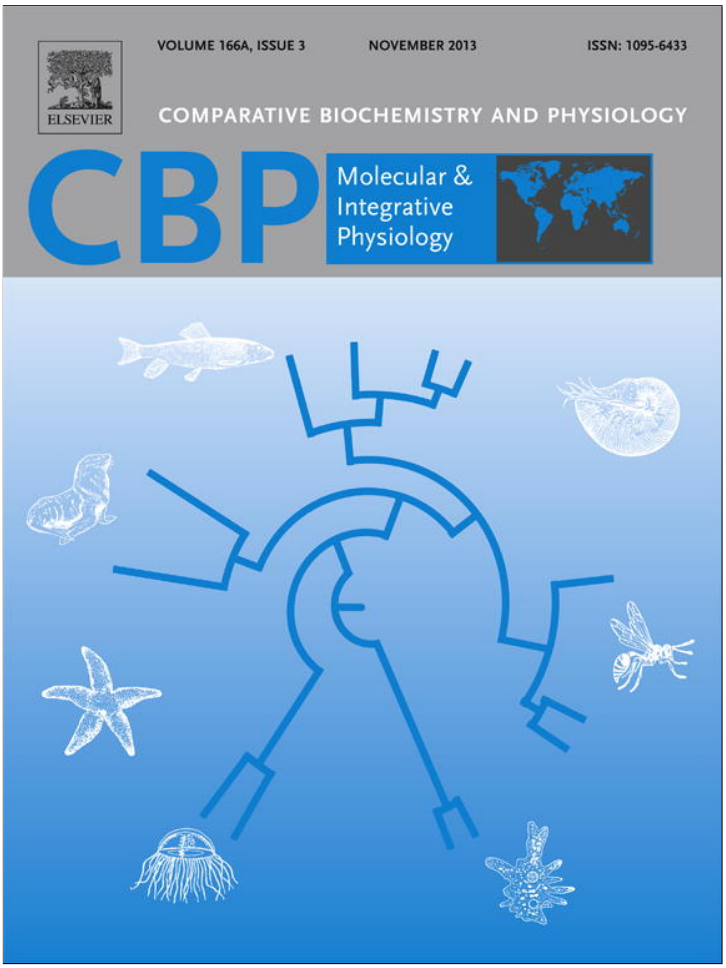

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Effects of sustained swimming on the red and white muscle transcriptome of rainbow trout (*Oncorhynchus mykiss*) fed a carbohydrate-rich diet

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ABSTRACT

Training at sustainable swimming speeds can produce changes in fish skeletal muscle that are important for aquaculture due to their growth-potentiating effects. Such changes may be even more relevant when fish are fed diets containing an increasing proportion of carbohydrates as an energy source. We evaluated the effects of moderate-intensity sustained swimming on the transcriptomic response of red and white muscle in rainbow trout fed a carbohydrate-rich diet using microarray and qPCR. Analysis of the red and white muscle transcriptome in resting or swimming (1.3 body lengths/s) fish for 30 days revealed significant changes in the expression of a large number of genes (395 and 597, respectively), with a total of 218 differentially expressed genes (DEGs) common for both muscles. A large number of the genes involved in glucose use and energy generation, contraction, development, synthesis and catabolism of proteins were up-regulated in red and white muscle. Additionally, DEGs in both muscles were involved in processes of defense response and apoptosis. Skeletal muscle contraction activates a transcriptional program required for the successful adaptation of both muscles to the changing demands imposed by swimming conditions. Future studies should further clarify the mechanisms involved in the adaptation of both tissues to exercise and assess possible benefits of such conditions for cultured fish.

1. Introduction

The optimization of culture conditions to maximize fish growth is an aspect of major importance for aquaculture's commercial production. Because fish growth is strongly influenced by environmental and nutritional factors (Haard, 1992; Lie, 2001; Palstra and Planas, 2011), it is essential to understand how swimming activity and the use of an alternative diet may affect the physiology of fish, particularly with regard to their skeletal muscle.

Skeletal muscle represents more than 60% of the fish body mass and plays an important role during swimming as well as in whole-body metabolic homeostasis. In fish, skeletal muscle is spatially segregated and consists of deep white fibers than constitute approximately 90% of the total muscle mass, covered by a thin superficial layer of red muscle fibers. Red and white muscle fibers differ in their structure and function as they are recruited in different proportions at different swimming velocities (Bone, 1979). Red fibers are active during sustained swimming and contain high numbers of lipid droplets and mitochondria, relying on aerobic metabolism. On the other hand, white fibers are recruited in an increasing proportion as swimming velocity increases, and they contain very few mitochondria, relying mainly on anaerobic glycolysis (Johnston, 1981; Altringham and Ellerby, 1999).

Skeletal muscle growth in fish involves a complex system of regulation influenced by several factors including the genotype and the environment (Kiessling et al., 2006; Johnston et al., 2011). Swim training has growth-potentiating effects in both white and red muscle by increasing their number of fibers and/or their size (Greer Walker and Emerson, 1978; Davison, 1997; Johnston, 1999; Ibarz et al., 2011). In addition to these modifications, swim training at sustainable speeds produces important metabolic adjustments in skeletal muscle (Davison and Goldspink, 1977; Farrell et al., 1991; Magnoni and Weber, 2007; Anttila et al., 2008; LeMoine et al., 2010).

Fish are able to cover the increased demands of ATP required during muscle contraction through hydrolysis of phosphocreatine, glycolysis and oxidative phosphorylation (Richards et al., 2002). Although there

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can be great variability in the source and quantity of energy available, this energy must be derived from the catabolism of lipids, carbohydrates or proteins (Weber, 2011), that ultimately have to be obtained from the diet. In terms of whole-body energy metabolism, carbohydrates and lipids appear to have preponderant roles as metabolic fuels, particularly at low to moderate-intensity swimming speeds (Moyes and West, 1995). In particular, sustainable swimming in rainbow trout at 1–2 body lengths per second (BL/s) is supported by approximately equal contributions of carbohydrate and lipid oxidation (Richards et al., 2002). Therefore, the metabolic changes produced in both red and white muscles by swimming activity are relevant, particularly when fish are fed diets containing an increased proportion of energy in the form of highly digestible carbohydrates. An increasing proportion of dietary carbohydrates can provide the energy needed for maintenance and to sustain swimming, so that a greater proportion of proteins can be spared to build up muscle tissue (Houlihan et al., 1995). In addition, supplementing the diet of fish with carbohydrates would improve the sustainability and reduce the environmental impact of the fish farming industry. Hence, there is a continuing interest to understand how the metabolic phenotype of fish can adapt to increased dietary carbohydrate availability. Nevertheless, rainbow trout (*Oncorhynchus mykiss*) are unable to use high levels of dietary carbohydrates because feeding the fish such a diet results in prolonged postprandial hyperglycemia (Moon, 2001; Hemre et al., 2002). However, we recently showed that when trout are fed a carbohydrate-rich diet (composed of 30% gelatinized starch) the capacity of glucose uptake and utilization by red and white muscle is increased during swimming (1.3 BL/s), decreasing the duration of hyperglycemia and improving the deposition of dietary protein for muscle growth (Felip et al., 2012).

In mammals, exercise has a clear effect in lowering plasma glucose (Hayashi et al., 1997), with skeletal muscle playing a key role in glucose homeostasis (Jessen and Goodyear, 2005). The rate-limiting step in glucose utilization by mammalian skeletal muscle appears to be its movement across the sarcolemma by carrier facilitative transport (Kubo and Foley, 1986), with glucose transporters (GLUT) 1 and 4 being the most preponderant in this tissue. Exercise increases glucose utilization in skeletal muscle by, at least in part, increasing the transcription of the GLUT4 gene (Neufer and Dohm, 1993; MacLean et al., 2002). Salmonid fish possess GLUT homologs that are expressed and regulated in response to hormonal treatment and feeding status in red and white muscle (Planas et al., 2000; Capilla et al., 2002; Díaz et al., 2007a, 2007b, 2009). Therefore, it is possible that the effects of swimming on glucose metabolism could be mediated by the up-regulation of GLUT1 and/or GLUT4 in rainbow trout skeletal muscle. The transcriptome of fish skeletal muscle is also altered by changing feed composition, resulting in modifications in energy use, protein metabolism, cell proliferation, apoptosis and immune response mechanisms (Tacchi et al., 2011, 2012) (Table 1).

In this study, we used microarray technology and quantitative real-time PCR (qPCR) to assess overall gene expression and to determine traits that may be modified in the red and white muscle of trout with exercise. Because this study was part of a broader study, our goal was to investigate the transcriptomic response of red and white muscles to swimming activity when using an energy-rich diet, integrating these results with those previously published on the use of nutrients as energy fuels for swimming (Felip et al., 2012). For that purpose, rainbow trout were fed a carbohydrate-rich diet and were swum or kept under a low speed current for 30 days. After that period, we analyzed changes in

Table 1
Sequences of primers used in gene expression analyses by qPCR.

Target gene	Abbreviation	GenBank ID	Primer sequence (5′–3′)	Amplicon size (bp)	Tm (°C)
Glucose transporter 1	GLUT1	AF247728	(F) AATATCGACAAGCCACGCTG (R) GAGAAGGAGCCGAAGATACC	270	62
Glucose transporter 4	GLUT4	AF247395	(F) CAGTGCTCCTTGTCTTCTTCCTCA (R) CAGCTCCATGTCCAGGTCCA	131	56
Hexokinase	HK	AY864082	(F) CTGGGACGCTGAAGACCAGA (R) CGGTGCTGCATACCTCCTTG	159	59
Glycogen synthase	GS	BT073381	(F) CGTGGTGAGAGGAAGGAACTGAGC (R) CCGTTGAGACCGTGGAGACA	236	56
Glucose-6-phosphate isomerase 1	G6PI	CA371363	(F) CGTAACCGCTCCAACACTCCCATT (R) TAAAGTGCGTAGTGGCGAGTGG	114	57
6-Phosphofructokinase	6-PFK	AM083785	(F) GATGGGAGTGGAGGCTGTGAT (R) CTGGAGGGTTGATGTGGGCTA	239	55
Glyceraldehyde-3-phosphate dehydrogenase 3	GAPDH	CB511095	(F) GGTTGGTTGCGTTACTCCTTGGT (R) CCAGTTACGACGCCATCAAGAAG	262	58
Beta-Enolase 2	B-Enol	DV670949	(F) CAAGGACATCGCTGCCAAAC (R) CCAGAGAAACGCCCAGGATG	140	55
Pyruvate kinase	PK	AF246146	(F) GTCCAATGACCCTACTGAGG (R) CCTGTCTTGAAGAAGCCCCT	306	59
Malate dehydrogenase mitochondrial 2	MDH	EG920179	(F) GAAGGGCGAGGATTTTGTTGCTAA (R) GGTGCCATTTTAAGTGACTGGACA	87	57
Citrate synthase	CS	TC89195	(F) CAACCAACCTCACTCATCACCATA (R) GCAGCAGAAGCAGCCCATAA	133	55
Lipoprotein lipase	LPL	AJ224693	(F) CAGGGAAGCCACTTCAAGAATAAC (R) GGAGGCAGAACATACAGCACAGG	155	55
Cluster differentiation 36	CD36	CA384638	(F) CTGCTATTTCTCCCGCTGCCTCA (R) GCTGCTACTTCTCACTTTGCCTCA	215	57
Carnitine-palmityl-transferase 1	CPT1	AF327058	(F) GCCGCAAACTAGAGAGAGGA (R) CCCGTAGTACAGCCACACCT	200	52
3_hydroxyacyl-CoA dehydrogenase	HOAD	BT074272	(F) GGACAAAGTGGCACCAGCAC (R) GGGACGGGGTTGAAGAAGTG	126	59
Very-long-chain acyl-CoA synthetase	VLACS	CA371001	(F) GCCAGCAGACAGAGAAGAAGAGAC (R) TCCACCTGAATGTATCTCCGACTC	138	55
Peroxisome proliferator-activated receptor γ coactivator 1α	PGC1α	CA368123	(F) TGTATAGGAGGCTCATTTTGATGGA (R) GGTTAACTTTTTTCCCCCAACAA	82	55
Parvalbumin 3	PARV	ES325822	(F) GCAAGTCCAGCGATGATGTGAAGA (R) GCCTTGGTCTCAGCGTCAGTCA	142	59
18S ribosomal RNA	18S	AF308735	(F) CGGAGGTTCGAAGACGATCA (R) TCGCTAGTTGGCATCGTTTAT	62	55

(F): forward, (R): reverse.

the expression of structural, regulatory, metabolic and immune related genes in the red and white muscle of trout.

2. Materials and methods

2.1. Animals and experimental conditions

Juvenile rainbow trout (*O. mykiss*) purchased from a local fish farm (Truites del Segre, Lleida, Spain) were held in the facilities of the School of Biology (University of Barcelona, Barcelona, Spain) in 1000 L tanks with fresh water in a semi-closed system (10% of water renovation daily) with physical and biological filters, ozone skimmers, continuous aeration and optimal water quality parameters at 15 °C and a 12 h light–12 h dark photoperiod. Fish with an average mass of 60 g were randomly distributed into four 200 L circular tanks at a density of 4 kg/m^3. Of these four tanks, two were kept in standard rearing conditions, with a water flow of 350 L/h and vertical water inflow. Fish in these conditions presented only spontaneous movements and were used as control (resting group). The other two tanks (swimming group) were kept in a circular, uniformly distributed flow of 700 L/h, induced by the perpendicular water entrance at the surface and a submerged water pump at the bottom of the tank, isolated from the free-living area. The shape of the tank prevented the fish from entering a central area of lower velocity, thus guaranteeing similar swimming velocities throughout the experiment. Consequently, water volume and fish density were the same as in the resting group. This design and water flow resulted in a uniformly distributed swimming velocity of 1.3 BL/s, measured with a low-speed mechanical flow meter (General Oceanics, Inc., Miami, FL, USA). All fish were kept in the same semi-closed circuit, guaranteeing that physico-chemical water parameters were the same for both groups, and they were fed twice daily to apparent satiety with a diet rich in digestible carbohydrates for 30 days, as described in Felip et al. (2012). Briefly, the high carbohydrate diet used in this study contained, as a percentage of dry matter, 44.39% protein, 10% lipid, 30% carbohydrate as gelatinized wheat starch and 39.16% digestible protein, with 16.82 kJ/g dry matter of digestible energy (Felip et al., 2012).

2.2. Muscle sampling, RNA isolation and cDNA synthesis

After 30 days, eight fish from the sustained swimming group and eight fish from the resting group were anesthetized in 3-aminobenzoic acid ethyl ester (0.1 g/L; Sigma-Aldrich, St. Louis, MO, USA) dissolved in fresh water and subsequently euthanized by sectioning the spinal cord. Red and white muscle tissues were dissected from regions established previously and using separate dissection equipment to avoid cross-contamination. Samples were frozen in liquid nitrogen and stored at −80 °C for analyses. Total RNA was isolated from Polytron-homogenized muscle samples (approximately 1 mg of tissue) using Trizol reagent (Invitrogen, Barcelona, Spain), assessed for quality and quantify with a NanoDrop ND-1000 (Thermo Scientific), treated with a RQ1 DNAse kit (Promega, Barcelona, Spain) and subsequently reverse-transcribed to cDNA using SuperScript III Transcriptase (Invitrogen), oligo(dT) primer and random hexamer primers (Promega), according to the manufacturer's instructions. The experimental protocols used for trout in this study have been reviewed and approved by the Ethics and Animal Welfare Committee of the University of Barcelona, Spain.

2.3. Microarray analyses

Microarray analyses were performed on muscle samples using a rainbow trout cDNA microarray platform (SFA2.0 immunochip) previously described and validated (Koskinen et al., 2004; Krasnov et al., 2005; MacKenzie et al., 2008; Crespo et al., 2010; Palstra et al., 2010), containing 1818 genes representing 366 functional categories and deposited in Gene Expression Omnibus under accession number (GEO ID: GPL6154). Equal amounts of total RNA (1 μg) from red and white skeletal muscle samples of individual rainbow trout from each group (resting fish, n = 8; swimming fish, n = 8), extracted as described before, were pooled and labeled with Cy3-dUTP and Cy5-dUTP (GE Healthcare, Barcelona, Spain) using SuperScript III reverse transcriptase (Invitrogen). The cDNA synthesis reaction was performed at 50 °C for 2 h in a 20 μL reaction volume, followed with RNA degradation with 0.2 M NaOH at 37 °C for 15 min and alkaline neutralization with 0.6 M Hepes. Labeled cDNA was purified with Microcon YM30 (Millipore, Madrid, Spain). We used a dye swap experimental design and each sample was hybridized to two microarrays. For the first slide, cDNA from resting and swimming fish was labeled with Cy5 and Cy3 respectively, and for the second array dye assignment was reversed. A total of four slides were used in this study. The slides were pretreated with 1% BSA (fraction V), 5 × SSC, 0.1% SDS (30 min at 50 °C) and washed with 2 × SSC (3 min) and 0.2 × SSC (3 min) at room temperature and hybridized overnight at 60 °C in a cocktail containing 1.3 × Denhardt, 3 × SSC, 0.3% SDS, 2.1 μg/μL polyadenylate and 1 μg/μL yeast tRNA. All chemicals were from Sigma-Aldrich (Madrid, Spain). After hybridization, slides were washed at room temperature in 0.5 × SSC and 0.1% SDS for 15 min, 0.5 × SSC and 0.01% SDS for 15 min, and twice in 0.06 × SSC for 2 and 1 min, respectively. Scanning was performed with ScanArray 5000 (GSI Lumonics) at 100% laser power, 10 μm resolution and with manual balancing of channels to account for differences in hybridization signal and background among different slides. Images were processed with GenePix Pro 5.0 (Axon). The measurements in spots were filtered by criteria I / B ≥ 3 and (I − B) / (SI + SB) ≥ 0.6, where I and B are the mean signal and background intensities and SI, SB are the standard deviations. After subtraction of median background from median signal intensities, the expression ratios (ER) were calculated. Locally weighted non-linear regression (Lowess) normalization was performed, first for the whole slide and then for twelve rows and four columns per slide using custom microanalysis software (Koskinen et al., 2004; Krasnov et al., 2005). The differential expression was assessed by the difference of the mean $\log_2$ ER from zero between the slides with reverse labeling (6 spot replicates per gene on each slide, Student's *t*-test, $p < 0.01$). Therefore, statistical analysis of gene expression changes between resters and swimmers was performed with technical replicates (n = 12). The $\log_2$ ER ranked up-regulated or down-regulated genes were analyzed interrogating the functional classes of Gene Ontology (GO) (Ashburner et al., 2000) and compared by the sums of ranked genes (Student's *t*-test, $p < 0.05$). Complete microarray results were submitted to Gene Expression Omnibus (GSE47141).

2.4. Quantitative real-time PCR (qPCR)

In order to quantify mRNA expression of particular individual genes not included in the microarray platform and to validate the microarray results, qPCR analysis was completed in red and white muscle samples of both experimental groups. Primer sequences, amplicon sizes and GenBank accession numbers of the target genes are presented in Table 2. The qPCR reactions contained 10 mL of SYBR GreenER qPCR SuperMix (Invitrogen), 500 nM of forward and reverse primers, 5 μL of cDNA at a 1:25 dilution or plasmid DNA as templates (absent in the no-template controls), and the addition of RNase/DNase-free water to reach a final volume of 20 μL. The reactions were run in a MyiQ Real-Time PCR Detection System (Bio-Rad, Madrid, Spain) using the following protocol: 2 min at 50 °C, 8 min at 95 °C, followed by 40 cycles of 15 s denaturation at 95 °C and 30 s at the temperature included in Table 2 for each pair of primers, and a final melting curve of 81 cycles from 55 °C to 95 °C (0.5 °C increments every 10 s). All the samples were run in triplicate and fluorescence was measured at the end of every extension step. Fluorescence readings were used to estimate the Ct values used to calculate the number of copies of the gene of interest. Changes in GLUT1 and GLUT4 gene expression by absolute qPCR were analyzed according to the method described by Whelan et al. (2003).

Table 2
Genes differentially regulated by exercise in red and white skeletal muscle of rainbow trout fed a carbohydrate-rich diet involved in muscle development and contraction, carbohydrate and lipid metabolsim and electron transport.

Clone name	Red muscle	White muscle
Muscle development/ contraction		
Actin, alpha skeletal 5	0.90	1.74
Calmodulin-2		1.01
Creatine kinase, M-2	1.48	1.30
Myosin heavy chain, cardiac muscle beta isoform	-0.90	1.03
Myosin heavy chain, skeletal, adult 1-2	-0.49	1.52
Myosin light chain 2-1	1.23	2.17
Parvalbumin alpha-3	4.55	1.67
Tropomyosin alpha 3 chain-1	0.92	1.44
Troponin I-1, fast skeletal muscle	1.15	2.14
Carbohydrate metabolism/ TCA cycle		
Glucose-6-phosphatase	-0.59	
Glucose-6-phosphate isomerase-1	-0.31	-0.76
Fructose-1,6-bisphosphatase isozyme 2		0.53
6-phosphofructokinase	0.39	-0.42
Glyceraldehyde-3-phosphate dehydrogenase-3	1.32	1.40
Beta-enolase	0.55	0.35
Malate dehydrogenase, mitochondrial-2	0.26	-0.96
Glycogen phosphorylase-1		-3.08
Lipid metabolism		
Acyl-CoA dehydrogenase 9, mitochondrial	-0.59	
Apolipoprotein E-2	-0.45	-0.99
CD36	-0.51	0.38
Fatty acid-binding protein-1	-0.40	
Fatty acid-binding protein-2		-1.17
Very-long-chain acyl-CoA synthetase	-0.42	-2.37
Electron transport		
ATP synthase beta chain-1	-0.38	-0.62
ATP synthase beta chain-2	0.83	-3.20
Cytochrome c oxidase subunit I-1		-0.85
Cytochrome c oxidase subunit I-2		-1.56
Cytochrome c oxidase subunit II	0.34	0.75
Cytochrome c oxidase subunit III-4		-1.17
Cytochrome c oxidase subunit VIIa-related		-1.78
NADH dehydrogenase subunit 2	-0.40	3.72
NADH dehydrogenase subunit 4		-0.43
NADH dehydrogenase subunit 5-1	-0.84	-0.54
NADH dehydrogenase subunit 5-2	-0.66	-0.40

> 2	1 to 2	0.6 to 1	0 to 0.6	0 to -0.6	-0.6 to -1	-1 to -2	< -2

A SFA 2.0 microarray platform was used for the gene expression analysis in muscles of swimming or resting trout, according to the procedure described in Materials and methods. Data shown represent mean $\log_2$ expression ratio. Significantly up- and down-regulated genes ($p < 0.01$, Student's test, 12 spot replicates per gene) are highlighted with a color scale showing intervals of $\log_2$ expression ratios. Missing values indicate lack of differential expression.

By this method, the Ct values were used to calculate copy numbers with standard plots constructed for each target gene using specific primers and serial dilutions (10^{-4} to 10^{-9}) of plasmid DNA corresponding to cloned PCR products into the pGEM-T Easy vector (Promega), previously quantified using a QubitTM fluorometer (Invitrogen). The Ct values were plotted against the logarithm of their initial template copy numbers, generating a standard curve by linear regression of the plotted points that are utilized for the calculations. For the remaining genes, changes in expression were analyzed using the relative quantification method by qPCR described by Livak and Schmittgen (2001). Using this method, values for each sample were expressed as fold change, calculated relative to the resting group and normalized for each gene against 18S ribosomal RNA as the reference gene (1:1000 dilution). The expression of 18S was not affected by exercise in red (Ct values of 17.17 ± 0.51 and 17.43 ± 0.36 in resters and swimmers, respectively) and white muscle (Ct values of 17.30 ± 0.63 and 17.45 ± 0.85 in resteres and swimmers, respectively). All the standard curves exhibited correlation coefficients higher than 0.99, and efficiencies were greater than 99%.

2.5. Statistical analysis

Differences between both groups were analyzed using a non parametric Mann–Whitney Rank Sum Test (Sigma Plot 9.0. Systat Software Inc.), and were considered statistically significant when $p < 0.05$. Results are expressed as means ± SE.

3. Results

This study was undertaken to understand the effects of sustained swimming in rainbow trout fed a diet containing a high proportion of digestible carbohydrates as an important source of metabolizable energy. In the first part of this study, we previously evaluated the changes produced by swimming activity on the relative metabolic use of carbohydrates and proteins by using stable isotopes as dietary tracers (Felip et al., 2012). To further characterize these observed metabolic effects, we subsequently investigated the transcriptomic changes taking place in the red and white muscles of trout by microarray and qPCR analyses.

3.1. Transcriptomic response of red and white muscle to swimming as assessed by microarray analysis

In order to evaluate the general effects of swimming on gene expression in white and red muscle of rainbow trout, we used a cDNA microarray platform (SFA2.0). Microarray analysis showed that a total of 395 differentially expressed genes (DEGs) were identified in the red muscle of swimming trout (Fig. 1A; Supplementary Table 1). A higher number of genes (597) were differentially expressed in the white muscle of swimming trout (Fig. 1A; Supplementary Table 2). A total of 218 differentially expressed genes were common for both white and red muscle. In red muscle, 156 and 239 genes were up- and down-regulated, respectively, in response to swimming (Fig. 1B). In white muscle, 287 and 310 genes were up- and down-regulated, respectively, in response to swimming (Fig. 1C). In white muscle 66% of the DEGs were strongly up- or down-regulated, compared with only 24% of the DEGs in red muscle ($\log_2$ expression ratio > 1).

A large number of functional annotation gene ontology (GO) classes were differentially ($p < 0.05$) expressed in the red or white muscle of swimming trout (Fig. 2; Supplementary Tables 3 and 4). However, to simplify the analysis we chose eight functional categories displaying a selection of DEGs representative of each category for both types of muscles (Tables 2 and 3). Our results evidence important differences in the transcriptomic profile between swimmers and resters, as determined by microarray analysis, and specifically show an increase in the number of DEGs involved in muscle development and contraction by swimming activity for both types of muscle (Fig. 2). In this particular functional category, a number of DEGs were up-regulated in red and white muscle in response to exercise and included several isoforms of actin, myosin, tropomyosin and troponin (Table 2; Supplementary Tables 1 and 2). Also within this functional category, DEGs up-regulated in both muscles included several isoforms of creatine kinase, a key enzyme in energy homeostasis, and calcium-binding proteins including calmodulin and parvalbumin. Only the genes encoding for myosin heavy chains (cardiac muscle beta, skeletal adult 1–1 and 1–2) and a collagen a3(I)-1 were found to be down-regulated in red muscle (Table 2; Supplementary Table 1).

Some of the genes involved in carbohydrate metabolism that were regulated by swimming in the red and white muscles of trout (Fig. 2) are included in Table 2. Within this diverse functional category we can identify several DEGs involved in gluconeogenesis, including glucose-6-phosphatase, glucose-6-phosphate isomerase-1, that were down-regulated in white or red muscle, with the exception of fructose-1,6-bisphosphatase 2 (FBP-2) which was up-regulated by swimming in white muscle (Table 2). Several DEGs involved in glycolysis, including glyceraldehyde-3-phosphate dehydrogenase-3 and beta-enolase were up-regulated in red and white muscle of swimmers, except for 6-phosphofructokinase (6-PFK), which was up-regulated in red muscle but down-regulated in white muscle. Malate dehydrogenase mitochondrial form 2 (MDH-m2), a gene involved in the tricarboxylic acid cycle,

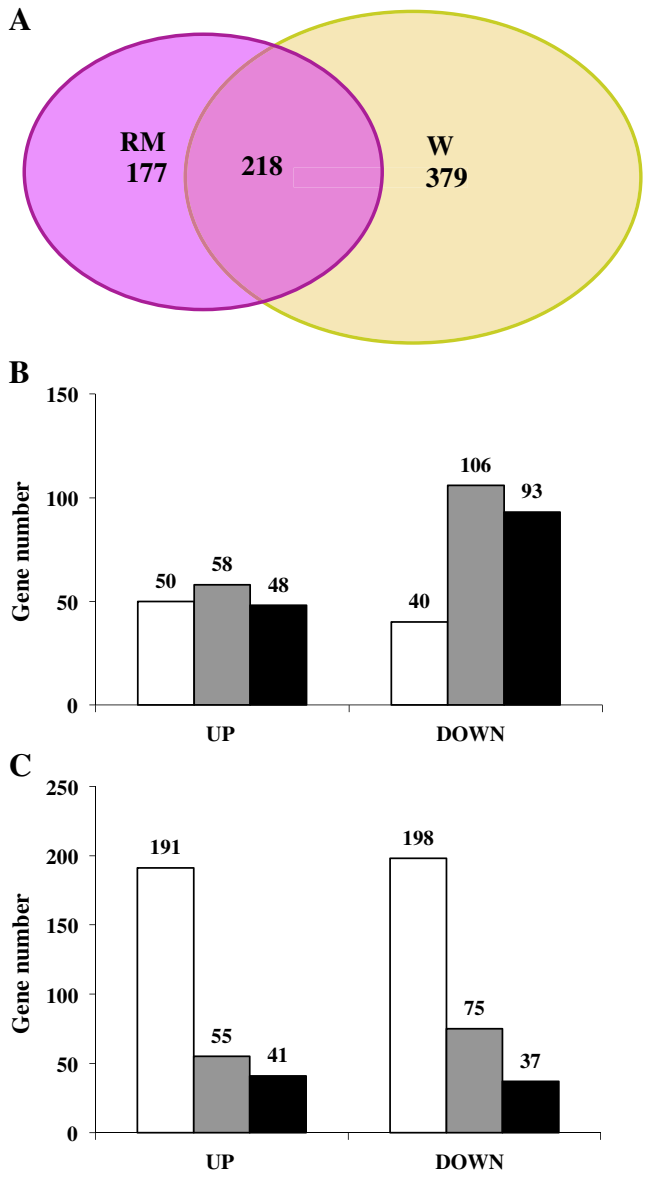

Fig. 1. Differentially expressed genes (DEGs) in response to sustained swimming of red (RM) and white (WM) muscles in rainbow trout fed a carbohydrate-rich diet as assessed by microarray analysis using the SFA2.0 platform ($p < 0.01$). (A) Total number of DEGs in RM and WM in response to sustained swimming. The overlap in the diagrams represents the number of the genes regulated in common between red and white muscle. In B and C, the number of up- and down-regulated DEGs separated by the intensity of change in RM and WM, respectively, is shown. White, grey, and black bars denote changes in gene expression of >1 $\log_2$ expression ratio (ER), 0.5–1 $\log_2$ ER, and 0–0.5 $\log_2$ ER, respectively ($p < 0.01$). See Supplementary Tables 1 and 2 for the complete microarray data.

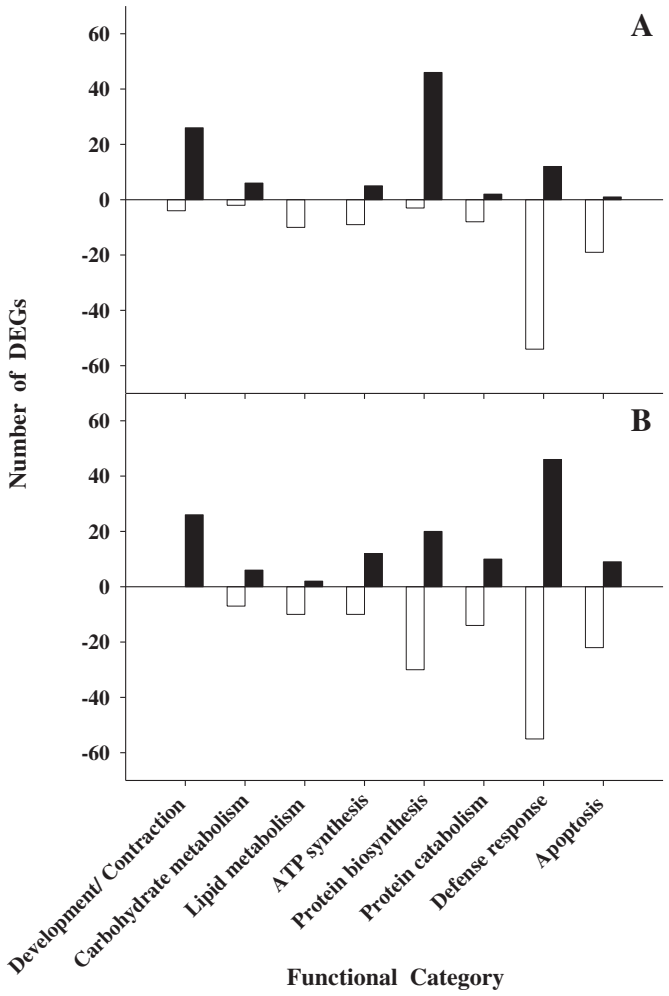

Fig. 2. Gene ontology analysis of differentially expressed genes (DEGs) in response to sustained swimming of red (A) and white muscles (B) in rainbow trout fed a carbohydrate-rich diet. Gene expression in the two types of muscle was assessed by microarray analysis using the SFA2.0 platform (see Materials and methods section). DEGs down- or up-regulated by exercise (white and black bars, respectively) were grouped by gene ontology functional categories. See Supplementary Tables 3 and 4 for the complete gene ontology analysis.

was down-regulated in white muscle but up-regulated in red muscle in response to swimming. The gene expression of glycogen phosphorylase-1 (GP-1), an enzyme which catalyzes glycogen hydrolysis, was down-regulated in white muscle by swimming, but no change was observed in red muscle.

The functional category lipid metabolism appeared to be mostly down-regulated in the two types of muscles (Fig. 2; Table 2). This category comprises genes involved in a wide range of physiological processes and functions including transport, synthesis and modification of lipids and fatty acids (FA) (Table 2). For example, apolipoprotein E, involved in lipid transport, was down-regulated in red and white muscle of fish subjected to swimming. Furthermore, fatty acid-binding protein-1 and -2, involved in FA transport, were down-regulated in red and white muscle, respectively, of fish subjected to swimming. However, in contrast to what was observed in red muscle, the gene expression of the FA transporter CD36 in white muscle was up-regulated in response to exercise. Very-long-chain acyl-CoA synthetase gene expression, an enzyme activating the breakdown of complex FAs, was down-regulated in red and white muscle of fish subjected to swimming. In parallel, acyl-CoA dehydrogenase 9, involved in oxidative phosphorylation and FA β-oxidation, was down-regulated in red muscle.

Microarray analysis evidenced changes in the expression of genes involved in electron transport and pathways for ATP generation in both red and white muscles as a result of swimming activity (Fig. 2; Table 2; Supplementary Tables 1 and 2). Several genes that participate in the respiratory chain, including ATP synthase beta chains 1 and 2, cytochrome c oxidase subunits I-1 and I-2, III-4, and VIIa-related and NADH dehydrogenase subunits 4, 5-1 and 5-2, were down-regulated in white muscle in response to swimming (Table 2). In comparison, the response in red muscle was less important in terms of the number of DEGs and more variable, because some genes were up-regulated by swimming (ATP synthase beta chain 2 and cytochrome c oxidase subunit II Table 2).

Transcriptomic profiling of white and red skeletal muscles from swimming trout evidenced significant changes in protein metabolism,

as seen by the differential expression of a large number of genes involved in protein biosynthesis as well as in protein catabolism (Fig. 2: Table 3). In particular, the functional category protein biosynthesis contained approximately 50 DEGs, including genes corresponding to 40S and 60S ribosomal proteins as well as translation elongation and initiation factors that were differentially regulated by swimming in both types of muscles. In red muscle of trout, 46 out of a total of 49 genes on this functional category were up-regulated in response to swimming (Fig. 2). In white muscle, however, ribosomal genes were either up-regulated or down-regulated and the eukaryotic translation initiation factor 3, subunit 5, was up-regulated, as in red muscle. Among genes involved in protein catabolism, we should highlight the increase in the expression of several cathepsins (C-2, C-3, D-1, F, and H) in white muscle and the decrease in the expression of calpain 2 specifically in red muscle of exercised trout.

Defense response was the GO functional category with the largest number of genes differentially expressed in response to swimming-induced exercise in red and white skeletal muscles (66 and 101, respectively; Fig. 2). DEGs in both muscles from swimming trout included cytokine receptors and signaling proteins, chemokines and their receptors, complement factors, immunoglobulins and antigen-presenting molecules (Table 3). Several tumor necrosis factor receptors were down-regulated in red and white muscle. Furthermore, different responses of immune-related genes to exercise were observed in white and red skeletal muscles of trout, with the most interesting differences residing in the general up-regulation of complement factors (B, C3, C9, D and H) and immunoglobins (Ig heavy and kappa chains) and down-regulation of major histocompatibility complex class I and II molecules in white muscle.

With deoxyribonuclease I-like-2 as the only exception, all the DEGs included in the GO functional category apoptosis were down-regulated in red muscle by exercise (Fig. 2; Table 3). However, the response in white muscle included a larger number of DEGs, and some of these apoptotic genes were up-regulated by exercise (e.g. growth arrest and DNA-damage-inducible GADD45) (Fig. 2; Table 3). We also found that other genes involved in cell signaling processes including several mitogen-activated protein kinases (MAPK) and other protein kinases were also differentially expressed in red and white muscles (Table 3).

3.2. Microarray validation

Validation of the microarray data was performed by qPCR of eight DEGs in common for both red and white muscle (Table 4). We selected a similar number of genes up- and down-regulated included in the functional category muscle contraction/development (PARV-3), carbohydrate metabolism (GAPDH-3, B-Enol-2, G6PI-1, and 6-PFK), lipid metabolism (VLCS and CD36), and TCA cycle (MDH-m2). In all cases, the direction and magnitude of change in expression were confirmed by qPCR.

3.3. Changes in red and white muscle gene expression to exercise as assessed by qPCR

To further characterize the metabolic response occurring at the transcriptome level, we analyzed possible changes in gene expression in red and white muscle of swimming trout to include key metabolic genes such as glucose transporters (GLUT1 and GLUT4) by absolute qPCR and hexokinase (HK), glycogen synthase (GS), piruvate kinase

Table 3
Genes differentially regulated by exercise in red and white skeletal muscle of rainbow trout fed a carbohydrate-rich diet involved in protein synthesis, protein modification and catabolism, defense response and apoptosis and cell signaling.

Clone name	Red muscle	White muscle
Protein synthesis		
40S ribosomal protein S11	0.62	0.91
40S ribosomal protein S9	0.92	0.44
60S ribosomal protein L10-1	0.59	-1.66
60S ribosomal protein L17	0.75	0.66
Elongation factor 1-beta	0.73	-1.68
Eukaryotic translation elongation factor 1 alpha 3	0.55	-0.50
Eukaryotic translation initiation factor 3 subunit 5	0.32	2.42
Ribosomal protein L13	1.19	-1.27
Ribosomal protein S12	0.92	-0.68
Protein modification/ catabolism		
Calpain 2, large [catalytic] subunit precursor	-0.36	
Cathepsin C-2		1.16
Cathepsin C-3		1.42
Cathepsin D-1		3.08
Cathepsin F	-0.61	1.01
Cathepsin H		1.30
Cathepsin S	0.92	
Serine protease-like protein-1	0.45	1.08
Ubiquitin	0.33	
Ubiquitin and ribosomal protein S27a-1	0.72	-0.94
Ubiquitin ligase protein CHFR	-0.70	-4.62
Defense response		
B-cell receptor-associated protein BAP37-1	-0.93	1.14
C-C chemokine receptor type 7 precursor	-0.63	0.55
CC chemokine SCYA106	-0.49	1.14
Chemokine receptor-like 1	-0.61	-1.87
Complement component C3 3-1		5.68
Complement factor D		1.85
Complement factor H-4		2.49
Cytokine receptor common gamma chain		-1.64
High affinity immunoglobulin epsilon receptor gamma	-0.44	-0.90
Ig heavy chain V-III region HIL		1.29
Ig kappa chain C region		1.21
Ig kappa chain V-IV region B17-1		1.16
Inter feron regulatory factor 1-1	1.21	1.40
Interferon regulatory factor 1-2	-0.90	-1.31
Interleukin-1 receptor-associated kinase 4	-0.38	2.18
Lysozyme g-1	-0.76	
Lysozyme g-2		5.30
Macrophage receptor MARCO	-0.80	-2.00
Mannan-binding lectin serine protease 2-2	-0.58	2.55
MHC class I antigen		1.31
MHC class II invariant chain-like protein 1		-1.03
MHC class II regulatory factor RFX1	-0.79	-2.06
TNF receptor associated factor 1		0.68
Toll-like receptor 3 -1		-2.18
Toll-like-receptor 1-2	-0.78	
Tum or necrosis factor receptor superfamily member 11B	-0.46	-3.63
Tumor necrosis factor receptor-2	-1.09	-1.38
Apoptosis/ cell signalling		
Apoptosis regulator Bcl-X	-0.46	
Inhibitor of apoptosis protein 3	-0.69	-1.76
Deoxyribonuclease I-like-2	1.34	-3.22
GADD45 beta		3.81
GADD45 gamma-2		1.51
MAPK 9-1		1.68
MAPK 9-2		-2.46
MAPK 13	-0.99	
MAPK 14b		0.98
MAPK/ERK kinase kinase 6	-0.73	0.59
NF-kappaB inhibitor alpha-2		-0.64
p53-regulated protein PA26	-0.58	
Programmed cell death 10		-1.66
Protein kinase C, alpha type	-0.75	-1.35
Protein tyrosine kinase 2 beta	-0.57	-0.46
Serine protease-like protein-3		-1.57
Tyrosine-protein kinase BTK	-0.69	-1.27
Tyrosine-protein kinase SYK	0.87	
Tyrosine-protein kinase HCK		2.02
Ubiquitin ligase protein CHFR	-0.70	-2.21

> 2	1 to 2	0.6 to 1	0 to 0.6	0 to -0.6	-0.6 to -1	-1 to -2	< -2

Note to Table 3:
A SFA 2.0 microarray platform was used for the gene expression analysis in muscles of swimming or resting trout, according to the procedure described in Materials and methods. Data shown represent mean $\log_2$ expression ratio. Significantly up- and down-regulated genes ($p < 0.01$, Student's test, 12 spot replicates per gene) are highlighted with a color scale showing intervals of $\log_2$ expression ratios. Missing values indicate lack of differential expression.

Table 4
Real-time PCR (qPCR) validation of microarray results from selected genes.

Clone name	Abbreviation	GenBank ID	White muscle		Red Muscle	
			Microarray	qPCR	Microarray	qPCR
Glyceraldehyde-3-phosphate dehydrogenase 3	GAPDH-3	CB511095	1.40	0.90	1.32	0.41
Beta-Enolase 2	B-Enol-2	DV670949	0.35	1.04	0.55	0.97
Glucose-6-phosphate isomerase 1	G6PI-1	CA371363	-0.76	-0.25	-0.31	-0.26
6-Phosphofructokinase	6-PFK	AM083785	-0.42	-0.70	0.39	0.70
Malate dehydrogenase mitochond rial 2	MDH-m2	EG920179	-0.96	-0.80	0.26	0.46
Very-long-chain acyl-CoA synthetase	VLCS	CA371001	-2.37	-2.09	-0.42	-0.34
Parvalbumin 3	PARV-3	ES325822	1.67	1.84	4.55	3.78
Cluster differentiation 36	CD36	CA384638	0.38	0.56	-0.51	-0.73

> 2	1 to 2	0.6 to 1	0 to 0.6	0 to -0.6	-0.6 to -1	-1 to -2	< -2

Data from qPCR is shown as $-\Delta\Delta Ct$ ($n = 8$, each performed in triplicate) and data from microarray as mean $\log_2$ ER (expression ratio). Genes are highlighted with a color scale showing intervals of $\log_2$ expression ratios.

(PK), citrate synthase (CS), lipoprotein lipase (LPL), carnitine palmitoyl transferase 1 (CPT1), 3-hydroxyacyl-CoA dehydrogenase (HOAD) and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α by relative qPCR.

In the present study, we observed changes in the expression of genes involved in glucose metabolic disposal and energy production in both red and white muscles as a result of swimming activity. The mRNA levels of GLUT4 and HK were up-regulated in both muscles, but no change was observed in GLUT1 expression (Fig. 3). The mRNA levels of PK, a key enzyme in the regulation of glycolysis, were up-regulated in white muscle of swimming fish, but not in red muscle. The mRNA levels of CS, an enzyme controlling one of the flux-determining steps of the TCA cycle, were up-regulated by swimming-induced exercise in both red and white muscle. We also observed an increase in the mRNA levels of glycogen synthase (GS) in red muscle, but not in white muscle, of swimming fish.

The mRNA levels of the enzyme lipoprotein lipase (LPL) were augmented by swimming-induced exercise in both types of skeletal muscle, reinforcing its pivotal role in lipid mobilization and its deposition (Fig. 4). The mRNA levels of PGC-1α were also up-regulated in both muscles of swimming trout, which is in accordance with its important function as a transcriptional regulator of genes involved in energy metabolism. However, the mRNA levels of the enzymes CPT1 and HOAD, involved in mitochondrial transport and oxidation of FA, were unaffected in both muscles of swimming fish.

4. Discussion

This is the first study to evaluate the effect of moderate-intensity sustained swimming on the transcriptomic response of red and white muscle in rainbow trout fed a carbohydrate-rich diet. A swimming regime of 1.3 BL/s for 1 month was used as a metabolic promoter to increase glucose utilization by the skeletal muscle of trout. We had previously reported that, in this same group of fish, swimming 1) increases food intake, 2) decreases plasma glucose levels and 3) increases deposition of glycogen and lipids in red and white muscle, respectively, as well as the deposition of protein in both types of muscle (Felip et al., 2012). In accordance with the described physiological effects, the transcriptomic analysis of red and white muscle performed in this study revealed significant changes in the expression of a large number of genes in both muscles from fish subjected to swimming, that are mostly related to alterations in their structural properties and metabolic profile. Furthermore, changes in gene expression were related not only to pathways involved in ATP generation from several metabolic precursors, but

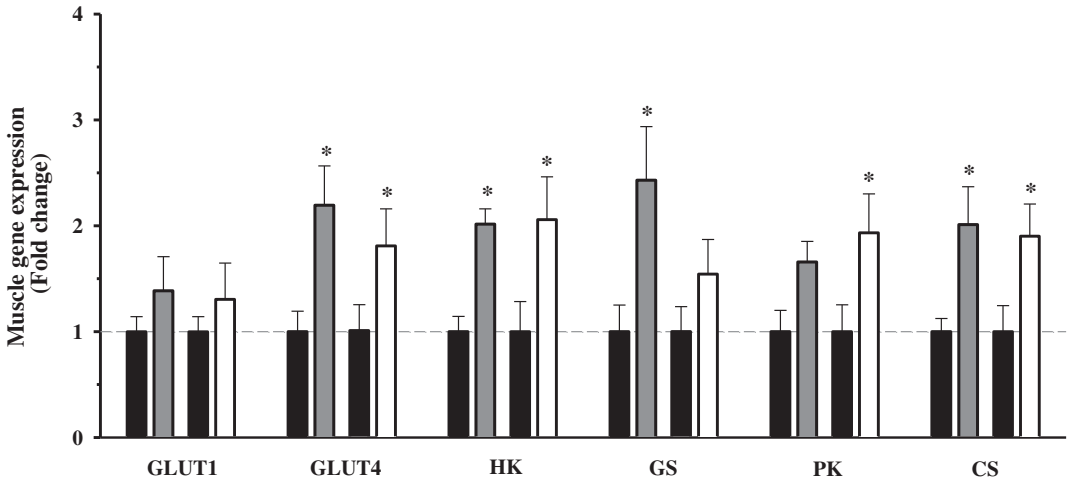

Fig. 3. Effects of swimming on glucose transporters 1 (GLUT1) and 4 (GLUT4), hexokinase (HK), pyruvate kinase (PK), glycogen synthase (GS) and citrate synthase (CS) mRNA expression levels in red (shaded bars) and white (open bars) muscle of rainbow trout fed a carbohydrate-rich diet. Total RNA from trout muscles was isolated and reverse transcribed to cDNA, and mRNA expression levels were determined by qPCR as described in Materials and methods. Results are expressed as fold stimulation above the resting group (black bars), which was set to 1, and shown as means ± SE of eight fish, each performed in triplicate. The asterisk indicates significant differences from the resting group ($p < 0.05$).

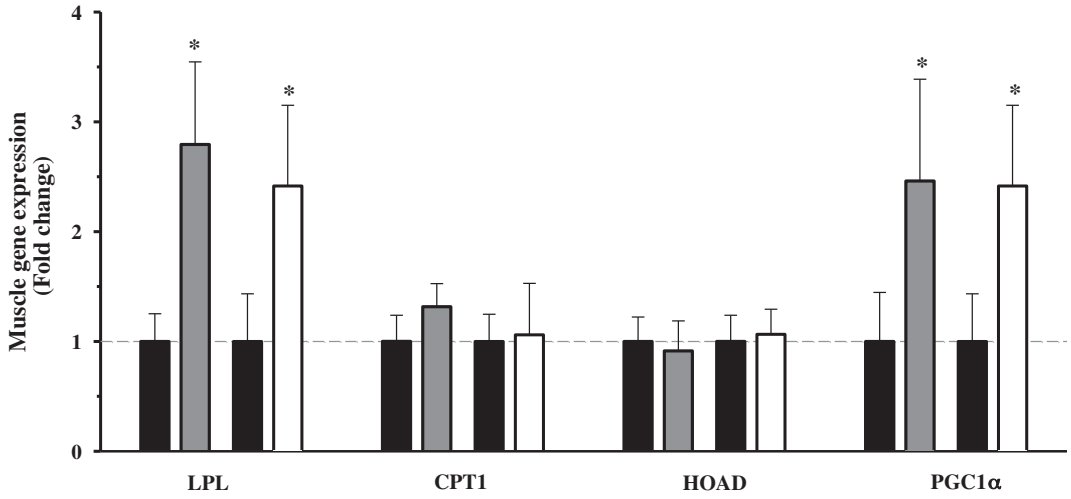

Fig. 4. Effects of swimming on lipoprotein lipase (LPL), carnitine palmitoyl transferase 1 (CPT1), 3-hydroxyacyl-CoA dehydrogenase (HOAD) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mRNA expression levels in red (shaded bars) and white (open bars) muscle of rainbow trout fed a carbohydrate-rich diet. Total RNA from trout muscles was isolated and reverse transcribed to cDNA, and mRNA expression levels were determined by qPCR as described in Materials and methods. Results are expressed as fold stimulation above the resting group (black bars), which was set to 1, and shown as means ± SE of eight fish, each performed in triplicate. The asterisk indicates significant differences from the resting group ($p < 0.05$).

they also included DEGs involved in contraction, development, synthesis and catabolism of proteins, together with processes of defense response in both types of muscles. Nevertheless, the transcriptomic response to the swim training regime used was more evident in white muscle than in red muscle of rainbow trout, as was shown by the larger number of DEGs observed in the former. In a similar way to this study, a differential response in proteins involved in carbohydrate metabolism, protein synthesis, and muscle contraction between red and white muscle was also observed in gilthead sea bream after sustained swim training (Martín-Pérez et al., 2012).

4.1. Changes in gene expression related to skeletal muscle contraction/development

In accordance with the experimental conditions, one of the categories with a large number of DEGs in the skeletal muscle of swimmers was muscle contraction/development. Red and white skeletal muscle showed a remarkably similar response, with almost all genes being up-regulated in the two types of muscles in exercised fish. Up-regulated genes included several alpha actins, myosin heavy and light chains, tropomyosin alpha and troponins C, I and T, all of them key components of the sarcomeric structure of skeletal muscle (Luther et al., 1995). A comparable response has been observed in skeletal muscle of zebrafish after endurance swim training, in which the increase in the expression of myosin and troponin C indicates a shift in phenotype (Van der Meulen et al., 2006). A shift in contractile properties of skeletal muscle and swimming performance in trout was also observed during smoltification, and has been related to the differential gene expression of myosin heavy chain, particularly in red muscle (Coughlin et al., 2001; Weaver et al., 2001). The mRNA levels of calmodulin, a Ca^{2+} binding protein associated with the sarcomere that regulates the release of this ion from intracellular stores and, therefore, plays an important role in the regulation of muscle contraction (Berchtold et al., 2000), was up-regulated in white muscle but did not significantly change in red muscle. Parvalbumin, a low molecular weight protein that buffers myoplasmic Ca^{2+}, was up-regulated at the mRNA level in white and red skeletal muscle of exercised fish. An increase in total parvalbumin content and shifts in the relative expression of parvalbumin isoforms has been shown to be associated with improvements in the relaxation rate of trout skeletal muscle (Coughlin et al., 2007). Furthermore, muscle creatine kinase, an enzyme that is associated with sarcomeric structures and whose function is to replenish ATP levels in muscle fibers by catalyzing the transfer of a phosphoryl group from phosphocreatine to ADP (Clark et al., 2002), was up-regulated at the mRNA level in both types of muscle in exercised fish. In particular, increases in creatine kinase activity and parvalbumin content in skeletal muscle have been linked to an improved swimming performance in zebrafish (Seebacher and Walter, 2012). Overall, the increase in the mRNA expression levels of important contractile elements of muscle fibers as well as regulators of its contractile activity in rainbow trout subjected to sustained swimming is consistent with the known stimulation of fiber hypertrophy in exercised fish (reviewed in Johnston et al., 2011; Palstra and Planas, 2011).

4.2. Changes in gene expression related to protein metabolism in skeletal muscle

An important consequence of swimming activity in trout fed a carbohydrate-rich diet is an improvement in protein deposition in skeletal muscle, as shown by an increase in the recovery of total dietary protein labeled (^{15}N) in red and white muscle of exercised fish (Felip et al., 2012). It is therefore believed that while following an exercise regime, dietary carbohydrates cause a protein-sparing effect that allows proteins to be used to promote growth rather than being used as fuel. In the present study, we show that sustained swimming alters protein metabolism of white and red skeletal muscle, as evidenced by the differential expression of a large number of genes involved in protein biosynthesis as well as in protein catabolism. In particular, our study shows that genes corresponding to 40S and 60S ribosomal proteins as well as translation elongation and initiation factor genes were overwhelmingly up-regulated in red muscle from exercised trout. However, in white muscle, similar numbers of genes were up- and down-regulated within this category.

Among genes involved in protein catabolism, cathepsins (C-2, C-3, D-1, F, and H) increased their expression in white muscle of exercised trout. Cysteine proteases participating in lysosomal protein degradation are known to be up-regulated by fasting in white muscle of rainbow trout (Rescan et al., 2007), but the increase in their mRNA levels in this study is seemingly in contrast to the increase in protein deposition in this tissue in response to swim training (Felip et al., 2012). One possible explanation for this apparent discrepancy is that protein turnover is a continuous and regulated process, which may be needed for the

metabolic and structural remodeling of white muscle under sustained swimming conditions, implying that protein synthesis can exceed the rate of its degradation, resulting in net protein deposition (Houlihan et al., 1995). This scenario appears to be supported by the DEGs involved in the activation of apoptotic and anti-apoptotic signals in skeletal muscle of swimming fish observed in this study (discussed in Section 4.4). In contrast to white muscle, the proteases cathepsin (D-2 and H) and calpain-2 decreased their expression at the mRNA level in red muscle of exercised fish, which is consistent with the prominent increase in the mRNA levels of sarcomeric components in this tissue. Ubiquitous calpains have been associated with processes of cellular apoptosis, myogenesis, and sarcomeric remodeling in mammals (Murphy, 2010). Particularly, calpain-2 is associated with muscle wasting and alterations of flesh quality in rainbow trout (Salem et al., 2005). To summarize, the vast majority of the observed transcriptional changes detected in our study are in accordance with the observed increase in the synthesis of skeletal muscle proteins (Felip et al., 2012). Although swimming in this same group of fish showed a tendency, although non-significant, towards increased growth rate that was coupled with increased food intake (Felip et al., 2012), increases in protein synthesis in skeletal muscle could be associated with the reported stimulation of the growth rate under swimming conditions (Houlihan and Laurent, 1987; Farrell et al., 1991).

4.3. Changes in gene expression related to energy storage and pathways of ATP generation in skeletal muscle

Swim training induced changes in the mRNA expression levels of genes involved in metabolic processes in red and white muscle, with distinct effects depending on the muscle type. Supporting the observations that exercise promoted glucose uptake and utilization in rainbow trout fed a carbohydrate-rich diet (Felip et al., 2012), significant changes in the expression of genes involved in carbohydrate metabolism were observed. These changes were revealed by an up-regulation in the expression of genes involved in glucose uptake and phosphorylation (GLUT4, HK), as well as in glycolysis (GAPDH, B-Enol, PK) in white and red muscle from exercising trout. High levels of dietary carbohydrate intake by its own has a low impact on the expression of genes related to glucose utilization (Seiliez et al., 2011), indicating that the transcriptional changes linked to glucose utilization reported in our study are most likely related to the induction of sustained swimming.

In fish, gluconeogenesis occurs primarily in the liver, with a negligible proportion taking place in the skeletal muscle (Moon and Johnston, 1980; Panserat et al., 2001). However, we found that the mRNA expression levels of several enzymes involved in gluconeogenesis were altered in skeletal muscle of trout under exercise conditions. As expected in a fish consuming a carbohydrate-rich diet, the expression of enzymes involved in gluconeogenesis (G6P and G6PI) was down-regulated in both types of muscle. Interestingly, the gene expression of FBP-2, a key enzyme regulating this metabolic pathway, was increased in red muscle of swimming trout. We also found that the gene expression of GP-1, the enzyme controlling the hydrolysis of glycogen, was down-regulated in white muscle, while the enzyme catalyzing glycogen synthesis (GS) was up-regulated in red and white muscles of swimming trout. Particularly, the transcriptional up-regulation of FBP-2 and GS may explain the increased glycogen deposition observed in red muscle from swimming trout (Felip et al., 2012).

Sustained swimming increases the lipid content in skeletal muscle of rainbow trout (Felip et al., 2012) and other salmonids (Davison and Goldspink, 1977; Totland et al., 1987). This increase is in accordance with the enhancement of lipoprotein lipase activity previously shown in red muscle of rainbow trout (Magnoni and Weber, 2007) and with the increase in the expression of this enzyme in skeletal muscle (this study). However, the expression of apolipoprotein E-2, important for the binding and catabolizing lipoproteins, was down-regulated in red and white muscle of fish subjected to sustained swimming. Furthermore, very-long-chain acyl-CoA synthetase and fatty acid binding proteins 1 and 2, two genes involved in the binding and transport of FAs, were down regulated in red and white muscle of exercised fish. Interestingly, exercise caused a down-regulation of the expression of the FA transporter CD36 in red muscle but not in white muscle, where it was up-regulated.

Because of the limited number of probes included in the microarray platform related to the generation of energy by oxidative metabolism and the use of FAs as metabolic fuels, we evaluated possible changes occurring in the mRNA expression levels of enzymes such as CS, CPT1 and HOAD by qPCR. In our study, the mRNA expression levels of CS, an enzyme linked to the oxidative capacity of the cell, were up-regulated in red and white muscles of trout subjected to swimming, as it was previously shown in white muscle of zebrafish after swim training (LeMoine et al., 2010). In agreement with these authors, our results indicate that CPT1 and HOAD expression remained unchanged in red and white muscle of swimming trout, suggesting that FA use during these experimental conditions is not up-regulated. However, we cannot discard post-transcriptional mechanisms operating in skeletal muscle of fish that may up-regulate the use of FAs in response to exercise. Interestingly, CPT1 and CD36 gene expression are reported to be down-regulated by insulin in red muscle of Atlantic salmon, which may explain the decreased skeletal muscle FA oxidation and the increase in lipid deposition produced by this hormone (Sánchez-Gurmaches et al., 2011).

Exercise produces not only an increase in the transcription of GLUT4, but also acts as a strong stimulus for mitochondrial biogenesis in the skeletal muscle of mammals (McGee and Hargreaves, 2006; Holloszy, 2011). Such effects appear to be mediated by changes in the levels of cytosolic Ca^{2+} and high energy phosphates, possibly by activating AMP-activated protein kinase (AMPK), which in turn activates and increases the expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Jäer et al., 2007; Irrcher et al., 2008; Lira et al., 2010). Therefore, the rapid induction of PGC-1α by exercise appears to be associated with the regulation of mitochondrial content, and this factor may exert its effect as a transcriptional regulator of several metabolic pathways engaged in energy homeostasis in mammalian tissues including oxidative metabolism, and anabolic processes including glycogen storage (Lin et al., 2005; Lee et al., 2006; Wende et al., 2007) and de novo lipogenesis (Summermatter et al., 2010). Overexpression of PGC-1α has been shown to turn anaerobic muscles into more oxidative muscles by increasing mitochondrial biogenesis and the expression of contractile proteins characteristic of aerobic myofibers (Lin et al., 2002). We have shown in this study that several genes related to electron transport in mitochondria (e.g. ATP synthases, cytochrome c oxidases and NADH dehydrogenases) were differentially expressed in both muscles and that PGC-1α gene expression was up-regulated in red and white muscle of swimming trout. We had recently reported that the activation of AMPK by synthetic compounds, used to simulate the effects of exercise, results in an increase in the expression of PGC1-α in trout myotubes (Magnoni et al., 2012). These observations are in line with previous studies showing a transient induction in the expression of PGC-1α in skeletal muscles of swimming zebrafish (McClelland et al., 2006; LeMoine et al., 2010), suggesting a common role for this co-activator in skeletal muscle of fish. However, an observed latency between the increases in gene expression of CS and PGC-1α in zebrafish led these authors to suggest that, in contrast with mammals, PGC-1α is not directly involved in the long-term changes in oxidative capacity of skeletal muscle in response to exercise, indicating additional regulatory steps. In this respect, current evidence supports that members of the p38 mitogen-activated protein kinase family (MAPK) are functionally required for endurance exercise-induced PGC-1α regulation, participating in a signaling cascade controlling cellular responses to cytokines and stress and involved in the processes of cell differentiation and apoptosis in mammals (Puigserver et al., 2001; Akimoto et al., 2005). In our study, we found that the several genes included in the p38

MAPK family were differentially expressed in red and white muscle of rainbow trout by swimming. Furthermore, MAPK 9-1, MAPK 14, and MAPK/ERK kinase kinase 6 gene expression were up-regulated in white muscle of rainbow trout by swimming, which may suggest a similar role than their mammalian counterparts. However, because the broad regulatory effects of PGC-1α and its interaction with exercise, further studies are needed to explore possible differences between fish and other vertebrates on the role that this co-activator may have on the modulation of different signaling pathways, particularly in skeletal muscle.

4.4. Changes in gene expression related to cellular apoptosis in skeletal muscle

Several DEGs were identified in the GO functional category cellular apoptosis, suggesting the activation of apoptotic and anti-apoptotic signals in skeletal muscle of swimming fish. Particularly, our results show several DEGs involved in a diverse array of signaling transduction cascades that regulate cell cycle progression and apoptosis. Among these genes we highlight the down-regulation of protein kinase C alpha in both muscles. Surprisingly, the expression of GADD45, a gene that is commonly associated with cell growth arrest and response to DNA damage, is up-regulated in white muscle. The expression of this nuclear protein is up-regulated in rat skeletal muscle after exercise as well, at times when p53 is active, and these mechanisms are believed to be important regulators of the cell cycle (Levine, 1997; Chen et al., 2002), which are believed to play a role in the adaptation of skeletal muscle to exercise conditions (Choi et al., 2005). Furthermore, other DEGs in red and white muscle of swimming trout included proteins related to the NF-kB and ubiquitin–proteasome signaling pathways, which have been advocated as important mechanisms regulating skeletal muscle remodeling in several pathologies of mammals (Siu and Alway, 2009).

4.5. Changes in gene expression related to defense response in skeletal muscle

In mammals, it is well known that exercise has an important effect on the immune system by modulating the immunological status of skeletal muscle (Wiendl et al., 2005) and by stimulating the production of immune mediators by skeletal muscle (i.e. myokines). Muscle myokines have important local metabolic effects involved in the adaptation of skeletal muscle to energy-demanding situations such as exercise, but also act systemically on other tissues exerting anti-inflammatory effects (Pedersen, 2011). However, very little is known regarding the immunological capability of skeletal muscle in fish or regarding the possible regulation of the production of muscle immune factors by swimming-induced activity. Our transcriptomic study on the effects of swimming on skeletal muscles has demonstrated significant changes in the expression of genes involved in the immune system of rainbow trout. Differentially expressed genes in white and red skeletal muscles from exercised trout included cytokine receptors and signaling proteins, chemokines and their receptors, complement factors, immunoglobulins and antigen-presenting molecules. Furthermore, different responses of immune-related genes to exercise were observed in white and red skeletal muscles of trout, with the most interesting differences residing in the general up-regulation of complement factors (B, C3, C9, D and H) and immunoglobins (Ig heavy and kappa chains) and down-regulation of major histocompatibility complex class I and II molecules in white muscle. These results show, for the first time, the potential effects of exercise on the expression of immune-relevant genes in skeletal muscle of rainbow trout. In line with reports on the lower mortalities experienced by exercised Atlantic salmon (Totland et al., 1987), it has been recently shown that exercise training increases survival against a pathogen challenge in Atlantic salmon juveniles (Castro et al., 2011, 2013), suggesting that swimming-induced activity could potentially improve disease resistance in fish.

5. Conclusions

Overall, transcriptomic profiling of skeletal muscle from trout subjected to sustained swimming provides molecular evidence that supports the physiological changes experienced by trout under these conditions. Namely, the increase in fiber hypertrophy that is the basis of the growth-promoting effects of exercise, the increase in the uptake and use of carbohydrates as fuel, the increase in protein deposition and the resulting protein-sparing effect as previously suggested by Felip et al. (2012). Furthermore, swim training according to the conditions used in this study appears to increase the use of dietary carbohydrates not only by increasing the expression of GLUT4 but also by up-regulating glycolysis in both white and red muscles in trout. Aerobic ATP generation from glucose also appears to increase, particularly in red muscle, making it seem possible to apply swimming conditions as 'metabolic promoters', increasing energy expenditure and growth rates in trout. In addition, our results also provide evidence for the first time of the potential immuno-modulatory effects of exercise in fish. Due to the important practical and economical considerations for aquaculture, it will be important to determine if the altered immune status of skeletal muscle in exercised trout is related to increased or decreased resistance to pathogens. As we have shown in this study, contractile activity can activate a transcriptional program required for the successful adaptation of skeletal muscle to swimming conditions. It is becoming increasingly clear that in the future further studies should evaluate the physiological response of skeletal muscle to metabolic demands imposed by different feed and swimming conditions, using a combination of transcriptomic, proteomic and metabolomic approaches.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2013.08.005.

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