaturations in delta 5 position of fatty acids	X
IGF2R	Insulin-like Growth Factor 2 Receptor	NM_1743522	IGF2 receptor activity	X
KIF3C	Kinesin family member 3C	NM_001078155.1	Subunit of the kinesin motor complex, involved in intracellular traffic	X
Locus *ASPH*	Aspartyl/Asparaginyl beta-hydroxylase	NM_174757.2	Produced by alternative splicing 3 proteins: AßH, JCTN, Junctin	X
THRAP3	Thyroid hormone receptor associated protein 3	XM_880877.1	Thyroid hormone receptor-associated protein	X
UCP2	Uncoupling protein 2	AF127029.1	Dissipates the proton electrochemical gradient across the inner mitochondrial membrane without coupling to an energy-producing process	X

presence/absence. Also, this trait has not been under selective pressure, as the lipid profile is an expensive trait to compute, but FA content is now becoming an issue for human health. As a consequence, selecting for a desired type of FAs has not been a common practice. No breed effect was found though, and this was the reason why the screened individuals that show extreme amounts of O3I belong indistinctly to the 4 beef breeds used in the study. As all animals were reared under the same conditions, differences for this trait can be accounted for by individual genetic differences.

DD-PCR is not a state-of-the-art technique, but when this study began the bovine genome was still not available and this open, easy, and flexible technique appeared to be a good alternative to candidate genes, allowing the identification of novel genes. A total of 24 genes were identified from excised polyacrylamide bands belonging to various functional categories.

Table 3. Average relative expression of the genes analyzed by real-time PCR (Q-PCR) (± standard error) and statistical significance (*P*) for each trait (*GDF8* genotype and omega-3 index, O3I) and their interaction (INT).

Gene symbol	Relative gene expression in compared groups						
	high O3I	low O3I	*P*	wild-type *GDF8*	null *GDF8*	P	INT
AANAT	65.46 ± 14.93	24.47 ± 15.44	**	38.19 ± 12.44	62.61 ± 17.51		
AHA1	5.28 ± 1.89	9.36 ± 1.89		5.67 ± 1.72	9.62 ± 2.04		
AIM1	9.68 ± 1.98	9.75 ± 2.05		12.23 ± 1.65	4.67 ± 2.32	**	
ASPH	17.35 ± 3.63	15.18 ± 4.12		19.55 ± 3.12	9.81 ± 4.51	*	
CAMK2A	12.68 ± 3.36	10.59 ± 3.47		13.89 ± 2.80	7.32 ± 3.93		
DAG1	10.61 ± 1.48	10.66 ± 1.54		10.91 ± 1.28	10.12 ± 1.71		**
FADS1	50.89 ± 16.11	42.53 ± 16.11		59.43 ± 14.71	28.90 ± 17.40		
GDH1	8.78 ± 1.77	7.72 ± 1.83		10.24 ± 1.47	4.38 ± 2.07	**	
IGF2R	9.89 ± 2.02	9.84 ± 2.09		11.72 ± 1.68	6.16 ± 2.37	*	
KIF3C	31.58 ± 8.39	23.67 ± 8.68		24.04 ± 6.99	35.58 ± 9.84		
MTCO3	9.34 ± 1.72	6.15 ± 1.77		8.05 ± 1.43	7.45 ± 2.01		
ND5	10.21 ± 2.85	12.60 ± 2.85		11.03 ± 2.60	11.93 ± 3.08		
THRAP3	7.48 ± 1.10	5.85 ± 1.60		7.03 ± 0.92	6.21 ± 1.71		
TNNT1	12.40 ± 2.51	13.18 ± 2.61		14.29 ± 2.17	10.03 ± 2.89		
UCP2	26.54 ± 6.17	13.58 ± 7.16	*	22.82 ± 4.91	16.29 ± 8.07		

*: $P < 0.05$; **: $P < 0.01$

Transcriptome variations associated with *GDF8* genotype measured by Q-PCR

The animals used here belong to an EU Project (QLRT-00-347) and some bear the *GDF8 nt821del11* (*mh*) mutation genotype, specifically the Asturiana de Valles breed, where the studied individuals were *mh/mh*. *GDF8* is a gene with a strong influence on many traits, as *GDF8*-null animals show hyperplasia, a low number of mitochondria, anaerobic energy metabolism, and reduced total amount of lipids in meat, but an increased percentage of O3I (Aldai et al. 2007). Due to differences in total FA content between *GDF8* genotypes, the relative proportion of PUFAs in total FAs significantly increases with increasing *mh* allele frequency (Aldai et al. 2006). The expression of some of the genes identified here and belonging to lipid pathways is inhibited in the *mh/mh* genotype, indicating that functional genes in wild-type individuals are not expressed in *GDF8*-null animals and hence other pathways must be activated.

The genes down-regulated in hypertrophic muscles are *GDH1*, *IGF2R*, *ASPH*, *FADS1* and *AIM1*, suggesting that myostatin can positively regulate these genes in normal muscles. The GDH1 enzyme catalyses oxidative deamination of L-glutamate to α-ketoglutarate, providing intermediates in the tricarboxylic acid cycle. A down-regulation of this gene fits with the glycolytic metabolism present in *mh/mh* muscles. The *IGF2R* gene is a multifunctional membrane receptor of *IGF2*. There is no previous report of any relationship between *IGF2R* and lipid metabolism in skeletal muscle, but polymorphisms in *IGF2* have been repeatedly reported both in bovine and porcine livestock as associated with the fat content of meat (Nezer et al. 1999; Sherman et al. 2008). Finally, *FADS1* (fatty acid desaturase 1), down-regulated in this muscle, is involved in the long-chain n-3 FA *de novo* production pathway (Schaeffer et al. 2006). Both *IGF2R* and *FADS1* expression reduction in *mh/mh* muscles could contribute to their attenuated lipid metabolism and reduced fat deposition. These results also suggest that high O3I in hypertrophic muscles is not a consequence of *de novo* synthesis.

Other genes not directly involved in lipid metabolism showed differential activity in *mh/mh* samples: The *ASPH* locus codes for various proteins due to alternative splicing and transcription variations. The sequence obtained by DD-PCR is located along exons 12-13, and Q-PCR was validated in this region, which matches 2 different isoforms: *AβH* (aspartyl/asparaginyl β-hydroxylase) or *JCTN* (junctate). These isoforms share common cytoplasmic, transmembrane, and calcium-binding domains (Feriotto et al. 2006). *AβH* has an exclusive domain that catalyses the hydroxylation of aspartate and asparagine residues in EGF-like domains; thus it is involved in post-translational regulation. *JCTN*, located in the junctional face of the triad, seems to have a role in the control of calcium homeostasis and muscular contraction (Treves et al. 2004). The second gene showing significant differences in expression between the two classes studied is *AIM1*, which has so far been described as belonging to a

superfamily of proteins specifically present in the vertebrate eye lens and has been associated with the control of tumorigenicity in human malignant melanoma models. No clear explanation can be made here for lipid levels.

Transcriptome variations associated with O3I measured by Q-PCR

Some of the gene expression differences observed in DD-PCR between extreme phenotypes for long-chain n-3 FAs were not validated by Q-PCR. This could be due to the existence of false positives in the acrylamide gels, but also to other regulation processes, not directly related to transcription variations (e.g. sequence polymorphisms). In addition, as the trait investigated here is multigenic, the small effects of many genes can contribute to the masking of subtle expression differences, and make the finding of clear associations between transcriptomic variations and composition of FAs in skeletal muscle more difficult.

Although Q-PCR turns out to be itself a technique subject to high variation, and there is an extra difficulty in finding of statistic associations between gene expression levels and one multigenic trait, Q-PCR is a sensible and very useful technique to validate gene expression variations in genes with a sufficient effect on the trait studied, as occurs with *AANAT*, *UCP2* or *AHA1*.

Significantly ($P < 0.05$) higher levels of *AANAT* transcription are found in high vs low O3I muscles and variations in *AANAT* expression in skeletal muscle have not yet been reported. This gene codes for a protein that catalyses the limiting step in the synthesis of melatonin from serotonin. Melatonin has been described as a protecting agent against lipid peroxidation affecting PUFAs (Leon et al. 2005; Fagali and Catala 2007; Leaden and Catala 2007). An increased melatonin production in skeletal muscle could explain a more effective protection against oxidative degradation, and thus a higher O3I. Linked to *AANAT* expression, *AHA1* (activator of heat shock protein A1) shows a suggestive down-regulation ($P < 0.07$). *AHA1* codes for a co-chaperone participating in heat shock protein heteromeric complexes and has the ability to increase the ATPase activity of *Hsp90*. Chaperones and co-chaperones increase their expression in response to oxidative or thermal stress (Liu et al. 2007). In low O3I tissue samples, a higher oxidative stress level is associated with a higher *AHA1* expression, due to a more intense oxidative degradation of PUFAs. The expression of other chaperones (i.e. *DNAJA1*, *HSPB1*) was previously found to be related to meat quality traits, such as tenderness, juiciness and flavor (Bernard et al. 2007).

Also, up-regulated *UCP2* transcription levels have been found in muscles with high O3I and seem to be related to *AANAT* and *AHA1*. *UCP2* produces a mitochondrial protein able to conduct a proton flux across the inner membrane along an electrochemical gradient without a coupled synthesis of ATP. *UCP2* has been described as being involved in cellular thermogenesis (Boss et al. 2000), and hence as a potential modulator of energy homeostasis; it has also been related to development of obesity and type II diabetes (Fleury et al. 1997). Our results suggest that the expression of this gene is involved in energy production included in lipid metabolism.

Finally, the observed interaction between O3I and *GDF8* genotypes for *DAG1*, as shown in Table 4, reveals a different behavior for the long-chain FA content depending on *GDF8* genotype.

In this study, we observed that *AANAT*, *UCP2* and *AHA1* transcription levels are related to O3I in skeletal muscle, giving us a clue to the reasons conditioning higher O3I and the pathways involved in the genesis of meat lipid profiles.

Our findings contribute to a better understanding of metabolic factors influencing long-chain n-3 FA quantities in meat, which therefore affect healthiness and sensorial traits, such as tenderness, flavor, juiciness, and marbling. We also contribute to a better knowledge of the transcriptomic and

Table 4. Effect of *GDF8* genotype (null vs wild-type) and omega-3 index (low vs high O3I) on *DAG1* expression in Spanish cattle.

GDF8 genotype × O3I	LS Mean	Standard error
null × high O3I	1.34[a]	0.30
null × low O3I	2.59[b]	0.30
wild-type × high O3I	2.40[b]	0.21
wild-type × low O3I	2.03[ab]	0.24
Main Effect		
null	1.97	0.23
wild-type	2.03	0.15
high O3I	2.21	0.19
low O3I	1.79	0.19
	P	
null	0.7515	
O3I	0.6907	
null × O3I	0.0269	

metabolic alterations observed in *longissimus dorsi* of doubled-muscled cattle.

Acknowledgements. The animals were sampled within the EU-QLRT00-347 Project. The study was supported by Santander-Complutense (grant no. PR27/05-13961).

REFERENCES

Aldai N, Najera AI, Dugan MER, Celaya R, Osoro K, 2007. Characterisation of intramuscular, intermuscular and subcutaneous adipose tissues in yearling bulls of different genetic groups. Meat Sci 76: 682–691.

Aldai N, Murray BE, Oliván M, Martínez A, Troy DJ, Osoro K, Nájera AI, 2006. The influence of breed and mh-genotype on carcass conformation, meta physico-chemical characteristics, and the fatty acid profile of muscle from yearling bulls. Meat Sci 72: 486–495.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. J Mol Biol 215:403–410.

Arthur PF, 1995. Double muscling in cattle: a review. Aust J Agric Res 46: 1493-1515.

Bassam BJ, Caetano-Anolles G, Gresshoff PM, 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196: 80–83.

Bernard C, Cassar-Malek I, Le Cunff M, Dubroeucq H, Renand G, Hocquette JF, 2007. New indicators of beef sensory quality revealed by expression of specific genes. J Agric Food Chem 55: 5229–5237.

Boss O, Hagen T, Lowell BB, 2000. Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. Diabetes 49:143–156.

Calder PC, 2006. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot Essent Fatty Acids 75:197–202.

Dannenberger D, Nuernberg G, Scollan N, Ender K, Nuernberg K, 2007. Diet alters the fatty acid composition of individual phospholipid classes in beef muscle. J Agric Food Chem 55; 452 –460.

De Smet S, Raes K, Demeyer D, 2004. Meat fatty acid composition as affected by fatness and genetic factors: a review. Anim Res 53: 81–98.

Enser M, Hallett KG, Hewett B, Fursey GAJ, Wood JD, Harrington G, 1998. Fatty acid content and composition of UK beef and lamb muscle in relation to production system and implications for human nutrition. Meat Sci 49: 329–341.

Fagali N, Catalá A, 2007. The effect of melatonin and structural analogues on the lipid peroxidation of triglycerides enriched in omega-3 polyunsaturated fatty acids. Life Sci 81:299–305.

Feriotto G, Finotti A, Breveglieri G, Treves S, Zorzato F, Gambari R, 2006. Multiple levels of control of the expression of the human A beta H-J-J locus encoding aspartylbeta-hydroxylase, junctin, and junctate. Ann N Y Acad Sci 1091:184–190.

Fernandez X, Monin G, Talmant A, Mourot J, Lebret B, 1999. Influence of intramuscular fat content on the quality of pig meat. 2. Consumer acceptability of *m. longissimus lumborum*. Meat Sci 53:67–72.

Fleury C, Neverova M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, et al 1997. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat Genet 15:269–272.

Laaksonen DE, Nyyssönen K, Niskanen L, Rissanen TH, Salonen JT, 2005. Prediction of cardiovascular mortality in middle-aged men by dietary and serum linoleic and polyunsaturated fatty acids. Arch Intern Med 165: 193–199.

Leaden PJ, Catalá A, 2007. Melatonin and N-acetyl serotonin inhibit selectively enzymatic and non-enzymatic lipid peroxidation of rat liver microsomes. Prostaglandins Leukot Essent Fatty Acids 77:29–35.

Leon J, Acuńa-Castroviejo D, Escames G, Tan DX, Reiter RJ, 2005. Melatonin mitigates mitochondrial malfunction. J Pineal Res 38:1–9.

Lin J, Zhang SM, Cook NCR, Lee LM, Buring JE, 2004. Dietary fat and fatty acids and risk of colorectal cancer in women. Am J Epidemiol 160: 1011–1022.

Liu DH, Yuan HY, Cao CY, Gao ZP, Zhu BY, Huang HL, Liao DF, 2007. Heat shock protein 90 acts as a molecular chaperone in late-phase activation of extracellular signal-regulated kinase 1/2 stimulated by oxidative stress in vascular smooth muscle cells. Acta Pharmacol Sin 28: 1907–1913.

Markham NR, Zuker M, 2005. DINAMelt web server for nucleic acid melting prediction. Nucleic Acids Res 33: W577–581.

Mir PS, McAllister TA, Scott S, Aalhus J, Baron V, McCartney D, et al. 2004. Conjugated linoleic acid-enriched beef production. Am J Clin Nutr 79: 1207S–1211S.

Nezer C, Moreau L, Brouwers B, Coppieters W, Detilleux J, Hanset R, et al. 1999. An imprinted QTL with major effect on muscle mass and fat deposition maps to the IGF2 locus in pigs. Nat Genet 21: 155–156.

Nürnberg K, Wegner J, Ender K, 1998. Factors influencing fat composition in muscle and adipose tissue of farm animals. Livest Prod Sci 56: 145–156.

Ohlund I, Hörnell A, Lind T, Hernell O, 2007. Dietary fat in infancy should be more focussed on quality than on quantity. Eur J Clin Nutr 62: 1058–1064.

Olsvik PA, Lie KK, Jordal AE, Nilsen TO, Hordvik I, 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol 6: 21.

Pérez R, Tupac-Yupanqui I, Dunner S, 2008. Evaluation of suitable reference genes for gene expression studies in bovine muscular tissue. BMC Mol Biol 9: 79.

Raes K, De Smet S, Demeyer D, 2004. Effect of dietary fatty acids on incorporation of long chain polyunsaturated fatty acids and conjugated linoleic acid in lamb, beef and pork meat: a review. Anim. Feed Sci. Technol 113: 199–221.

Rozen S, Skaletsky H, 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365–386.

Scollan ND, Choi NJ, Kurt E, Fisher AV, Enser M, Wood JD. (2001). Manipulating the fatty acid composition of muscle and adipose tissue in beef cattle. Br J Nutr. 85:115–24.

Scollan ND, Enser M, Gulati SK, Richardson I, Wood JD, 2003. Effects of including a ruminally protected lipid supplement in the diet on the fatty acid composition of beef muscle. Br J Nutr 90: 709–716.

Schaeffer L, Gohlke H, Müller M, Heid IM, Palmer LJ, Kompauer I, et al. 2006. Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. Hum Mol Genet 15: 1745–1756.

Sherman EL, Nkrumah JD, Murdoch BM, Li C, Wang Z, Fu A, Moore SS, 2008. Polymorphisms and haplotypes in the bovine neuropeptide Y, growth hormone receptor, ghrelin, insulin-like growth factor 2, and uncoupling proteins 2 and 3 genes and their associations with measures of growth, performance, feed efficiency, and carcass merit in beef cattle. J Anim Sci 86: 1–16.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, et al. 1999. Housekeeping genes as internal standards: use and limits. J Biotechnol 75: 291–295.

Treves S, Franzini-Armstrong C, Moccagatta L, Arnoult C, Grasso C, Schrum A et al. 2004. Junctate is a key element in calcium entry induced by activation of InsP3 receptors and/or calcium store depletion. J Cell Biol 166: 537–548.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F, 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: research 0034.1– 0034.11.

Von Schacky C, 2000. N-3 fatty acids and the prevention of coronary atherosclerosis. Am J Clin Nutr 71: 224S–227S.

Webb EC, O'Neill HA, 2008. The animal fat paradox and meat quality. Meat Sci 80: 28–36.

Wheeler D, Bhagwat M, 2007. BLAST QuickStart: example-driven Web-based BLAST tutorial. Methods Mol Biol 395: 149–176.

Wood JD, Enser M, Fisher AV, Nute GR, Richardson RI, Sheard PR, 1999. Manipulating meat quality and composition. Proc Nutr Soc 58: 363–370.