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Changes in white muscle transcriptome induced by dietary energy levels in two lines of rainbow trout (*Oncorhynchus mykiss*) selected for muscle fat content

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Energy intake and genetic background are major determinants of muscle fat content in most animals, including man. We combined genetic selection and dietary energy supply to study the metabolic pathways involved in genetic and nutritional control of fat deposition in the muscle of rainbow trout (*Oncorhynchus mykiss*). Two experimental lines of rainbow trout, selected for lean (L) or fat (F) muscle, were fed with diets containing either 10 or 23 % lipids from the first feeding, up to 6 months. At the end of the trial, trout exhibited very different values of muscle fat content (from 4·2 to 10·1 % wet weight). Using microarrays made from a rainbow trout multi-tissue cDNA library, we analysed the molecular changes occurring in the muscle of the two lines when fed the low-energy or high-energy diet. The results from microarray analysis revealed that eleven metabolism-related genes were differentially expressed according to the diet while selection resulted in expression change for twenty-six genes. The most striking observation was the increased level of transcripts encoding the VLDL receptor and fatty acid translocase/CD36 following both the high-fat diet and upward selection for muscle fat content, suggesting that these two genes are relevant molecular markers of fat deposition in the white muscle of rainbow trout.

Muscle lipid: Genetic selection: Dietary treatment: Fatty acid translocase/CD36: VLDL receptor

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Muscle lipid storage provides an important energy source for muscle functions under specific conditions such as prolonged muscular exercise[1,2] or during fasting[3]. In the animal farming industry, muscle lipid levels also affect meat quality in terms of its nutritional value and sensory properties[4,5]. Dietary manipulation and genetic selection are the two main tools to manage muscle fat levels in farm animals. Genetic factors and energy intake are known to affect muscle fat content in man[6]. However, the molecular mechanisms controlling muscle lipid deposition are not fully understood.

Salmonids have a high ability to store fat in white muscle[7] and are known to exhibit a large range of variations in muscle fat content under dietary manipulation (from 3 % to as much as 18 % fresh matter)[4,8,9], thus making these species particularly suitable for the study of mechanisms involved in muscle fat deposition and mobilisation. Using divergent selection on muscle fat content, we have been able to generate two lines of rainbow trout (*Oncorhynchus mykiss*) having either a fatty muscle (F) or a lean muscle (L)[10]. We combined the use of these two lines with long-term feeding of diets of different energy levels in order to obtain animals having very different levels of muscle fat content (from 4·2 to 10·1 % wet weight) depending on their genetic background and the level of dietary energy.

As in man and birds, endogenous lipids in fish are mainly synthesised in the liver, and then transported to peripheral tissues by the bloodstream[11,12]. Muscle lipid content in these species thus results from the metabolic balance among dietary fat supply, *de novo* fatty acid synthesis in the liver, uptake of plasma TAG by muscle, and subsequent partitioning of fatty acids toward storage or oxidation for meeting the energy requirements. Studying gene expression and activity levels of selected key enzymes of lipid metabolism, glycolysis and energy production provided the first information on the metabolic changes induced in the liver and the white muscle by the selection process and changes in dietary energy[13]. The high-energy (HE) diet repressed the activity of the lipogenic enzymes and stimulated those involved in β-oxidation and glycolysis in the liver but induced minor changes in the muscle enzymes we tested. The change in muscle fat content induced by selection and dietary treatment was not associated with a change in activity and expression of lipoprotein lipase

Abbreviations: ADT, ATP/ADP translocase; CEL, carboxyl ester lipase; F, fat muscle line; FAT/CD36, fatty acid translocase/CD36; HE, high-energy; L, lean muscle line; LE, low-energy; LPL, lipoprotein lipase; SDH, serine dehydratase.

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(LPL) or enzymes involved in fatty acid oxidation in muscle. In order to identify the molecular mechanisms responsible for the difference in muscle fat content induced by the selection process and dietary treatment, we used in the present study an overall approach at the transcriptome level. Our aims were: (1) to assess the overall metabolic changes induced in white muscle of rainbow trout by long-term feeding of a HE/high-fat diet; (2) to identify the metabolic differences induced in muscle as a consequence of the selection procedure; (3) to evaluate whether the two factors (genetic *v.* nutritional) used to modify muscle fat content act through the same metabolic pathways.

Materials and methods

Experimental animals and diets

The animals used in the present study were the same as those described in Kolditz *et al.* [13]. The two lines of rainbow trout, designated L (lean muscle line) and F (fat muscle line), were obtained after three generations of divergent selection for high or low muscle fat content, evaluated using a non-destructive method (Distell Fish Fatmeter) in live fish. Trout were reared in the INRA's experimental facilities (PEIMA, Sizun, France) at a constant water temperature of 11·5 ± 0·5°C. Just before the first feeding time, fish of each line were distributed into six tanks of 500 animals (mean body weight 0·16 g). Triplicate groups of each genotype were fed to satiation two diets containing either 100 (low-energy (LE) diet) or 230 (HE diet) g lipid/kg DM from the first feeding for 6 months. The two diets were made from the same fishmeal-based mixture, and about 15 % fish oil was added to the HE diet to create a wide difference in lipid content between the two diets. The increase in dietary crude fat content (+135 %) was accompanied by a decrease in the proportions of protein (−11 %) and starch (−23 %) in the HE diet (Table 1). At the end of the 6-month feeding trial, and 24 h after the last meal, all fish were anaesthetised with 2-phenoxyethanol at the recommended dose (0·2 ml/l), and individually measured for weight and length. From each tank ten fish were sampled, pooled, ground and freeze-dried before being analysed for fat content of the whole body. From each tank nineteen fish were killed by a sharp blow on the head. Viscera were weighed in order to calculate the viscero-somatic index (VSI):

$$\text{VSI}\,(\%) = 100 \times (\text{total viscera weight}/\text{body weight}).$$

Fillets from the left side of the fish were kept after trimming and skin withdrawal as samples for analysis of lipid content. Also, three additional fish per tank were sampled for liver

Table 1. Chemical composition of experimental low-energy (LE) and high-energy (HE) diets

Diet...	LE	HE
DM (%)	93·0	93·3
Protein (% DM)	57·6	51·1
Lipid (% DM)	9·8	23·1
Starch (% DM)	12·1	9·2
Energy (kJ/g DM)	21·0	23·8

and white muscle under RNAse-free conditions to perform gene expression analysis. All the tissue samples were frozen in liquid N_2 and stored at −80°C until analysis. The experiment was conducted following the *National Guidelines on Animal Care of the French Ministry of Research* and authorisation to experiment (no. 5228) was delivered to the F. M. by the French Ministry of Agriculture and Fisheries.

RNA extraction

The RNA extracts used for the present microarray and real-time PCR analyses were the same as those described by Kolditz *et al.* [13] for expression analysis of candidate genes. Total RNA was extracted from eight individual muscles per experimental condition using the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA). Total RNA was quantified using spectrophotometry based on absorbance at 260 nm and integrity was monitored using the Agilent 2100 Bioanalyser (Agilent Technologies, Kista, Sweden).

cDNA microarray production

Nylon microarrays were obtained from INRA-GADIE Biological Resources Centre (http://www.crb.jouy.inra.fr; Jouy-en-Josas, France). A total of 9023 rainbow trout cDNA originating from a normalised multi-tissue library[14] were spotted after PCR amplification onto Hybond N+ membranes as previously described[15]. Positive (luciferase) and negative (water) controls were also spotted on each microarray. Positive controls allowed checking that hybridisation ran smoothly and negative (water) spots were used for checking the absence of non-specific hybridisation and for calculation of the background level.

Microarray hybridisation and signal processing

Five muscle RNA samples from each experimental condition were used for microarray hybridisation at the INRA UMR 1067 transcriptome facility (St-Pée-sur-Nivelle, France). Initial hybridisation was performed at 42°C for 48 h using a ^{33}P-labelled oligonucleotide sequence (TAATACGACTCACTATAGGG) present at the extremity of each PCR product to monitor the amount of cDNA in each spot. After three washes (three times for 1 h at 42°C, 0·1 × saline sodium citrate (SSC), 0·2 % SDS), arrays were exposed for 48 h to phosphor-imaging plates before scanning using a phosphorimager (Fuji BAS 5000; Fujifilm Corp.; Tokyo, Japan). The oligo probe was then removed from the arrays (three times for 1 h at 68°C, 0·1 × SSC, 0·2 % SDS) and all the membranes were checked for effective stripping of the oligo probe. Arrays were then prehybridised for 1 h at 65°C in hybridisation solution (5 × Denhardt's solution, 5 × SSC, 0·5 % SDS). Labelled cDNA samples were prepared from 3 μg of RNA by simultaneous reverse transcription and labelling for 1 h at 42°C in the presence of 1·85 MBq (50 μCi) [α-^{33}P]dCTP, 5 μM-cold dCTP, 0·8 mM each dATP, dTTP, dGTP and 200 units SuperScript™ III RT (Invitrogen, Carlsbad, CA, USA) in 30 μl final volume. RNA was degraded by treatment at 68°C for 30 min with 1 μl 10 % SDS, 1 μl 0·5 M-EDTA and 3 μl 3 M-NaOH, and then equilibrated at room temperature for

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15 min. Neutralisation was performed by adding 10 µl 1 M-2-amino-2-hydroxymethyl-propane-1, 3-diol-HCl plus 3 µl 2 M-HCl. Then 2 µg PolydA 80mers were added to the solution to saturate the polyA tails. Arrays were incubated with the corresponding denatured labelled cDNA for 40 h at 65°C in hybridisation solution. After three washes (three times for 1 h at 68°C; 0·1 × SSC, 0·2 % SDS), arrays were exposed for 60 h to phosphor-imaging plates before scanning using a phosphorimager (Fuji BAS 5000; Fujifilm Corp.).

Microarray signal processing

Signal intensities were quantified using AGScan software[16], and normalisation was performed using BASE software (BioArray Software Environment), a MIAME-compliant database available at the SIGENAE bioinformatics facility (http://www.sigenae.org/). Spots with an oligonucleotide signal lower than three times the background level (calculated as the median value of the negative controls for each membrane) were excluded from the analysis. After correction, signal processing was performed using the oligonucleotide vector data to correct each spot signal according to the actual amount of DNA present in each spot. After correction, the signal was normalised by dividing each gene expression value by the median value of the array before log transformation. Microarray data from the present study have been deposited in the GEO database (GSE16577)[17].

Statistical analysis of microarray data

A total of 7688 clones out of 9023 (85 %) passed through the background filter and were kept for further analysis. To evaluate potential interactions of diet and genotype, variations in gene expression were analysed for each gene by two-way ANOVA ($P<0·01$) using Tiger TMEV 3.1 software[18], with dietary treatment and genotype as independent variables. When differences were significant, means were compared using the Student–Newman–Keuls test.

Data mining

Rainbow trout sequences originating from INRA AGENAE[19] and USDA[20] expressed sequence tags sequencing programs were used to generate publicly available contigs (Sigenae: http://www.sigenae.org). The 8th version (Om.8, released January 2006) was used for BlastX[21] comparison against the Swiss-Prot database (January 2006) (Swiss-Prot: http://www.expasy.org/sprot/). This was performed automatically for each expressed sequence tag spotted onto the membrane and used to annotate the 9023 clones of the microarray. For all genes identified as differentially expressed in the transcriptome analysis, ontologies were obtained using GoMiner software (http://discover.nci.nih.gov/gominer/) and completed when necessary with information from the literature. When feasible, functional categories were allocated as they relate to muscle biology.

Gene expression analysis: quantitative RT-PCR

Eight individual samples per experimental condition, including the five samples used for microarray hybridisation, were used as biological replicates. Real-time PCR measurements were performed as described in Kolditz *et al.*[13]. Specific primer pairs were designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) according to the transcript sequences that were spotted onto the array. The Genbank accession numbers, the sequences of the forward and reverse primers and the corresponding annealing temperature used for each gene tested for its expression are summarised in Table 2. Relative quantification of the target gene transcript with the elongation factor Iα (*ef1α*) reference gene transcript[22] was performed according to the ΔΔCT method described by Pfaffl[23]. The effects of dietary treatment, line and line–diet interaction on real-time PCR data were tested using SAS® statistical software (SAS Institute, Inc., Cary, NC, USA) by a two-way ANOVA. Differences were considered significant when the probability level was $<0·01$. When differences were significant, means were compared using the Student–Newman–Keuls test.

Results

Growth parameters and body lipid content of fish are reported in Fig. 1. Feeding the HE diet led to an increased whole-body weight and length ($P=10^{-4}$) irrespective of the line. As expected, whole-body and muscle lipid content as well as the viscero-somatic index were higher in fish fed the HE diet compared with fish fed the LE diet ($P=10^{-4}$ and $P<10^{-4}$, respectively), irrespective of the genetic background. At the end of the 6-month trial, the whole-body weights and lengths of the trout of the lean muscle line (L) were higher ($P=10^{-4}$) than those of the fat muscle line (F). Whole-body lipid content was similar for the two lines fed the same diet ($P=0·27$). Muscle lipid content was significantly higher in fish of the F line ($P<10^{-4}$), whereas visceral fat deposition was increased in the L line ($P<10^{-4}$). A line × diet interaction was observed for muscle fat level ($P=0·003$), with a marked increase in F fish fed the HE diet. Muscle fat content thus ranged from 4·2 % wet weight in the L line fed the LE diet to 10 % wet weight in the F line fed the HE diet at the end of the feeding trial (mean fish weight 60–90 g). The other two groups (L-HE and F-LE) had similar muscle fat contents (6·4 % wet weight).

Muscle transcripts differentially expressed between the dietary treatments

Analysis of microarray findings showed that thirty-five genes were differentially expressed between the two dietary treatments ($P<0·01$); fifteen genes were up-regulated and twenty were down-regulated in the white muscle from the HE-fed group, compared with that from the LE-fed group. The categories of biological function represented in this set of genes are summarised in Fig. 2(A). The complete list of genes that were differentially expressed is presented in Table 3. The identity and biological function of four of them are unknown. About one-third (eleven genes) of the differentially expressed genes were related to metabolic processes: seven were involved in protein and amino-acid metabolism, three in lipid metabolism, and one in the antioxidant pathway. In particular, two clones exhibiting significant sequence homology with mammalian transcripts

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Table 2. Accession numbers and primer sequences of genes selected for analysis by real-time RT-PCR

Target gene	Abbreviated gene name	Databank	Accession number	Forward primer	Reverse primer	Annealing temperature (°C)	Amplicon size (bp)
VLDL receptor	*vldlr*	Genbank	BX077158	GTTTTGGACAGATGGGAGA	AGCCTTCTCATTGCACCAGT	60	160
Fatty acid translocase/CD36	*fat/cd36*	Genbank	BX300637	CCACTGAAGTTGAGCCATGA	TGCTAGACTCATGCCGTGTC	60	121
L-Serine dehydratase	*sdhl*	Genbank	CA354115	CAACAACCCTGGATGGATCT	TCCACCTCCAACACAGACAACA	60	136
Carboxylester lipase	*cel*	Genbank	BX073438	ACCGCGATGTCTCCAAATAC	ATTTGGCGTTGATCTCCAG	60	145
PPARα	*pparα*	Genbank	BX300814	CCCTCATTGCTGAGTTCCAT	ACATGATGGAGCCCAAGTTC	60	133
ATP/ADP translocase	*adt2*	Genbank	BX865617	ACAAAGGGATCATGGACTGC	CTTCTGGTCCACTCCTCCAA	60	175
Elongation factor Iα	*ef1α*	Genbank	AF498320	TCCTCTTGGTCGTTTCGCTG	ACCCGAGGGACATCCTGTG	59	159

encoding the VLDL receptor and the fatty acid translocase (FAT/CD36), both involved in lipid uptake in muscle and adipose tissue, were significantly more abundant in fish fed the HE diet than in those fed the LE diet. The HE diet also increased the expression of the gene encoding lipoic acid synthase, involved in the detoxification of activated oxygen species. Concomitantly, genes encoding proteins involved in the regulation of ubiquitin-dependent proteasomal proteolysis (ubiquitin carboxyl-terminal hydrolase 2, ubiquitin carboxyl-terminal hydrolase L1, 26S proteasome non-ATPase regulatory subunit 7), amino-acid catabolism (L-serine dehydratase (SDH)) and peroxisomal fatty acid oxidation (peroxisomal 3, 2-trans-enoyl-CoA isomerase) were under-expressed in the HE-fed group.

Transcripts expressed differentially between the two lines

The two-way ANOVA ($P<0{\cdot}01$) showed that eighty-two genes were differentially expressed between the two rainbow trout lines. In the muscle of the F line sixty genes were expressed at a higher level and twenty-two at a lower level compared with that of the L line. The list of the genes that were differentially expressed in the muscle of the two lines is presented in Table 4, and Fig. 2(B) summarises the different categories of biological functions represented by this set of genes. The identity and biological function of eleven of them are unknown. The largest number of genes differentially expressed between the two genotypes were those related to metabolic processes (twenty-six genes; 32 %). Of these, seven were involved in lipid and sterol metabolism, five in energy production, two in the antioxidant pathway, six were related to protein metabolism, four had functions in amino acid metabolism, one in carbohydrate metabolism and one in Cu metabolism. Transcript levels of ATP/ADP translocase (ADT), cytochrome c oxidase sub unit 3, isocitrate dehydrogenase (NADP dependent) isoform 2, involved in mitochondrial energy production, and cardiolipin synthase, the last enzyme involved in the synthesis of cardiolipin phospholipid, an important component of the inner mitochondrial membrane, were significantly higher in the F line than in the L line. Transcript levels corresponding to carboxyl ester lipase (CEL) and prosaposin, a precursor of sphingolipid activator proteins required for lysosomal degradation of sphingolipids, were also higher in the F line than in the L line.

Transcripts encoding PPARα, microsomal PGE synthase 2 and two transcripts encoding enzymes involved in the antioxidant pathway (ATP-binding cassette transporter 7 and cytochrome P450 2K1) were expressed at lower levels in the F line than in the L line.

Transcripts involved in line–diet interactions

We detected nineteen transcripts for which the effects of the diet were dependent on genotype. The different categories of biological function represented are summarised in Fig. 2(C) and the corresponding list of genes is presented in Table 5. Of these, four have unknown functions, one encoded a protein involved in the cell cycle, one in vesicle-mediated transport, one cell structure, five encoded transcription factors and two were involved in metabolic pathways. The latter two had

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functions in purine metabolism and encoded P2X purinoceptor 3, which was significantly lower in the L-HE group than in the other three groups, as was deoxyuridine 5′-triphosphate nucleotidohydrolase, which was concomitantly increased in the L-HE group. It is worth noting that most of the significant line–diet interactions observed (fourteen out of the nineteen) consisted of increased or decreased transcript levels in the L-HE group. Their biological relevance was difficult to interpret in the light of the phenotype traits recorded, especially regarding how they relate to muscle fat content.

Confirmation of microarray findings with real-time RT-PCR

We then selected a set of candidates from the gene lists described above and proceeded with a real-time RT-PCR analysis of their transcript levels using eight RNA extracts, including the five used for microarray hybridisation. These genes were those encoding VLDL receptor and FAT/CD36, due to their function in cellular uptake of blood-derived lipids, PPARα, due to its key role in lipid metabolism, CEL, also known as bile-salt activated lipase, ADT, a rate-limiting enzyme of the respiratory chain, and finally SDH, a key enzyme of amino acid catabolism. It is of note that the PPARα sequence assayed in the present experiment was not the same as those analysed in the previous study[13].

The diet-induced changes detected in the expression of VLDL receptor, FAT/CD36 and SDH in the microarray analysis were confirmed by real-time RT-PCR analysis. There were higher transcript levels for VLDL receptor and FAT/CD36 ($P=10^{-3}$ and 10^{-4}, respectively) and concomitant lower transcript levels for SDH ($P<10^{-4}$) in fish fed the HE diet than in fish fed the LE diet (Fig. 3). In addition,

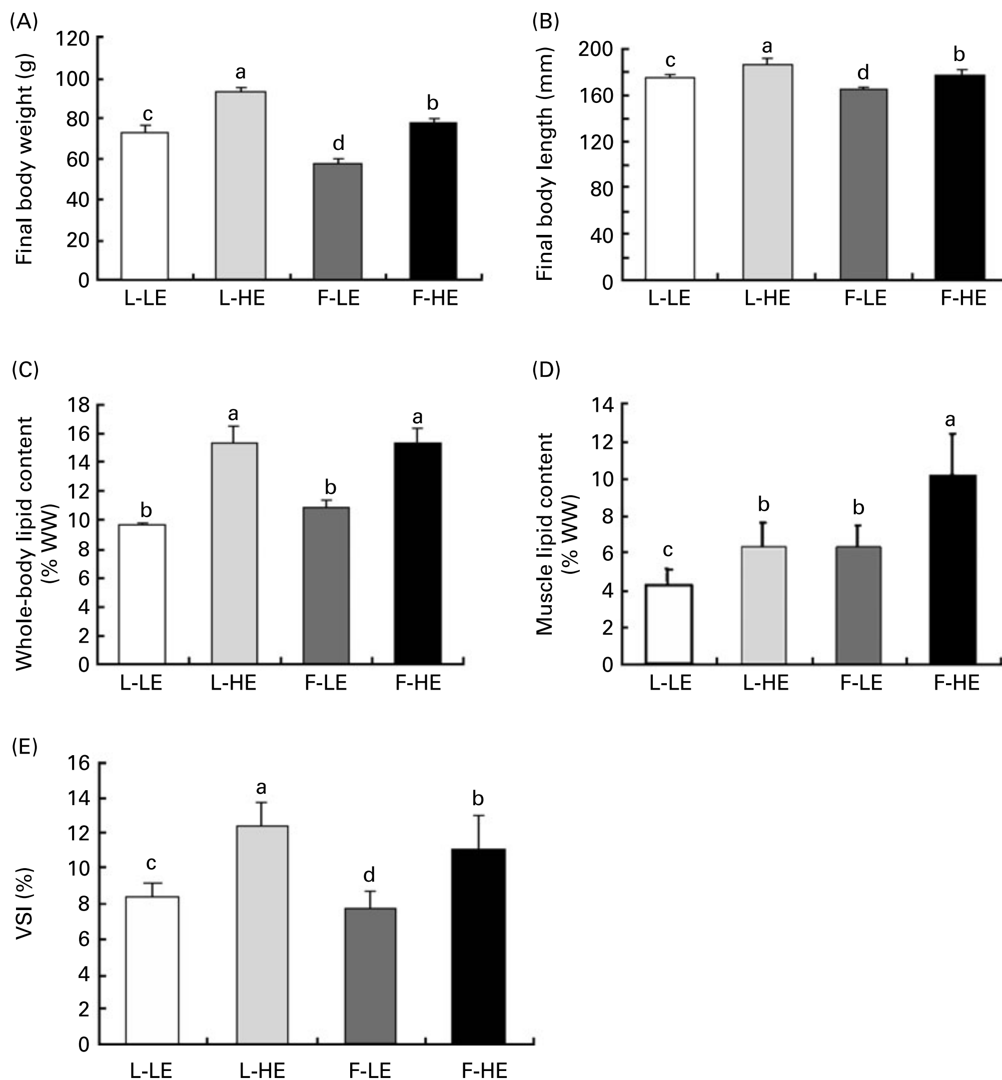

Fig. 1. Body composition of trout (*Oncorhynchus mykiss*) from the lean muscle line (L) and fat muscle line (F) fed the low-energy (LE) and high-energy (HE) diets for 6 months. Values are means, with standard errors represented by vertical bars (*n* 57 individuals in all groups, except for muscle lipid content, for which *n* 30). (A) Final body weight (g). HE > LE ($P=10^{-4}$); L > F ($P=10^{-4}$) (two-factor ANOVA). (B) Final body length (mm). HE > LE ($P=10^{-4}$); L > F ($P=10^{-4}$) (two-factor ANOVA). (C) Whole-body lipid content (% wet weight (% WW)). HE > LE ($P=10^{-4}$); F = L ($P=0.27$) (two-factor ANOVA). (D) Muscle lipid content (% WW). HE > LE ($P<10^{-4}$); F > L ($P<10^{-4}$) (two-factor ANOVA). (E) Viscero-somatic index (VSI; %). HE > LE ($P=10^{-4}$); L > F ($P=10^{-4}$) (two-factor ANOVA). [a,b,c,d]Mean values with unlike letters were significantly different ($P<0.05$; ANOVA).

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real-time RT-PCR measurement of VLDL receptor and FAT/CD36 transcript levels revealed a significant line effect ($P < 10^{-2}$ and $< 5 \times 10^{-3}$, respectively) with higher expression in the F line than in the L line (Fig. 3), an effect that was not revealed in the microarray analysis. In real-time RT-PCR analysis, hybridisation conditions (annealing temperature, primer probe design) were optimised according to the sequence of the target genes, making this technique generally more reliable, sensitive and specific for quantification of mRNA expression than microarray.

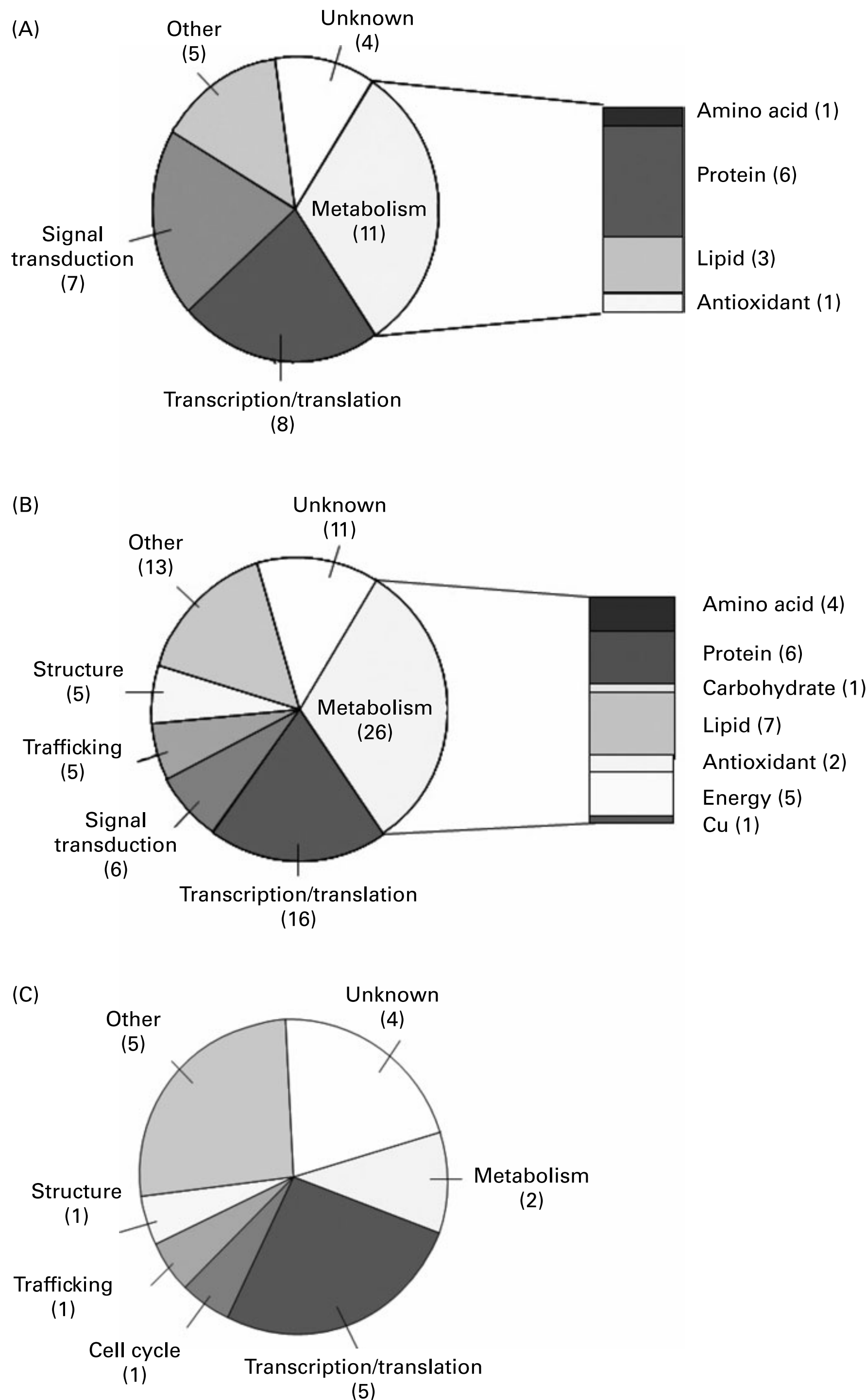

Fig. 2. Biological functions of the genes differentially expressed according to ANOVA ($P < 0{\cdot}01$) (A) between the two dietary treatments (high-energy *v.* low-energy) and (B) between the two genetic lines (fat muscle line *v.* lean muscle line) of rainbow trout (*Oncorhynchus mykiss*). (C) Genes involved in a significant line–diet interaction effect.

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Table 3. Muscle transcripts exhibiting differential expression between the two dietary treatments (high-energy (HE) *v.* low-energy (LE)) in both lines of rainbow trout (*Oncorhynchus mykiss*), as identified by ANOVA analysis ($P<0{\cdot}01$)

Biological function and clone name		HE:LE ratio	
		L line	F line
Metabolism (31 %)			
Lipid metabolism (8·9 %)			
BX077158	Very low-density lipoprotein receptor precursor (VLDL-R)	1·4	1·4
BX300637	Fatty acid translocase/antigen cd36 (FAT/CD36)	1·4	1·4
BX867423	Peroxisomal 3,2-trans-enoyl-CoA isomerase	−1·5	−1·5
Amino-acid derivative metabolism (2·9 %)			
CA354115	L-Serine dehydratase (SDS)	−1·5	−1·2
Protein-folding/synthesis/breakdown (17 %)			
BX861348	Ubiquitin carboxyl-terminal hydrolase 2	−1·4	−1·1
CA348824	Ubiquitin carboxyl-terminal hydrolase isozyme L1	−1·2	−1·5
CA343951	26S proteasome non-ATPase regulatory subunit 7	−1·6	−1·3
CA384189	Putative serine protease F56F10·1 precursor	1·5	1·3
CA365863	ATP-dependent Clp protease proteolytic subunit, mitochondrial precursor	1·7	1·2
BX872771	Augmenter of liver regeneration	−1·5	−1·3
Xenobiotic and oxidant (2·9 %)			
CA373646	Lipoic acid synthetase	1·3	1·8
Regulation of transcription/translation			
BX303258	DNA-directed RNA polymerase III largest subunit	1·2	1·5
CA353007	Translation initiation factor eIF-2B subunit β	1·1	1·3
BX298782	Polycomb group RING finger protein 4	1·2	1·3
CA365421	Homeobox protein Hox-A2b	−3·6	−1·6
BX087067	Methyl-CpG-binding domain protein 3	−1·6	−1·2
CA343779	CCAAT/enhancer-binding protein δ (C/EBPδ)	−1·8	−1·2
CA349800	Nuclear pore complex protein Nup93	−1·4	−1·3
CA347895	Transcription factor 21	−1·4	−2·3
Signal transduction			
CA346554	Mitogen-activated protein-binding protein-interacting protein	1·3	1·2
CA385556	Ras GTPase-activating protein 2	1·2	1·5
CA355642	Regulator of G-protein signalling 18	−1·4	−1·4
CA344992	Frizzled-7 precursor (Fz-7)	−1·1	−1·6
CA344649	Ras-related C3 botulinum toxin substrate 2 precursor	−1·3	−1·2
CA385863	Brother of CDO precursor (Protein BOC)	−1·2	−1·4
CA364621	RAC-α serine/threonine-protein kinase	−1·4	−1·3
Miscellaneous			
BX888224	Calponin homology domain-containing protein 1	1·4	1·4
CA355259	Annexin A13	1·8	1·3
BX867827	Acid sphingomyelinase-like phosphodiesterase 3a precursor	1·4	1·2
CA367722	Hsp90 co-chaperone Cdc37	1·2	1·3
CA342763	YTH domain family protein 2	1·2	1·5
Unknown			
BX084012	Protein C14orf4 homologue	1·2	1·4
CA358365	Hypothetical protein ywlC	1·8	1·1
CA354428	Unknown	1·3	1·7

L, lean muscle line; F, fat muscle line.

Real time RT-PCR results also confirmed the higher transcript levels observed in the F line for ADT ($P<10^{-4}$) (Fig. 3). In agreement with the microarray data, no significant difference in ADT transcript level was observed between the two dietary groups. In contrast, the specific primer pairs we designed for PPARα and CEL did not allow sufficient amplification of these transcripts for effective detection, indicating a very low level of expression of these transcripts in the muscle. This disparity may have resulted from a false-discovery error or from cross-hybridisation of transcripts to regions of similarity of the PPARα and CEL cDNA spotted onto the arrays.

Discussion

Muscle fat content in farm animals has effects both on muscle physiology and on meat quality. However, relatively little is known about the molecular mechanisms that determine muscle fat deposition. Because the white muscle of rainbow trout as other salmonids generally has high but variable fat content[4,8,9] and is capable of mobilising these fat reserves (as for instance during migration), these species can be considered as particularly suitable for the study of mechanisms involved in muscle fat deposition and utilisation. The present study is the first ever attempt to analyse the molecular mechanisms involved in lipid deposition in the white muscle of rainbow trout. Muscle transcriptome analysis was applied as a high-throughput technology for identification of unappreciated molecular actors that responded to the dietary treatments and the selection procedure. We then performed quantitative RT-PCR to measure mRNA levels of the genes that were identified as differently expressed from analyses of microarray data by two-way ANOVA ($P<0{\cdot}01$), and that

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Table 4. Muscle transcripts exhibiting differential expression between the two lines (fat muscle line (F) *v.* lean muscle line (L)) of rainbow trout (*Oncorhynchus mykiss*) in both dietary groups, as identified by ANOVA analysis ($P < 0·01$)

Biological function and clone name		F:L ratio	
		LE diet	HE diet
Metabolism (32 %)			
Lipid metabolism			
BX311641	Cardiolipin synthetase-1·1	1·1	2·5
BX073438	Carboxyl ester lipase	1·3	2·2
CA352463	2-Hydroxyphytanoyl-CoA lyase	1·2	1·2
CA347411	Proactivator polypeptide precursor	1·2	1·3
BX302544	Protein ARV1	1·3	1·3
BX300814	PPARα	−1·2	−2·0
CA366030	PGE synthase 2	−1·5	−1·3
Generation of precursor metabolites and energy			
CA345607	Isocitrate dehydrogenase (NADP) 2	1·3	1·5
BX865617	ADP/ATP translocase	1·2	1·4
CA352391	Cytochrome c oxidase subunit 3	1·8	1·4
CA379757	Decaprenyl-diphosphate synthase subunit 2	1·3	1·3
CA365064	Molybdenum cofactor biosynthesis protein 1 B	1·2	1·2
Amino-acid derivative metabolism			
CA357276	3-Mercaptopyruvate sulfurtransferase	1·2	1·3
CA345917	Guanidinoacetate N-methyltransferase	1·3	1·7
CA345342	Serine-pyruvate aminotransferase, mitochondrial precursor	−1·3	−1·6
CA344534	Kynureninase	−1·7	−1·7
Protein-folding/synthesis/breakdown (17 %)			
BX297091	Ubiquitin carboxyl-terminal hydrolase 28	1·2	1·5
CA346736	Acetohydroxy-acid synthase	1·3	1·8
CA341937	T-complex protein 1 subunit theta	1·1	1·6
CA342154	Peptidyl-prolyl cis-trans isomerase B precursor	1·1	1·4
CA346477	α-2,8-Sialyltransferase 8F	1·4	1·3
BX303582	F-box only protein 6	1·2	1·5
Xenobiotic and oxidant metabolism			
BX074191	ATP-binding cassette sub-family B member 7	−1·5	−1·1
CA348235	Cytochrome P450 2K1	−1·3	−1·2
Carbohydrate metabolism			
BX084018	Trehalase precursor (*EC* 3.2.1.28)	−1·1	−1·5
Cu metabolism			
BX305643	Copper-transporting ATPase 1	1·2	1·7
Regulation of transcription/translation			
CA363479	Cysteinyl-tRNA synthetase, cytoplasmic (*EC* 6.1.1.16)	1·9	1·3
CA358296	Mitochondrial 39S ribosomal protein L39	1·2	1·4
CA344147	Eukaryotic translation initiation factor 3 subunit 2	1·5	1·1
CA365421	Homeobox protein Hox-A2b	1·6	3·8
CA377690	Nuclear transcription factor Y subunit β	1·3	1·4
CA378568	Pre-mRNA-splicing factor syf2	1·3	1·4
BX314809	DNA-directed RNA polymerase III subunit F (*EC* 2.7.7.6)	1·1	2·2
CA341803	Eukaryotic translation initiation factor 3 subunit 4	1·4	1·4
CA343545	40S ribosomal protein S13	1·4	1·3
BX875597	40S ribosomal protein S17	1·3	1·4
CA343637	60S acidic ribosomal protein P2	1·3	1·4
CA345781	60S ribosomal protein L24	1·2	1·5
CA342765	Arginyl-tRNA synthetase, cytoplasmic (*EC* 6.1.1.19)	−1·4	−1·4
CA351058	RNA polymerase II elongation factor ELL	−1·3	−1·2
CA362326	Zn finger protein-like 1	−1·3	−1·6
BX297360	Homeobox protein GBX-1	−1·2	−1·6
Signal transduction			
CA378399	TNF receptor-associated factor 2	1·3	1·3
CA364863	Serine/threonine-protein kinase 19	1·4	1·2
CA357556	Guanine nucleotide-binding protein γ-3 subunit	1·4	1·3
BX866310	Mitogen-activated protein kinase kinase kinase kinase 2	−1·2	−1·4
CA341936	Serine/threonine-protein kinase D3	−1·3	−1·6
BX320696	Casein kinase II subunit β	−1·2	−1·3
Cellular trafficking			
CA346849	Unc-13 homologue C	1·5	1·2
CA376723	Gelsolin precursor	1·4	1·2
CA351559	Latrophilin-2 precursor	1·8	1·2
CA360465	B-cell receptor-associated protein 29	1·5	1·7
CA358906	γ-Aminobutyric acid receptor-associated protein	1·5	1·5
Structure			
CA363233	Spondin-1 precursor	1·5	1·2
CA363745	Tubulin-specific chaperone A	1·2	1·3

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Table 4. *Continued*

Biological function and clone name		F:L ratio	
		LE diet	HE diet
BX860799	Bullous pemphigoid antigen 1 (hemidesmosomal protein)	1·1	1·9
CA345128	Cellular myosin heavy chain, type A	1·5	2·1
Miscellaneous			
CA387939	Chromosome region maintenance 1 protein homologue	1·6	1·5
CA358005	Hb subunit α	1·4	1·9
CA346506	Osteopetrosis-associated transmembrane protein 1 precursor	1·3	1·3
CA379313	DNA repair protein RAD51 homologue A	1·5	1·3
CA342488	Polypyrimidine tract-binding protein 2	1·3	1·6
BX080425	Zygotic DNA replication licensing factor mcm6	1·4	1·5
CA361755	Cell division cycle 5-related protein		
BX869597	Uromodulin-like 1 precursor	−1·4	−1·2
CA352407	H-2 class II histocompatibility antigen, α chain precursor	−1·8	−1·2
BX877741	Retrovirus-related Pol polyprotein from transposon 17·6	−1·2	−1·5
CA343383	Nocturnin	−1·5	−1·4
BX301281	TBC1 domain family member 8	−1·2	−1·7
Unknown			
CA345936	Hypothetical 29·3 kDa protein	1·3	1·1
CA350374	Ankyrin repeat domain-containing protein 39	1·3	1·3
CA376426	Adrenal medulla 50 kDa protein	1·4	1·2
CA356186	AFG3-like protein 2	1·3	1·3
BX076268	Neutrophil cytosol factor 2	1·4	1·8
CA354044	Sulfatase-modifying factor 2 precursor	1·3	1·2
BX877019	WD repeat protein 35	−1·6	−1·6
BX074012	THAP domain-containing protein 11	−1·3	−1·3
BX876294	Protein KIAA0415	−1·3	−1·3

LE, low-energy; HE, high-energy.

were thought to be involved in metabolic pathways related to the observed phenotypes.

Long-term effects of a high-energy and high-lipid diet on muscle gene expression profile

The microarray data showed little diet-induced change in the muscle transcriptome, although muscle fat content in fish fed the HE diet was 1·5-fold greater than that in fish fed the LE diet. This is, however, consistent with the results derived from a previous study conducted on the same animals, in which we analysed several key enzymes involved in lipid metabolism, glycolysis and energy production at gene expression and activity levels[13]. An interesting finding here is that the level of two transcripts encoding proteins involved in mediating blood-derived lipid delivery to the cell, i.e. FAT/CD36 and the VLDL receptor, was significantly increased in muscle following long-term feeding of the HE diet. SDH, a key enzyme of amino acid catabolism that transforms serine into pyruvate, and transcripts involved in the regulation of the proteasome-dependent proteolysis process were under-expressed in fish fed the HE diet, compared with those fed the LE diet. Fish swiftly use proteins as oxidative substrates to meet energy requirements[24,25]. It has been fully established in fish that increasing non-protein energy-yielding nutrients such as lipids generally leads to a protein-sparing effect, probably by redirecting dietary protein and amino acids from energy production toward tissue deposition[26,27]. White muscle protein accretion which determines somatic growth rate[28] results from the balance between protein synthesis and protein degradation rates. The present results suggest a decrease in muscle protein breakdown and subsequent amino acid catabolism associated with increased availability of dietary fat for energy purposes. This is consistent with the enhanced growth observed in fish fed the HE diet and especially the higher protein efficiency ratio in the same group of fish[13].

Taken together, these results suggest that increasing the energy content of the diet induced minor changes in white muscle metabolism of rainbow trout, and that transporter-facilitated uptake of circulating lipids by muscle is likely to be the major mechanism responsible for the higher muscle fat content in salmonids fed HE diets. This is in agreement with the close correlation generally observed between dietary and muscle fatty acid composition in fish[29] as in most single-stomached animals[30–32].

Muscle transcripts affected by the selection process

Compared with L line muscle, F line muscle exhibited higher levels of transcripts encoding ADT and cytochrome c oxidase subunit 3. Since ADT and cytochrome c oxidase subunit 3 are both rate-controlling enzymes of mitochondrial oxidative phosphorylation, we can hypothesise that higher ATP production occurred in F line muscle than in L line muscle. In a previous study using the same animals[13], we evidenced higher activity and gene expression of both hexokinase-I and pyruvate kinase in muscle of the F line compared with the L line, suggesting greater glycolytic activity in the muscle for the former. We did not find any significant effect on key enzymes of peroxisomal and mitochondrial fatty acid oxidation in muscle induced by selection, except for one

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Table 5. Muscle transcripts involved in a significant line–diet interaction identified by ANOVA analysis ($P<0·01$)
(Mean values and standard deviations)

Biological function and clone name		Diet							
		L-LE		L-HE		F-LE		F-HE	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Metabolism									
BX856197	P2X purinoceptor 3	0·014[a]	0·013	−0·152[b]	0·036	0·008[a]	0·028	0·043[a]	0·028
CA342307	Deoxyuridine dUTP pyrophosphatase	−0·107[b]	0·016	0·081[a]	0·035	0·012[a,b]	0·35	−0·027[a,b]	0·050
Regulation of transcription/translation									
CA342029	Zn-binding protein A33	0·077[a,b]	0·055	0·031[a,b]	0·021	−0·127[a]	0·042	0·127[b]	0·055
BX872208	MORC family CW-type Zn finger protein 2A	−0·099[b]	0·019	0·153[a]	0·031	0·020[a,b]	0·031	−0·008[b]	0·051
BX314809	DNA-directed RNA polymerase III subunit F	0·005[a,b]	0·039	−0·136[b]	0·063	−0·003[a,b]	0·042	0·207[a]	0·078
CA387701	Poly (ADP-ribose) polymerase 14	−0·032[a,b]	0·022	0·217[a]	0·092	0·005[a,b]	0·023	−0·090[b]	0·023
CA351413	Histone H1-β, late embryonic	−0·075[b]	0·035	0·157[a]	0·054	0·049[a,b]	0·045	−0·016[a,b]	0·032
Vesicle-mediated transport									
CA370385	Kinesin-like protein KIF1B	−0·080[b]	0·045	0·164[a]	0·056	0·033[a,b]	0·019	−0·068[b]	0·040
Structure									
CA343182	γ-Adducin	−0·005[a,b]	0·040	0·165[a]	0·060	0·092[a,b]	0·038	−0·101[b]	0·042
Cell cycle									
CA361563	NGF-inducible anti-proliferative protein PC3	−0·013[a,b]	0·035	0·104[a]	0·039	0·014[a,b]	0·014	−0·127[b]	0·062
Miscellaneous									
CB486388	Phosphorylase b kinase γ catalytic chain, skeletal muscle isoform	0·150[a]	0·045	−0·116[b]	0·041	−0·072[b]	0·027	0·058[a,b]	0·066
BX084629	γ-Aminobutyric-acid receptor subunit α-1	−0·043[b]	0·042	0·173[a]	0·025	−0·015[b]	0·030	−0·006[b]	0·039
BX082422	Globoside α-1,3-N-acetylgalactosaminyltransferase 1	−0·116[b]	0·034	0·171[a]	0·046	−0·030[b]	0·034	0·020[a,b]	0·041
BX082670	Protein odd-skipped-related 1	−0·054[b]	0·024	0·026[a,b]	0·042	0·130[a]	0·010	−0·017[a,b]	0·036
CA346811	Retrotransposable element Tf2 155 kDa protein type 1	0·014[a,b]	0·032	0·150[a]	0·031	0·011[a,b]	0·035	−0·097[b]	0·028
Unknown									
BX882876	Scrapie-responsive protein 1 precursor	−0·078[b]	0·024	0·208[a]	0·055	0·018[b]	0·031	0·002[b]	0·027
BX860222	Hypothetical oxidoreductase yulF	−0·010[a,b]	0·035	−0·163[b]	0·056	0·015[a,b]	0·052	0·231[a]	0·069
BX869292	Unknown	0·036[a]	0·017	−0·156[b]	0·025	−0·015[a]	0·028	−0·012[a]	0·025
BX297572	Unknown	−0·001[a,b]	0·032	−0·071[b]	0·027	−0·042[b]	0·035	0·125[a]	0·022

L, lean muscle line; LE, low-energy; HE, high-energy; F, fat muscle line.
[a,b] Mean values with unlike superscript letters were significantly different ($P<0·01$).

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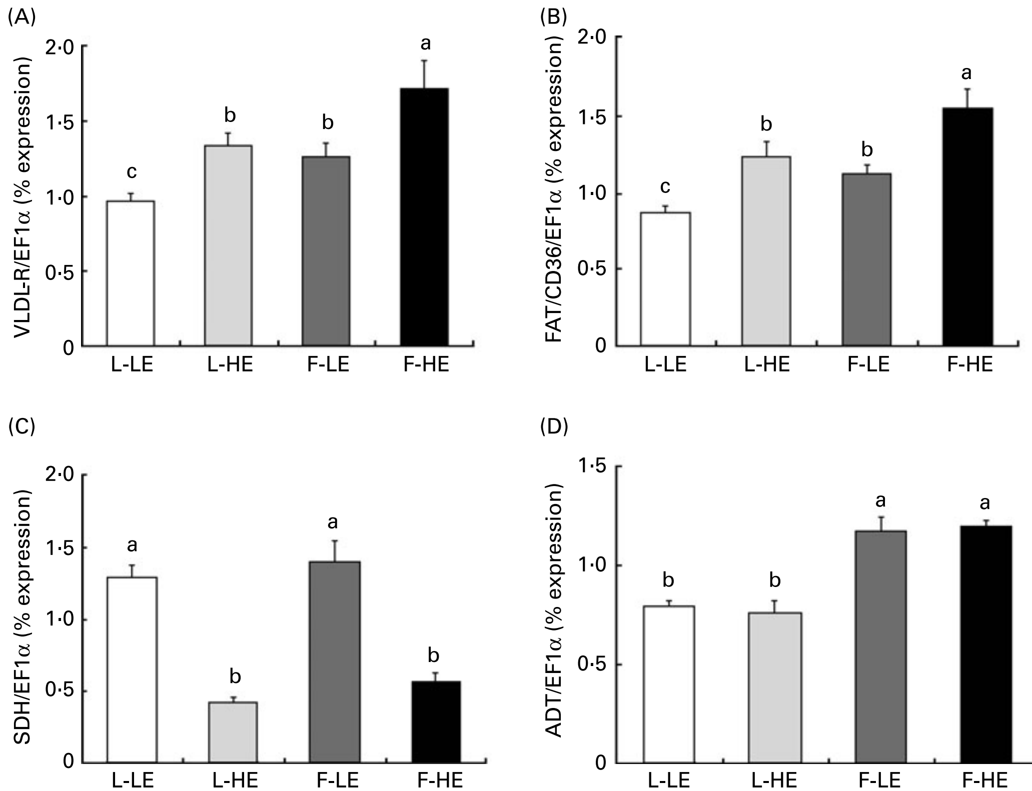

Fig. 3. Gene expression of selected genes measured by real-time quantitative RT-PCR (controls of the microarray data) in the white muscle of rainbow trout (*Oncorhynchus mykiss*) from the lean muscle line (L) and fat muscle line (F) fed the low-energy (LE) and high-energy (HE) diets for 6 months. Data are means of eight samples performed in triplicate, with standard errors represented by vertical bars. Expression values were normalised with elongation factor Iα (EF1α)-expressed transcripts. (A) Gene expression measurement of VLDL receptor (VLDL-R). HE > LE ($P=10^{-4}$); F > L ($P<5\times10^{-3}$) (two-factor ANOVA). (B) Gene expression measurement of fatty acid translocase/CD36 (FAT/CD36). HE > LE ($P=10^{-4}$); F > L ($P<10^{-2}$) (two-factor ANOVA). (C) Gene expression measurement of L-serine dehydratase (SDH). HE > LE ($P<10^{-4}$) (two-factor ANOVA). (D) Gene expression measurement of ATP/ADP translocase (ADT). F > L ($P<10^{-4}$) (two-factor ANOVA). [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$; ANOVA).

isoform of carnitine palmitoyl-transferase I (CPTIb), the mRNA level of which was higher in the F line than in the L line. The white muscle of rainbow trout is mainly composed of fast glycolytic fibres, which utilise fatty acids as fuel rather poorly except during recovery from exhausting swimming[2]. Hence, the enhanced mitochondrial oxidative phosphorylation activity in the F line suggested in the present study can be related to a greater reliance of the F line muscle on glycolysis for energy production as evidenced by the higher activity and gene expression of hexokinase I and pyruvate kinase observed in the muscle of the F line[13].

A high level of expression of a transcript exhibiting high sequence similarity with CEL was detected in muscle of the F line. CEL, also known as bile-salt activated lipase, is a non-specific lipolytic enzyme capable of hydrolysing cholesteryl esters, acylglycerols and phospholipids[33]. Although this enzyme is synthesised primarily in the pancreatic cells and lactating mammary glands of mammals, CEL expression was also detected in the muscle of two pig breeds (Duroc and Taoyuan) through a transcriptome analysis[34]. CEL transcript level was higher in the muscle of the Chinese breed, which displayed a greater level of fatness than the Western breed. This leads us to believe CEL to be a potential genetic marker for muscle fat content. However, no further amplification of CEL could be obtained using RT-PCR performed with probes specifically designed according to the sequence that was spotted on the array in the present study. The same was observed for the PPARα transcript assessed in the present study when another PPARα-specific primer pair allowed us to detect an expression in the previous study with the same samples[13]. One possible reason for these discrepancies between the results from microarray and from RT-PCR may be that other transcripts with high sequence similarity (i.e. other isoforms) may be functionally expressed in white muscle and that the primers used in the present study were too specific to allow their amplification and effective detection. This is a major pitfall to be taken into account when working with rainbow trout, due to the many potential paralogues for a still unappreciated number of genes arising from the recent salmonid genome duplication[35].

The most striking result was undoubtedly provided by the real-time RT-PCR measurement of FAT/CD36 and VLDL receptor gene expression, since it revealed that, in addition to being significantly enhanced by the high-fat diet, muscle VLDL receptor/vitellogenin receptor and FAT/CD36 transcript levels were also increased in trout selected for higher muscle fat content. Despite the fact that the range of changes was limited, the transcript level paralleled the muscle fat content of fish, suggesting that long-term moderate changes in expression of genes related to lipid uptake might significantly affect muscle fat content.

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VLDL receptor and fatty acid translocase/CD36 as potential molecular markers for muscle fat deposition in rainbow trout

VLDL receptor is a member of the LDL receptor superfamily. These receptors mediate the internalisation and degradation of lipoproteins. In mammals, VLDL receptor is expressed predominantly on the capillary endothelium[36] of heart, muscle and adipose tissue[37]. VLDL receptor binds with high affinity to VLDL, intermediate-density lipoproteins and chylomicrons, but not to LDL[38,39]. Recent studies using VLDL receptor-deficient mice have provided compelling evidence that in mammals VLDL receptor has a role in the metabolism of TAG-rich lipoproteins, and is important for postprandial plasma TAG clearance and storage in peripheral tissues[40–42]. In contrast, VLDL receptor homologue expression in oviparous animals was thought to be restricted to ovarian tissue where it definitely has a key function in oocyte development by specifically binding to vitellogenin. It was therefore renamed vitellogenin receptor[43]. In fact it has been demonstrated that at least two vitellogenin receptor variants co-exist in the chicken, as well as in rainbow trout[44,45], which are distinguished by the presence or absence of an O-linked sugar domain. Whereas the O-linked sugar domain-lacking variant has been found to be highly and exclusively expressed in the ovary, the O-linked sugar-containing variant is expressed as a major form in heart and muscle[44,45]. Because studies of vitellogenin receptor/VLDL receptor in egg-laying species such as rainbow trout have so far focused merely on oocyte development, the role played by the somatic form of this receptor and its ligand-binding properties in these species have received little attention. Nevertheless, considering that this receptor is expressed in almost the same tissues as in mammals, it could be argued that this receptor is likely to perform similar functions in oviparous species as in mammals. The present results support the hypothesis, as VLDL receptor was highly expressed in the white muscle of immature rainbow trout, and its mRNA level correlated with the muscle fat level observed across the different experimental groups.

With regard to the physiological role of FAT/CD36, studies in human subjects and mice genetically deficient in FAT/CD36 have indicated that this transporter determines long-chain fatty acid uptake and lipid metabolism in the heart, muscle and adipose tissue[46–48]. Expression of FAT/CD36 and VLDL receptor is notably induced during adipocyte differentiation[49]. As we worked with the whole white muscle tissue, we cannot assert whether the increase in VLDL receptor and FAT/CD36 mRNA levels observed therein was derived from adipocytes located between the fibres, from the white muscle cells or from both. However, since fat deposition in white muscle in fish mainly occurs as lipid accumulation within adipocytes located in connective tissue surrounding the fibres, it seems reasonable to suggest that this increase in expression of FAT/CD36 and VLDL receptor may have mostly occurred in adipose cells.

Taken together, the present results suggest that enhanced lipid uptake is the major mechanism regulating muscle fat in rainbow trout, irrespective of the means used to tailor muscle fat content. Plasma lipids taken up by muscle are transported in the bloodstream either as TAG-rich lipoproteins (i.e. chylomicrons and VLDL) or as NEFA bound to albumin. TAG-rich lipoproteins are hydrolysed by LPL which generates NEFA, 2-monoglycerols and remnant lipoprotein particles. NEFA are then transported across the plasma membrane into muscle cells by simple diffusion or by membrane-associated transporters, FAT/CD36 being among the best characterised to date. Remnant lipoprotein particles can be also taken up into cells by receptors such as VLDL receptor. LPL is thus generally considered as the rate-limiting enzyme in the uptake of fatty acids derived from circulating lipoproteins in peripheral tissues[50]. In the present study, neither transcript levels nor activity of LPL were modified by the dietary treatment, or by the selection procedure used to modify muscle lipid content[13]. This is in line with the lack of correlation observed in muscle between LPL activity and TAG-derived lipid uptake in rats treated with a PPARγ agonist[51], suggesting that the extent of muscle TAG-derived lipid uptake could be independent of changes in LPL *per se*. When LPL activity is not limiting, the increase of expression of VLDL receptor and FAT/CD36 could be sufficient to mediate an increase in muscle lipid uptake. Some studies have reported a strong relationship between LPL and VLDL receptor in mammals[42,52], suggesting functional cooperation of these proteins in the control of postprandial delivery of lipoprotein-derived fatty acids to tissues. In addition to mediating the internalisation of remnant lipoprotein into the muscle cells, VLDL receptor may also act by facilitating the hydrolysis of TAG-rich lipoproteins by maintaining them in close interaction with LPL.

Conclusions

The present study provides evidence for changes in lipid uptake mechanisms in response to dietary or genetic determinants. The increase in muscle fat deposition in rainbow trout resulting from feeding a HE diet seems more likely to be related to enhanced transport and uptake of fatty acids than to changes in intermediary metabolism in the muscle. This is also consistent with the close relationship generally observed between the dietary fatty acid profile and the muscle fatty acid composition. Our data also suggest that VLDL receptor in trout is not only related to vitellogenesis, but may also play a major role in muscle lipid uptake as in mammals. The higher lipid content induced in the muscle by the HE and high-fat diet and upward selection for muscle fat content was associated with a concomitant increase in transcript levels of FAT/CD36 and VLDL receptor, thus making these genes potential molecular markers for understanding molecular mechanisms underlying intramuscular fat deposition in the white muscle. According to the present results, VLDL receptor and FAT/CD36, and not LPL, could be the rate-limiting enzymes for circulating lipid uptake in rainbow trout white muscle. The relevance of VLDL receptor and FAT/CD36 as molecular markers of muscle fattening needs to be confirmed by further studies with muscle samples from various origins and characterised by different lipid content.

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C. I. K. performed sample preparation, microarray hybridisations, integrated the data and drafted the manuscript. E. P.-J. contributed to the microarray hybridisations. E. Q. supervised the production of the two divergent lines. F. M. supervised the whole study together with F. L., conceived of the experimental design, formulated diets, performed quantitative RT-PCR and participated in writing the manuscript. All authors read and approved the final manuscript.

All authors declare no conflict of interest.

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