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Functional genomics of the muscle response to restraint and transport in chickens[1]

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ABSTRACT: In the present study, we used global approaches (proteomics, transcriptomics, and metabolomics) to assess the molecular basis of the muscle response to stress in chickens. A restraint test, combined with transport for 2 h (RT test) was chosen as the potentially stressful situation. Chickens (6 wk old) were either nontreated (control chickens) or submitted to the RT test (treated chickens). The RT test induced a 6-fold increase in corticosterone concentrations, suggesting hypothalamic-pituitary-adrenal axis activation. The RT test decreased the relative abundance of several hexose phosphates [glucose-1-P (G1P), glucose-6-P (G6P), fructose-6-P (F6P), and mannose-6-P (M6P)] in thigh muscle. In addition, 55 transcripts, among which 39 corresponded to unique annotated genes, were significantly up- (12 genes) or downregulated (27 genes) by treatment. Similarly, 45 proteic spots, among which 29 corresponded to unique annotated proteins, were overexpressed (11 proteins), underexpressed (14 proteins), or only expressed in treated chickens. Integrative analysis of differentially expressed genes and proteins showed that most transcripts and proteins belong to 2 networks whose genes were mainly related with cytoskeleton structure or carbohydrate metabolism. Whereas the decrease in energetic metabolites suggested an activation of glycogenolysis and glycolysis in response to the RT test, the reduced expression of genes and proteins involved in these pathways suggested the opposite. We hypothesized that the prolonged RT test resulted in a repression of glycogenolysis and glycolysis in thigh muscle of chickens. The down-expression of genes and proteins involved in the formation of fiber stress after the RT test suggests a reinforcement of myofibrils in response to stress.

Key words: carbohydrate metabolism, cytoskeleton structure, data integration, proteomics, transcriptomics

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INTRODUCTION

The adaptive response to stressful environmental challenges involves behavioral coping processing (for review see Ramos and Mormède, 1998) and the activation of 2 major neuroendocrine systems: the hypothalamic-pituitary-adrenocortical (**HPA**) axis leading to the secretion of corticosteroids by the adrenal glands and the sympathoadrenal system leading to the release of catecholamines (for reviews see, respectively, Mormède et al., 2007, and Carrasco and Van der Kar, 2003; Wong, 2006). Corticosteroids and catecholamines act directly in various important biological processes such as carbohydrate metabolism, energy expenditure, and lipid and protein metabolism (Dallman et al., 1993) and thus contribute to brain and muscle functions and

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to behavioral response developed in response to stress (Sapolsky et al., 2000).

In livestock production, many studies report that preslaughter conditions influence meat quality. In poultry, increased temperatures, crating and transport, and an increased delay on the shackle line (Kannan et al., 1997; Debut et al., 2003, 2005) may decrease meat quality. Physiological and behavioral measurements performed during these studies suggested that responses to stress occur during preslaughter handling and may affect meat quality. Until now, most of the studies concerning the effects of preslaughter stress on meat quality mainly focused on the mobilization of energetic compounds in muscle (Fernandez and Tornberg, 1991). However, mechanisms by which muscle responses to stress influence meat quality are only partly understood.

Our objective was to better understand the molecular mechanisms underlying muscle response to stress in chickens. We developed generic approaches to assess molecular bases of muscle response to stress in chickens by analyzing muscle transcriptome, proteome, and metabolome.

MATERIALS AND METHODS

The experiments described here fully comply with legislation on research involving animal subjects according to the European Communities Council Directive of November 24, 1986 (86/609/EEC). Investigators were certificated by the French governmental authority for carrying out these experiments (agreement n°31–165).

Animals and Housing

Six-week-old standard broiler chickens (Ross PM3; n = 30) were used in this study. The animals were reared and experiments were conducted at the INP Toulouse experimental research farm of Borret (Poucharramet, France). Animals were housed in collective pens on a 16 h light, 8 h dark cycle (light on at 0630 h) with food and water ad libitum. Two experimental groups of 15 chickens were randomly set up and placed in collective pens (3 × 4.5 m) 2 wk before the experiments.

Treatment and Sampling

Chickens were either nontreated (control chickens) or submitted to a restraint test combined with transport (**RT** test; treated chickens). Restraint test consisted of placing individual chickens in a "crush-cage" in the form of a wooden box (30 cm length × 15 cm width × 25 cm height) closed at the top by a netting cover. Restrained chickens were then immediately driven for a period of 2 h in a truck. This period was chosen because corticosteroid concentrations have been reported to be maintained at maximal concentrations throughout a 2-h restraint period (Hazard et al., 2008), and second to ensure a maximum effect of the restraint and transport test on mRNA and protein abundance. Indeed, changes in gene or protein expression or both have been measured in the brain or in the peripheral tissues or both after a 2-h immobilization stress (Kvetnanský et al., 1995; Smith et al., 1995; Moncek et al., 2001) or after a 2-h glucocorticoid treatment (Vandenborne et al., 2005). In accordance with approved slaughter methods, chickens were euthanized by decapitation immediately after capture from their pen (control chickens) or after a 2-h RT test. Experiments were performed between 0800 and 1200 h. Blood samples (2 mL) were collected directly from each chicken in tubes containing EDTA (2 mg/mL of blood) at euthanasia. The blood was kept on ice until centrifugation (2,000 × *g* for 15 min, 4°C) and plasma frozen at −80°C until measurement of total corticosterone using a specific RIA (Etches, 1976). The thigh muscles (tensor fascia latae and biceps femoris) were also collected at euthanasia, frozen immediately on dry ice, and then stored at −80°C until RNA, protein, and metabolite isolation.

Biochemical Analyses

Muscle glycogen, glucose, and lactate contents were measured by enzymatic procedures according to Dalrymple and Hamm (1973) and Bergmeyer (1974). Briefly, about 1 g of frozen muscle tissue was homogenized in 10 mL of 0.5 *M* perchloric acid. Aliquots (0.5 mL) of the homogenate were taken for enzymatic determination of total glucose after glycogen hydrolysis with amyloglycosidase (Dalrymple and Hamm, 1973). The rest of the homogenate was centrifuged (20 min at 2,500 × *g*, 4°C) and the supernatant was used for determination of free glucose and lactic acid (Bergmeyer, 1974). The glycogen content was calculated as the difference between total and free glucose. The results were expressed in micromoles per gram of fresh muscle.

Total RNA Isolation and Purification

Muscle samples from each animal were ground into a fine powder in a mortar cooled by liquid nitrogen, and 100 mg was added to 1 mL of prechilled TRIzol Reagent (Invitrogen, Cergy-Pontoise, France). Total RNA extractions were carried out according to manufacturer's directions and were further purified by passage through RNeasy mini-columns (RNeasy MinElute kit, Qiagen, Courtaboeuf, France) according to manufacturer's protocols for RNA cleanup. The DNA was digested using an RNase-free DNase set (Qiagen) during RNA purification. Total RNA was quantified by spectrophotometer (NanoDrop, Labtech, Palaiseau, France) and its integrity was assessed on an Agilent 2100 Bioanalyser (RNA 6000 Nano LabChip, Agilent Technologies, Massy, France).

Microarray Analysis

Array Slides. Gene expression was analyzed by hybridization of the chicken 20K microarray produced

by the Biological Resources Center GADIE (Genomic for Domestic Animals of Economic Importance, INRA, Jouy en Josas, France). The DNA microarray design was obtained from ARK-Genomics (http://www.ark-genomics.org; published in Gene Expression Omnibus data sets with the platform name GPL8199) and consisted of 20,460 oligonucleotides (Désert et al., 2008).

mRNA Labeling and Hybridization. Five micrograms of each RNA sample were reverse transcribed and Cy3 or Cy5 fluorescent-labeled using the ChipShot direct labeling kit (Promega, Charbonnieres, France). Microarray analyses were performed on 6 animals of each experimental group selected on the basis of their corticosterone concentrations (i.e., the most representative animals of the mean of each experimental group). Each fluorescent dye was used to label cDNA from 3 control animals and 3 treated animals (dye switch method). The Cy5 or Cy3-labeled cDNA from control animals was hybridized to the microarray with Cy3 or Cy5-labeled cDNA from treated animals, respectively, according to the Biochips platform of the Genopole Toulouse Midi-Pyrénées procedure (http://genopole-toulouse.prd.fr). Hybridization experiments were carried out with an automatic hybridization chamber (Discovery from Ventana Medical System, Strasbourg, France). Briefly, microarray was prehybridized with a solution of 1% BSA, 2× SSC, 0.1% SDS over 30 min at 42°C. After automatic washing, the microarray was hybridized overnight at 42°C in 200 μL of ChipHybe buffer containing 10 μL of each labeled cDNA purified (approximately 2,000 pmol of each incorporated dye). Slides were then manually washed twice for 2 min in a 2× SSC, 0.1% SDS solution and for 2 min in 0.1× SSC.

Data Acquisition. The microarray slides were scanned using a laser scanner (GenePix 4000B from Axon Instruments) at 532 nm for Cy3 fluorescence (green signal) and 635 nm for Cy5 fluorescence (red signal) keeping a PMT difference between both channels less than 100. The 2 images generated for each slide were then analyzed with GenePix Pro 6.1 software (Axon Instruments, Molecular Devices France, Saint Grégoire, France).

Microarray Data Filtering, Normalization, and Statistical Analysis. Data filtering, normalization, and statistical analysis were performed using the Bioconductor package Limma (Linear Models for Microarray Analysis; http://www.bioconductor.org, Smyth, 2005) in the R environment (http://www.cran.r-project.org). A filter procedure eliminated non-informative spots according to 2 criteria: 1) the genepix flag automatically performed by Genepix Pro 6.1 software, which indicated spots found or not, 2) the signal-to-noise ratio provided also by Genepix Pro 6.1 software, which was set to 0. The mean percentage of spots discarded was 50% for both criteria taken together. The homogeneity of the red and green background was systematically checked on each microarray by boxplot and imageplot procedures of the Limma package. Background was not subtracted in the normalization process. One array, which presented a greater percentage of spots discarded after filtering and background heterogeneity, was excluded from subsequent analysis.

Statistical analysis was performed on spots for which signal was present in the 5 microarrays after the filtering and normalization procedures. The ratio Cy5/Cy3 used was expressed as the Log2 of the ratio of median pixel of intensity of the 2 red and green spots. Log2 median ratio values were then normalized on each individual array (ratio Cy5/Cy3 centered on zero) according to the hypothesis that the majority of gene expressions do not differ between 2 samples. The centering was performed by the Robustspline procedure (Smyth and Speed, 2003), which was intermediary between the Print Tip Loess (locally weighted scatterplot smoothing by block) and Global Loess (locally weighted scatterplot smoothing) procedures. Robustspline normalization takes into account the intensity fluorescence bias. Only spots conforming to the filtering step were used for the normalization.

A linear model and a "moderated" Student test (Smyth, 2004) were used to test the effect of treatment (i.e., treated vs. control). False discovery rate (**FDR**) was determined using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). The complete data set is publicly available on the NCBI Gene Expression Omnibus (accession number: GSE19644; http://www.ncbi.nlm.nih.gov/geo/).

Functional Annotation. Transcripts differentially expressed were annotated using the annotations of the corresponding oligonucleotides obtained by a bioinformatics procedure developed by SIGENAE group (INRA, France) available on the website http://www.sigenae.org. Oligonucleotides without annotation, but corresponding to differentially expressed transcripts, were further annotated by comparison using the NCBI Basic Local Alignment Search Tool (BLAST) against nucleotide collection databases. Oligonucleotides showing complete similarity with known *Gallus gallus* transcripts (4 oligonucleotides) or high similarity (more than 80% identity) with known *Homo sapiens* transcripts (2 oligonucleotides) were further annotated.

Real-Time Quantitative Reverse-Transcription PCR Assay. A set of 6 genes present on the microarray was chosen for confirmation by quantitative reverse-transcription PCR, the 6 genes were significantly differentially expressed between control and stress animals. In addition 3 candidate genes (2 not present on the microarray and 1 present, but not significantly differentially expressed) for which the corresponding proteins were differentially expressed were also measured by quantitative reverse-transcription PCR.

Reverse transcription was carried out using the high-capacity cDNA archive kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's protocol. Briefly, 20 μL of each reaction mixture containing 2 μL of 10× reverse transcription buffer, 0.8 μL of 25× dNTP, 2 μL of 10× random primers, 1 μL of MultiScribe Reverse Transcriptase (50 U/μL), 1 μL of

Table 1. Gene-specific primers used for quantitative reverse-transcription PCR[1]

Gene symbol	Forward primer sequence	Reverse primer sequence	Accession No.
Trim63	GACCGCATCCAGACCATCAT	GCGCTGAGAACGCATCAAA	ENSGALT00000015210
Xirp1	AAGAAACATTCAACACCACGTCC	CTCCCGCATGGTAGATGCA	ENSGALT00000038916
Pdk4	CGGATGCTGATGAACCAACA	ACTACCTCAACAACATCACAGCAAG	ENSGALT00000015790
Ipp	CTGTGAAGTCTGTAAATCTCCCAAAG	CTGCTGTCACTCCAACGTCCT	ENSGALT00000016739
Pgk1	CCACAGTGGCTTCAGGCATT	ACTAT TTGTTTGGCCCTTCCC	ENSGALT00000012893
Ndufa4	GTACGTCATGCGTTTGGCAG	GGACGATCCTTTTTGAGCTTACTGTA	ENSGALT00000017426
Acta1	GCGTGGCTATTCCTTTGTGAC	GGAGGATGACGAAGCAGCTG	ENSGALT00000016005
Hsbp1	GTGGTGCAGACGTTGCTTCA	CGGCAGCTCATGTCATCAAG	ENSGALT00000033760
Eef2	TCTGGTCTCGTATCAACTGGCTT	GTTCAACGTAGCGGCCCAT	ENSGALT00000002831
RN18S	TTGTGCCGCTAGAGGTGAAAT	CGAACCTCCGACTTTCGTTC	AF173612.1

[1]Gene-specific primers were designed based on sequences provided under the accession numbers listed in Ensembl or National Center for Biotechnology Information nucleotide databases. *Trim63*, tripartite motif-containing 63; *Xirp1*, xin actin-binding repeat containing 1; *Pdk4*, pyruvate dehydrogenase kinase, isozyme 4; *Ipp*, intracisternal A particle-promoted polypeptide; *Pgk1*, phosphoglycerate kinase 1; *Ndufa4*, NADH dehydrogenase (ubiquinone) 1 α subcomplex, 4, 9 kDa; *Acta1*, actin, α 1, skeletal muscle; *Hsbp1*, heat shock factor binding protein 1; *Eef2*, eukaryotic translation elongation factor 2; *RN18S*, 18S ribosomal RNA gene.

RNase inhibitor, and 2 μg of total RNA were incubated for 10 min at 25°C followed by 2 h at 37°C and 5 min at 85°C. The resultant cDNA was diluted 1:50 with nuclease-free water. Five microliters of diluted cDNA was used in subsequent PCR reactions. All primers were designed based on nucleotide sequences in Ensembl or GenBank using the Primer Express software (Applied Biosystems; Table 1). Intron-exon organization of the chicken genes was used to choose primers from 1 pair in distinct exons of the corresponding gene. Reaction efficiency of PCR was calculated for each primer pair with five 10-fold serial dilution points of the calibrator sample (pool of the cDNA samples) to validate primers. A melting curve program was performed for each gene to check the presence of a unique product with specific melting temperature. Each real-time PCR reaction consisted of 1× Power SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μ*M* forward and reverse primers, and cDNA to a total volume of 20 μL. Reactions were carried out on an ABI PRISM 7900 Sequence detection system (Applied Biosystems) for 1 cycle at 95°C 10 min and 40 additional cycles (95°C for 15 s, 60°C for 1 min). Quantitative real-time PCR analysis was done in each out of the 6 animals constituting an experimental point and measurements were done in triplicate. The fold change in expression of each gene was calculated using the ΔΔCt (**Ct**, threshold cycle) method with the abundance of 18S RNA as an internal control; as determined by quantitative reverse-transcription PCR, the abundance of 18S did not change depending on treatment in our study (data not shown). So, for a same sample, gene expression could be normalized relative to 18S expression as follows: gene normalized Ct = Ct-Gene – Ct18S = ΔCt. For each gene, the n-fold gene expression difference between 2 conditions (control vs. stress) was expressed as follows: fold change = $2\ \exp(-\Delta\Delta Ct)$ with $\Delta\Delta Ct = \text{mean}(\Delta Ct)_{\text{stress}} - \text{mean}(\Delta Ct)_{\text{control}}$, where $(\Delta Ct)_i$ are the mean of the gene normalized Ct of the different samples of the condition i. The significance of expression differences between control and stress conditions were analyzed by ANOVA on the basis of the gene normalized CT values. The linear model was $y_{ij} = \mu + \alpha_i + e_{ij}$, where y_{ij} represents the gene normalized Ct values of the jth subject for the treatment i, μ is the overall mean, α_i is the effect of treatment, and e_{ij} represents the residual assumed to be normally distributed with zero mean and variance σ^2.

Metabolomic Semi-Quantification of Target Metabolites

The identification and relative quantification of most of the phosphorylated metabolites of key pathways and of the components of the Krebs cycle was carried out using liquid chromatography tandem mass spectrometry analysis of cellular extracts to which labeled substrates were added (Van Dam et al., 2002; Mashego et al., 2003). Muscle metabolome was previously extracted using boiling water (MilliQ-grade water +100°C; El Rammouz et al., 2010). The relative abundance of targeted metabolites was expressed as the ratio of experimental peak area to the peak area of labeled substrate. The focus was put on the following metabolite families: sugar phosphates [glucose-1-P (**G1P**), glucose-6-P (**G6P**), fructose-1-P (**F1P**), fructose-6-P (**F6P**), mannose-6-P (**M6P**), fructose-1,6-biP (**F1,6bP**), ribose-1-P (**R1P**), ribose-5-P (**R5P**), and erythrose-4-P (**E4P**)] phosphorylated organic acids [6-phosphogluconate (**6PG**), 2 and 3 phosphoglycerate (**2/3PG**), phospho-enol-pyruvate (**PEP**), and glyceraldehyde-1,3-biP (**1,3bPG**)], nucleotides (AMP, ADP, ATP, IMP), and organic acids [fumarate, succinate, oxaloacetic acid, malate, α-ketoglutarate (**αKG**), and citrate].

Proteomic Analysis

2-Dimensional Electrophoresis Gels. Soluble proteins were extracted from thigh muscles according to a modified procedure of Sayd et al. (2006). Muscles previously ground in liquid nitrogen were homogenized

in 40 m*M* Tris (pH 8), 2 m*M* EDTA, and 2.5% (vol/vol) protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO; ratio muscle/buffer 1:4). After 1 h at 4°C, samples were centrifuged at 10,000 × *g* for 10 min at 4°C. Supernatants were quickly removed. Protein concentration was then determined by using the method of Bradford (1976).

Samples were then solubilized in a solution containing 8.75 *M* urea, 2.5 *M* thiourea, 5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 50 m*M* dithiothreitol, 0.2% ampholytes 5–8 (Bio-Rad Laboratories Inc., Hercules, CA). Sample preparation and migration conditions for 2-dimensional electrophoresis (**2-DE**) separation were based on those described by Görg et al. (2004). Strips containing an immobilized pH gradient (**IPG**; pH 5 to 8, 17 cm, Bio-Rad Laboratories Inc.) were used for isoelectrofocusing. Each sample was performed in duplicate. Samples (900 μg of protein/strip) were then loaded onto strips including protein extract in rehydration solution. Rehydration of strips was performed at 50 V for 14 h at 20°C. First dimension was done with an IEF Cell (Bio-Rad Laboratories Inc.). Intensity was limited to 50 μA per strip, and the temperature was set at 20°C. First dimension conditions were as follows: 25 min at 250 V, 3 h to reach 10,000 V, and 10,000 V until 80,000 Vh were finally cumulated. The IPG strips were immediately frozen at the end of the isoelectrofocusing step and were stored at –20°C until the 2nd dimension was performed.

A Protean XL tank (Bio-Rad Laboratories Inc.) was used for the second dimension. The IPG strips were first equilibrated for 15 min in a 1.5 *M* Tris-Cl buffer (pH = 8.8) containing 1% dithiothreitol, 6 *M* urea, 30% glycerol, and 2% SDS. They were then placed for 15 min in a 1.5 *M* Tris-Cl buffer (pH = 8.8) containing 4.8% iodoacetamide, 6 *M* urea, 30% glycerol, and 2% SDS. Migration occurred on continuous SDS-PAGE containing 12% acrylamide (2.6% C) at 35 mA constant/gel. The temperature was set at 10°C. At the end of the second dimension, gels were stained with Coomassie colloidal blue procedure according to the method described by Molloy et al. (1998).

Data Analysis of 2-DE Gels. All the gels were analyzed with the software Image Master 2D Platinum (GE Healthcare, Uppsala, Sweden) to point out proteins of interest. Detected and matched spots were standardized by expressing the relative intensity of each spot as the ratio of the intensity of the individual spot on the total intensity of valid spots. Spots of interest were determined by using the procedure of Meunier et al. (2005). False positive proteins, proteins found differentially expressed when considering a 2-fold change ratio but not differentially expressed when a *t*-test is performed ($P > 0.05$), were removed.

Protein Identification by Mass Spectrometry. Spots of interest were excised from gels and treated as described previously (Laville et al., 2009). Briefly, the peptides obtained after hydrolyze by trypsin were analyzed with a MALDI-TOF DE PRO mass spectrometer (Applied BioSystems). Then, peptide mass fingerprints observed were compared using Mascot Software (Matrix Sciences London, V2.2, home license), to those from NCBInr databases, *Gallus gallus* (32977 seq, 06012009) or Aves (102448 seq, 18122008). The databases searches were carried out with, a maximum peptide ion mass tolerance of 25 ppm, one miscleavage, and variable modifications of methionine (oxidation) and of cysteine (carbamidomethylation). Identification was considered positive if the Mascot score was greater than significant Mascot score ($P < 0.05$) given by software according to the database used (58 and 63, respectively, *Gallus gallus* and Aves).

Statistical Analysis

The corticosterone data were transformed to base-10 logarithmic scores and analyzed by ANOVA to assess the effects of treatment. The linear model was $y_{ij} = \mu + \alpha_i + e_{ij}$, where y_{ij} represents the corticosterone concentrations of the jth subject for the treatment i, μ is the overall mean, α_i is the effect of treatment, and e_{ij} represents the residual, assumed to be normally distributed with zero mean and variance σ^2. Results are given as the mean ± SE.

RESULTS

Corticosterone Concentrations

Comparison of plasma corticosterone concentrations in control and treated chickens showed significant effect of the RT test ($P < 0.0001$). Corticosterone concentrations in restrained and transported chickens (30.8 ± 1.9 ng/mL) were 6-fold greater than basal corticosterone concentrations in control chickens (5.9 ± 1.1 ng/mL). A subgroup of 6 control chickens and a subgroup of 6 treated chickens have been constituted to perform a transcriptomic approach. Corticosterone concentrations were of 4.8 ± 1.7 ng/mL and 31.3 ± 4.17 ng/mL in control and treated subgroups, respectively.

Metabolites

Glucose and lactate concentrations were significantly decreased ($P < 0.0001$ and $P < 0.05$, respectively) in response to the RT test (Table 2). Glycogen concentrations did not significantly differ ($P = 0.12$) between control and treated chickens.

The relative abundance of nucleotides, organic acids, and metabolites from the pentose pathway was not significantly ($P > 0.1$) affected by the RT test (Figure 1). There was, however, a significant effect of the RT test on the relative abundance of several hexose phosphates. Indeed, the muscle contents of G1P, G6P, F6P, and M6P were significantly reduced by −20, −40, −40, and

Table 2. Effect of a 2-h restraint-transport (RT) test on glycogen, glucose, and lactate content in thigh muscles of broiler chickens (n = 15 per treatment, mean ± SE)[1]

Metabolite	Control	RT test	*P*-value
Glycogen, μmol/g	5.75 ± 1.1	3.70 ± 0.7	0.12
Glucose, μmol/g	1.84 ± 0.1	1.01 ± 0.1	<0.0001
Lactate, μmol/g	36.13 ± 1.6	28.71 ± 2.6	0.021

[1]Glycogen, glucose, and lactate concentrations in thigh muscles were measured in control chickens and in chickens submitted to a 2-h restraint test combined with transport (RT test).

−35%, respectively, in treated compared with control chickens ($P < 0.01$ for each metabolite).

Genes Differentially Expressed

Using filtering and normalization processes described in the Materials and Methods section, 7,296 of the 20,460 oligonucleotides present on the chicken microarray (approximately 35%) were found to be expressed in thigh muscles under our experimental conditions. Few transcripts (i.e., oligonucleotides) were identified to be significantly differentially expressed in response to the treatment RT test using the cut-off FDR value of 0.05, probably because of the number of repetitions (n = 5). Therefore, the gene list was expanded to include those genes with *t*-test *P*-values less than or equal to 0.01 (i.e., FDR ≤ 0.2) and an absolute fold change greater than or equal to 1.25. A similar procedure has been described previously by Jaffrézic et al. (2007). We identified 55 transcripts to be significantly upregulated or downregulated by the RT test (Supplemental Table 1; http://jas.fass.org/content/vol89/issue9/). Among these, 39 transcripts corresponded to unique annotated genes and 16 remained unknown. These 39 genes were ordered in the biological process in which they are involved (Figure 2). Among these, 12 genes were significantly upregulated and 27 downregulated by the RT test. The major functional categories for these genes included transport (e.g., proteins, phospholipides), transcription regulation, glycolysis, actin cytoskeleton organization, signal transduction, proteolysis, and cell growth/maintenance.

Validation by Quantitative Reverse-Transcription PCR

Six genes significantly affected by treatment in microarray analysis were selected for further examination by quantitative reverse-transcription PCR (Table

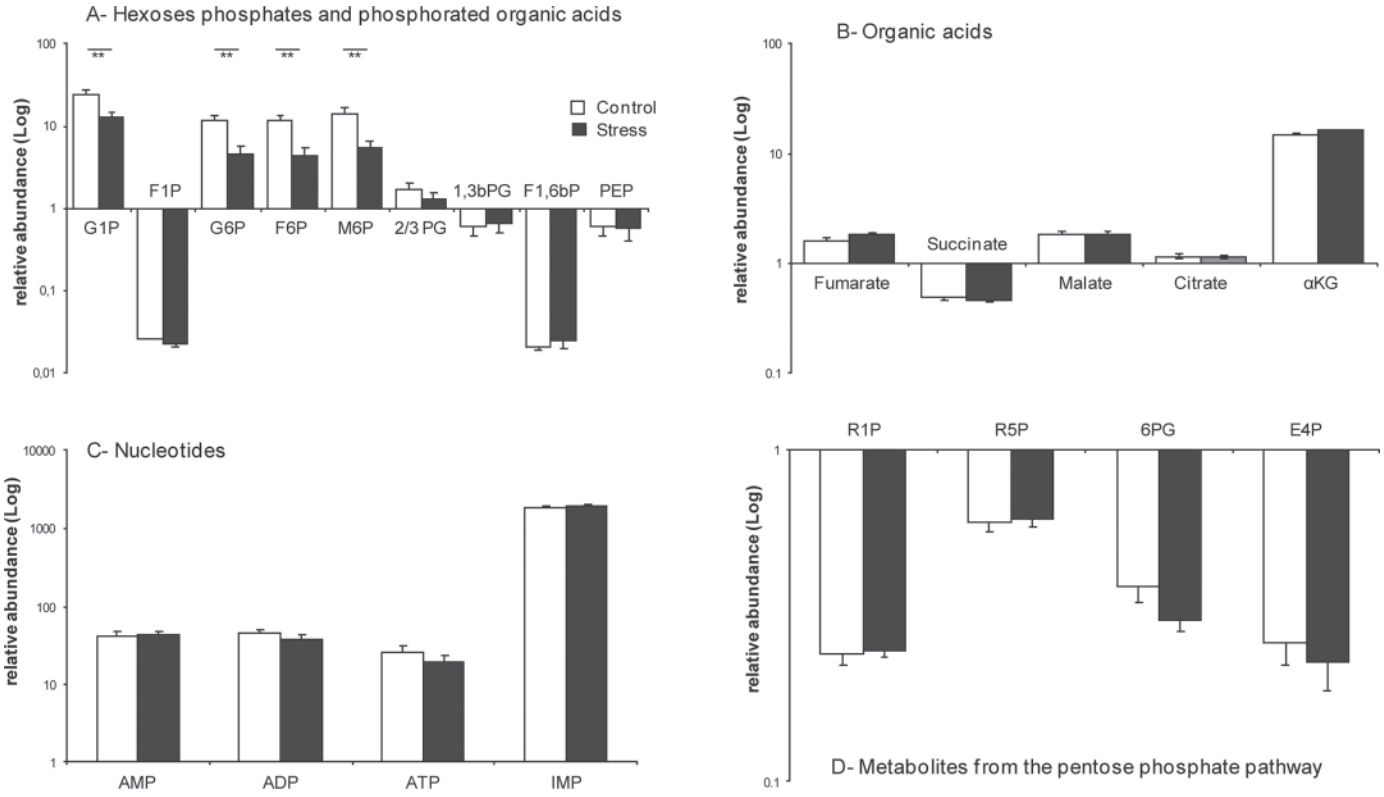

Figure 1. Comparison of metabolic pool sizes in muscles of control and stressed chickens. The relative abundance corresponds to the ratio between the peak area of ^{12}C metabolites and the peak area of the corresponding labeled substrate (^{13}C) added in the same quantity in all samples. Metabolites: glucose-1-P (G1P), fructose-1-P (F1P), glucose-6-P (G6P), fructose-6-P (F6P), mannose-6-P (M6P), 2 and 3 phosphoglycerate (2/3PG), glyceraldehyde-1,3-biP (1,3bPG), fructoses-1,6-biP (F1,6bP), ribose-1-P (R1P), ribose-5-P (R5P), erythrose-4-P (E4P), 6-phosphogluconate (6PG), α-ketoglutarate (αKG), and phospho-enol-pyruvate (PEP). Nucleotides: adenosine monophosphate (AMP) and inosine monophosphate (IMP). **Significant effect of stress on relative abundance ($P < 0.01$).

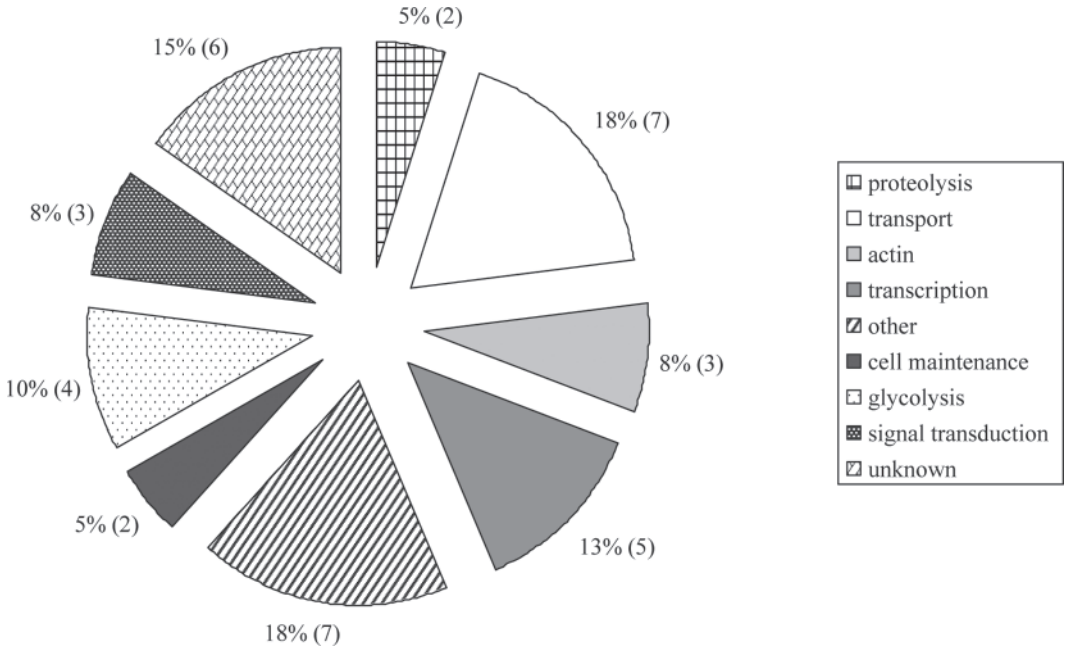

Figure 2. Functional classification of differentially expressed genes according to the biological process in which they are involved. The numbers of genes with differential mRNA expression are indicated in each category.

3). Changes in transcripts abundance were confirmed for the 3 upregulated genes chosen (*Trim63*, *Xirp1*, *Pdk4*). The magnitudes of the changes were greater in the reverse-transcription PCR than in the microarray for these upregulated genes that seemed dampened on the microarray. Similar differences in magnitudes of the changes have been previously reported (Désert et al., 2008). The 3 genes (*Ipp*, *Pgk1*, *Ndufa4*) found to be downregulated by microarray were not found to be differentially expressed based on reverse-transcription PCR. Three additional genes for which corresponding proteins were differentially expressed but either not found to be significantly affected by the RT test or not present on the array were selected for further examination by real-time PCR. The absence of significant changes in the microarray study for *Eef2* gene was confirmed by reverse-transcription PCR, whereas the corresponding protein was differentially expressed. The *Hsbp1* and *Acta1* genes not present on the array, for which proteins were downregulated by the RT test, were not found differentially expressed or upregulated by real-time PCR, respectively.

Table 3. Measurement of treatment effect on transcript accumulation by relative quantitative real-time PCR and comparison with microarray and proteome data[1]

	Real-time PCR		Microarray		Proteome	
Gene	Fold change (stress/control)	Treatment effect (*P*-value)	Fold change (stress/control)	Treatment effect (*P*-value)	Fold change (stress/control)	Treatment effect (*P*-value)
Trim63	7.9	<0.0001	2.3	9.39×10^{-6}		NS
Xirp1	6.8	<0.0001	1.9	1.08×10^{-4}		NS
Pdk4	8.4	<0.0001	1.3	4.42×10^{-3}		NS
Ipp	0.9	NS	0.8	1.04×10^{-2}		NS
Pgk1	1.1	NS	0.7	1.83×10^{-4}		NS
Ndufa4	1.3	NS	0.7	6.01×10^{-4}		NS
Acta1	1.9	0.02	Absent		Control	6.30×10^{-6}
Hsbp1	1.2	NS	Absent		Control	2.40×10^{-2}
Eef2	1.1	NS	1.0	NS	Control	4.80×10^{-4}

[1]The changes in expression for 6 genes shown by microarray analysis to be significantly regulated by the restraint-transport test were quantified by real-time PCR. For comparison, relative expression values for each gene were determined in each animal (fold change, n = 6 chickens per experimental point). Changes in expression were also analyzed for 3 additional genes of great interest for our study according to proteome results whereas they were either shown by microarray analysis to be not significant (NS, $P > 0.05$) or not determined because they were not included on the microarray (absent). Control indicates that protein was found only in control chickens. *Trim63*, tripartite motif-containing 63; *Xirp1*, xin actin-binding repeat containing 1; *Pdk4*, pyruvate dehydrogenase kinase, isozyme 4; *Ipp*, intracisternal A particle-promoted polypeptide; *Pgk1*, phosphoglycerate kinase 1; *Ndufa4*, NADH dehydrogenase (ubiquinone) 1 α subcomplex, 4, 9 kDa; *Acta1*, actin, α 1, skeletal muscle; *Hsbp1*, heat shock factor binding protein 1; *Eef2*, eukaryotic translation elongation factor 2.

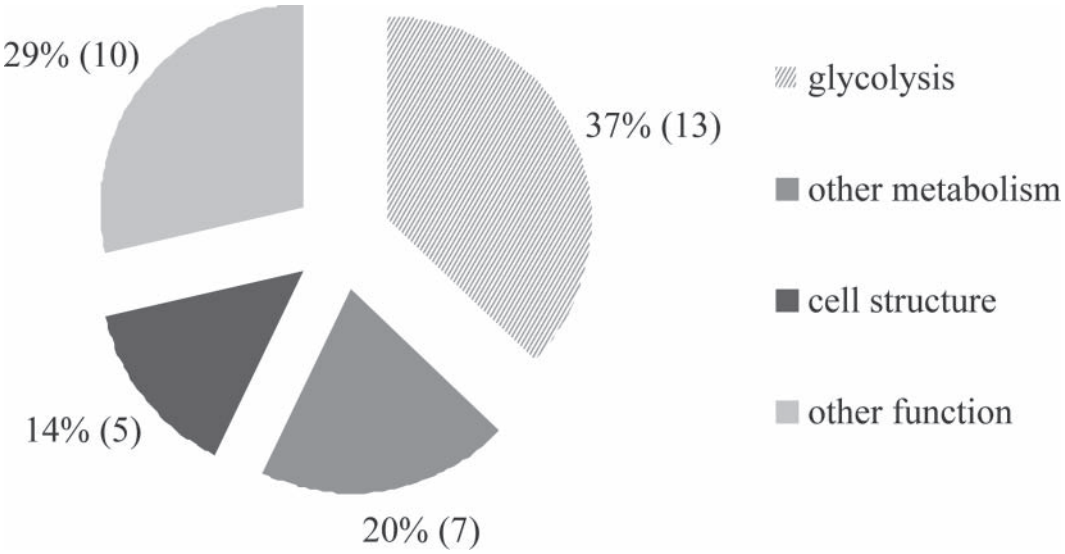

Figure 3. Functional classification of differentially expressed proteins according to the biological process in which they are involved. The numbers of proteins with a differential expression are indicated in each category.

Proteomic Analysis

On 2-DE gels, out of 265 matched spots, 45 proteic spots were differentially expressed between control and treated chickens. Thirty-five proteic spots were successfully identified by mass spectrometry and corresponded to 29 individual proteins (Supplemental Table 2; http://jas.fass.org/content/vol89/issue9/). Figure 3 synthesizes identification information about proteins that were differentially expressed. Among these, 11 proteic spots were overexpressed and 14 underexpressed in treated chickens. Moreover, 20 proteic spots were only present in control chickens.

DISCUSSION

We used a comprehensive gene expression profiling by microarray analysis to identify groups of genes differentially expressed in response to the RT test. This is to our knowledge the first gene array analysis investigating in vivo muscle response to psychobiological stress in birds. Present results show that the RT test affected gene expression in chicken muscle (39 identified genes differentially expressed). A proteomic analysis was also used to determine the differentially expressed muscle soluble proteins by the RT test. We identified 35 proteins that were differentially expressed in control and treated chickens.

When comparing proteins and transcripts differentially expressed, no common genes have been found. de Nobel et al. (2001), Chen et al. (2002), and Liu et al. (2009) reported a lack of overlap between transcriptome and proteome analysis in various tissue or organisms. In the present study, we used a generic chicken microarray that was not designed for muscle tissue. Consequently, genes involved in other tissues and functions were represented on the microarray. For the proteomic analysis, the focus was set on soluble proteins issued from a prefractionation of the whole muscle proteins, which excluded contractile and cytoskeletal proteins. Moreover, time-courses necessary to reveal differences in proteins and transcripts expression may explain the observed lack of redundancy (Liu et al., 2009).

Nevertheless, transcriptome and proteome analyses provided complementary information. The data of the 2 approaches were biologically integrated by using Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc., Redwood City, CA). This analysis revealed that most of both transcripts and proteins differentially expressed by the RT test belong to a major network (Figure 4) that could be subdivided into 2 subnetworks whose actors were mainly related to cytoskeleton structure or carbohydrate metabolism.

Carbohydrate Metabolism

Chickens that were exposed to the RT test showed a decreased abundance of glycogen (even though it was not significant), G1P, G6P, F6P, and M6P compared with control chickens. Therefore, those results could suggest an increase in the glycogenolysis and glycolysis activities in chickens submitted to the RT test, resulting in a decrease of muscular energetic stores. These results were consistent with literature showing the mobilization of energetic compounds in muscle in response to stress (Fernandez and Tornberg, 1991). In addition, consistent with the decreased carbohydrate content in thigh muscle of treated chickens, pH at 20 min and 24 h postmortem were also less in treated chickens compared with control chickens (pH 20 min = 6.82 ± 0.13 and 6.52 ± 0.74, respectively, $P < 0.0001$ and pH 24 h = 6.39 ± 0.23 and 6.21 ± 0.11, respectively, $P < 0.001$). There were no differences ($P > 0.1$) in metabolic indicators, gene and protein expression, or pH values in

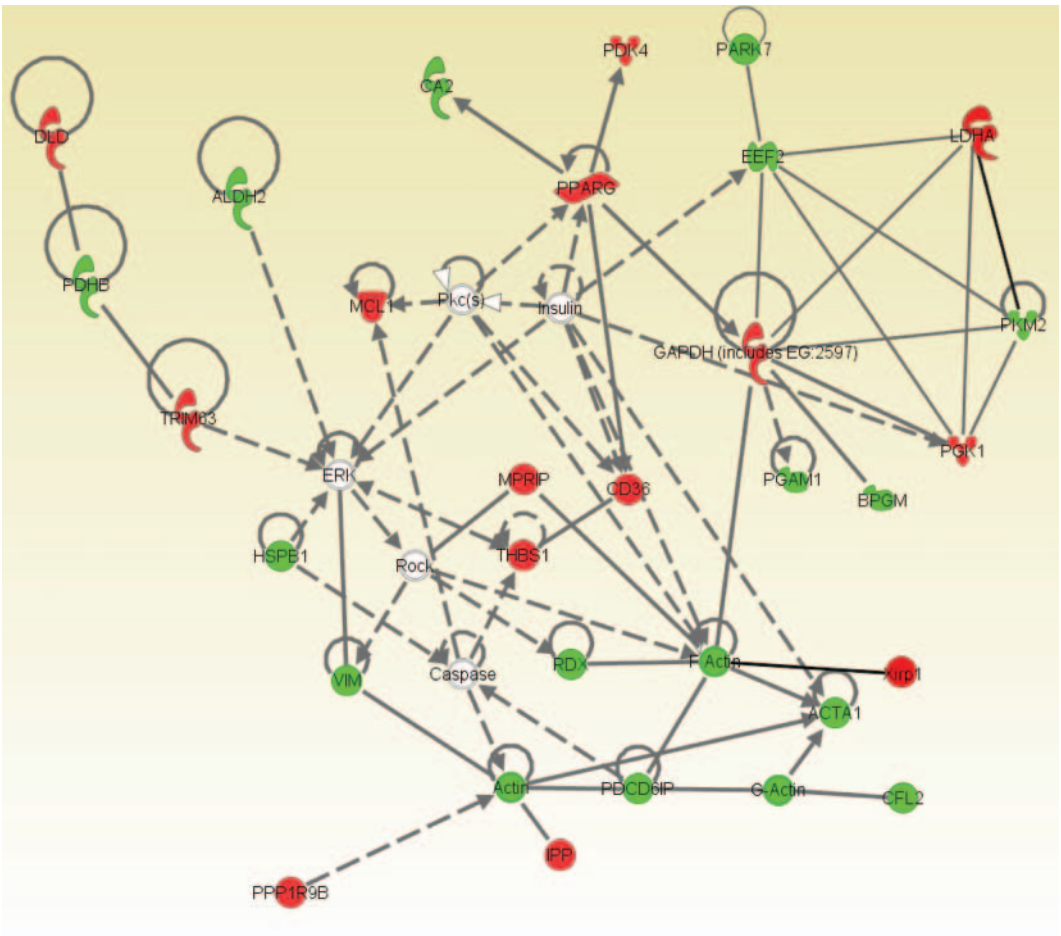

Figure 4. Ingenuity (IPA, Ingenuity Systems Inc., Redwood City, CA) network analysis revealed 2 subnetworks. The first one is involved in glycolysis and transcription regulation and the second one in actin polymerization and stabilization. Red indicates transcripts differentially expressed and green, proteins differentially expressed of the associated genes. Genes not colored indicate no change in expression or genes not detected, but they are highly linked to genes that did change. Solid lines indicate a direct interaction between genes, whereas dashed lines indicate an indirect interaction between the genes. *ACTA1*, actin α 1; *ALDH2*, aldehyde dehydrogenase 2 family; *BPGM*, 2,3-bisphosphoglycerate mutase; *CA2*, carbonic anhydrase II; *CD36*, CD36 molecule; *CFL2*, cofilin 2; *DLD*, dihydrolipoamide dehydrogenase; *EEF2*, eukaryotic translation elongation factor 2; *ERK*, extracellular signal regulated kinase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HSPB1*, heat shock 27 kDa protein 1; *IPP*, intracisternal A particle-promoted polypeptide; *LDHA*, lactate dehydrogenase A; *MCL1*, myeloid cell leukemia sequence 1; *MPRIP*, myosin phosphatase Rho interacting protein; *PARK7*, Parkinson disease 7; *PDCD6IP*, programmed cell death 6 interacting protein; *PDHB*, pyruvate dehydrogenase (lipoamide) β; *PDK4*, pyruvate dehydrogenase kinase, isozyme 4; *PGAM1*, phosphoglycerate mutase 1; *Pkc*, protein kinase C; *PKM2*, pyruvate kinase; *PPARG*, peroxisome proliferator-activated receptor gamma; *PPP1R9B*, protein phosphatase 1, regulatory (inhibitor) subunit 9B; *RDX*, radixin; *THBS1*, thrombospondin 1; *TRIM63*, tripartite motif-containing 63; *VIM*, vimentin; *Xirp1*, xin actin-binding repeat containing 1. Color version available in the online PDF.

breast muscle (data not shown). While chickens were restrained in the crush cage, their physical activity was very limited. Chickens submitted to the RT test could still move their legs to stand up or lie down for instance, or could be immobile but with their thigh muscles still active at least to maintain their vertical position during the restraint and transport phase. Thus, we cannot exclude that metabolic differences observed here in the thigh muscles could be related to the presence of physical activity during the RT test. However, the RT test did not induce any change in phosphorylation of AMP-activated protein kinase, a valuable indicator of physical activity (Witczak et al., 2008; data not shown). This last result suggests that metabolic activation in muscle of chickens submitted to the RT test is not mainly due to physical activity.

Greater corticosterone concentrations measured in chickens submitted to the RT test indicated activation of the HPA axis by the RT test. Such neuroendocrine activation has been previously reported in birds submitted to a similar paradigm (Hazard et al., 2008). Indeed, we previously reported in quail that glucocorticoid concentrations induced by restraint in a crush cage were at high concentrations throughout a 2-h restraint period (Hazard et al., 2008). Thus, increased corticosterone concentrations measured at the end of the RT test period may reflect activation of HPA axis throughout the RT test period. Activation of the HPA axis is associated among other things with a generalized response to perceived stresses (Selye, 1936), and although the interpretation of such measures requires caution, it remains a standard approach to investigate stress in farm

animals (Mormède et al., 2007). The decrease of muscular energetic stores in chickens submitted to the RT test was thus consistent with previous studies reporting that stress induced muscular glycogen mobilization (reviewed by Fernandez and Tornberg, 1991).

Catecholamines and glucocorticoids have been reported to have a critical role in metabolic changes induced by stress (Dallman et al., 1993), and more particularly to increase the use of energy stores during the stress response (Coderre et al., 1991; Sapolsky et al., 2000). Thus, after exposure to stressful stimuli, catecholamines (epinephrine, norepinephrine), which are the first stress neuroendocrine mediators released, increase the use of energy stores by activating hepatic and muscular glycogenolysis (Bassett, 1970; Halter et al., 1984; Scheurink and Steffens, 1990). Even if catecholamine concentrations had not been measured in the present study, similar regulation could be hypothesized in muscle and could also be consistent with the decrease of muscular energetic stores in chickens submitted to the RT test. Viguerie et al. (2004) have reported that genes involved in glycogen synthesis were repressed by epinephrine in muscle, whereas genes involved in glycogenolysis were overexpressed. Glucocorticoids have been reported to inhibit glycogen synthase activity in muscle (Ekstrand et al., 1996; Henriksen et al., 1999). Interestingly, in our study, genes and proteins involved in glycogen synthesis were not affected by the RT test, whereas glycogen phosphorylase protein involved in glycogenolysis was repressed by the RT test. Similarly, several genes (*Dld*, dihydrolipoyl dehydrogenase; *Gapdh*, glyceraldehyde 3P dehydrogenase; *Ldh*, L-lactate dehydrogenase; *Pgk*, phosphoglycerate kinase) and proteins (ALDH, aldehyde dehydrogenase; BPGM, bisphosphoglycerate mutase; GP, glycogen phosphorylase; PGAM, phosphoglycerate mutase; PDH, pyruvate dehydrogenase; PK, pyruvate kinase; TPI, triose phosphate isomerase) involved in glycolysis were repressed by the RT test. These decreases in expression of genes and proteins involved in glycogenolysis and glycolysis are consistent with the increased concentrations of glucocorticoids measured in response to the RT test. Nevertheless, one has to keep in mind that glucocorticoids and epinephrine action on glycogenolysis or glycogenesis or both are dependent on muscular glycogen content (Frolow and Milligan, 2004). Therefore, prolonged RT test (2 h) may result in reduced glycogenolysis and glycolysis in muscle by decreasing either gene or protein expression (Figure 5). Finally, we hypothesized that the increase in the use of muscular energy stores by the RT test, as indicated by the decrease in the metabolic levels, may have occurred mainly at the onset of the RT test period. On the contrary, in the present study, the reduced expression of genes and proteins involved in glycogenolysis and glycolysis pathways may be considered as an adaptive response to a prolonged stress situation which placed the muscle tissue into a "sparing" profile with respect to energy stores. It has long been reported, at least in domestic animals, that the

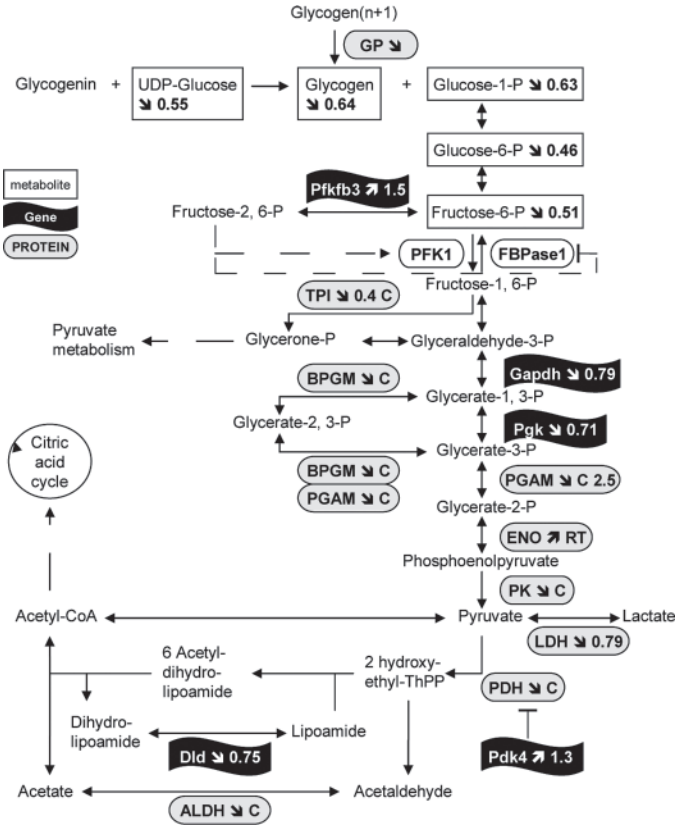

Figure 5. Effect of the restraint test combined with transport (RT) on metabolite concentrations and on the expression of genes and proteins involved in carbohydrate metabolism in chicken thigh muscle. Arrows indicate the effect of the RT test, followed by the value of the ratio of RT/control, or followed by C or RT if the protein was only found in control chickens or chickens submitted to the RT test, respectively. ALDH, aldehyde dehydrogenase; BPGM, bisphosphoglycerate mutase; Dld, dihydrolipoyl dehydrogenase; ENO, enolase or phosphopyruvate hydratase; FBPase1, fructose-1,6-bisphosphatase; Gapdh, glyceraldehyde 3P dehydrogenase; GP, glycogen phosphorylase; LDH, L-lactate dehydrogenase; PDH, pyruvate dehydrogenase; Pdk4, pyruvate dehydrogenase kinase isozyme 4; PFK1, 6-phosphofructo-1-kinase; Pfkfb2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase isoform 3; PGAM, phosphoglycerate mutase; Pgk, phosphoglycerate kinase; PK, pyruvate kinase; TPI, triose phosphate isomerase.

restoration of energy stores in stress-depleted muscles is a long metabolic process lasting more than 24 h that requires favorable environmental conditions (e.g., see review by Fernandez and Tornberg, 1991). Thus, in our case, glycogen reserves have most likely been depleted during the 2-h RT stress, thus leading to a physiological adaptation toward a reduced level of energy catabolism (e.g., glycolysis), whereas the glycogen reserves were not yet restored.

Further differences in gene expression were consistent with the hypothesis of a reduced glycolysis. The gene *Pfkfb3* was overexpressed in chickens submitted to the RT test (1.5-fold, data not shown due to filtering procedure). The corresponding enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase is a homodimeric bifunctional enzyme involved in the precise regulation of the quantity of fructose 2,6-bisphosphate, which is a potent allosteric activator of 6-phosphofructo-1-kinase and reduces the activity of the key regulatory enzyme

of gluconeogenesis, fructose-1,6-biphosphate (F1,6bP) (Okar et al., 2001). Interestingly, the *Pfkfb2* gene has been reported to be the most highly upregulated gene in human muscle after epinephrine infusion (Viguerie et al., 2004). The gene *Pfkfb4* has also been reported to be upregulated by glucocorticoids (Almon et al., 2007). Moreover, *Pdk4*, a gene coding for an inhibitor of pyruvate dehydrogenase (**PDH**), was also overexpressed in chickens submitted to the RT test, which was consistent with the decrease in the expression of PDH.

Concerning lactate concentrations, chickens exposed to the RT test showed decreased concentrations of lactate compared with control chickens, whereas they used more glycogen. Such a difference may involve a greater release of the lactate by muscle. A further explanation could also be an increase in the oxidation process of pyruvate by tricarboxylic acid cycle in response to stress. The decrease observed in the expression of lactate dehydrogenase gene (involved in the reduction of pyruvate to lactate) in chickens submitted to the RT test supports the latter hypothesis.

Actin Polymerization and Stabilization

The second subnetwork deals with actin polymerization and stabilization (Figure 4). The transcripts and proteins actin-binding Rho-activating protein (ABRA), actin alpha 1 (ACTA1), cofilin 2 (CFL2), heat shock 27kDa protein 1 (HSPB1), intracisternal A particle-promoted polypeptide (IPP), myosin phosphatase Rho interacting protein (MPRIP), Parkinson disease 7 (PARK7), programmed cell death 6 interacting protein (PDCD6IP), radixin (RDX), thrombospondin 1 (THBS1), tripartite motif-containing 63 (TRIM63), vimentin (VIM), and xin actin-binding repeat containing 1 (XIRP1) are related with this subnetwork. The transcripts and proteins VIM, ABRA, TRIM63, and XIRP1 were overexpressed in chickens submitted to the RT test. The other components (ACTA1, RDX, HSBP1, IPP, PDCD6IP, MPRIP, and CFL2) were underexpressed.

Several proteins differentially expressed can have physical links with ACTA1: RDX (Luciani et al., 2002), XIRP1 (Cherepanova et al., 2006), CFL2 (Ono, 2003), TRIM63 (Witt et al., 2008), ABRA (Barrientos et al., 2007), VIM (Cízková et al., 2009), PDCD6IP (Pan et al., 2006), IPP (Kim et al., 1999; VanHouten et al., 2001), and MPRIP (Riddick et al., 2008). In skeletal muscle, ACTA1 is involved in contractile activity. However, the actin cytoskeleton is also involved in cell motility and mitosis as well as regulation of transcription and gene expression (Barrientos et al., 2007). The protein RDX belongs to a proteic complex with ezrin and moesin called ERM. This complex cross links actin with plasma membrane. The protein complex ERM is emerging as key regulator of actin cytoskeleton via Rho (Hall, 1998). The protein XIRP1 is thought to be involved in the remodeling of actin cytoskeleton of muscle during sarcomere assembly (Jung-Ching Lin et al., 2005), cardiac morphogenesis (Jung-Ching Lin et al., 2005), and actin filament stabilization (Hawke et al., 2007). The protein CFL2 belongs to a complex of proteins with actin depolymerizing factor (**ADF**). This complex is essential for the polymerization/depolymerization of actin filaments. Actin-binding activity is negatively regulated by the phosphorylation of CFL (Yahara et al., 1996). Moreover, various stresses activate CFL by inducing dephosphorylation (Yahara et al., 1996). The striated muscle activator of Rho signaling (STARS) or ABRA has a role in transcription regulation and gene expression either by direct association with nuclear chromatin remodeling proteins or by cytoplasmic changes in actin cytoskeleton dynamics (Barrientos et al., 2007). Furthermore, ABRA action prerequires the activation of the Rho GTPase and actin polymerization (Barrientos et al., 2007). The protein VIM belongs to the intermediate filaments and is involved in numerous functions such as cell adhesion, migration, and signaling (for review, see Ivaska et al., 2007). This protein is present in newly differentiated muscle cells and in regenerating muscle fibers (Cízková et al., 2009). The protein PDCD6IP has several functions in cells including apoptosis, endocytic membrane trafficking, and cytoskeletal remodeling (for review see Odorizzi, 2006). In particular, PDCD6IP may play a positive role in the F-actin bundling step in stress fiber assembly because it directly interacts with α-actinin and promotes its association with F-actin (Pan et al., 2006). The protein HSBP1 or HSP27 has no physical link with ACTA1, but in vitro, HSBP1 acts as a phosphorylation-regulated F-actin capping protein able to inhibit actin polymerization (Guay et al., 1997). In stressful conditions, HSBP1 becomes phosphorylated with p-38 as the major upstream, and consequently, actin filament is stabilized (Guay et al., 1997).

The subnetwork involved in actin polymerization and stabilization showed various proteins or transcripts upregulated or downregulated. The upstream regulation of this phenomenon could be due to Rho-GTPases. Indeed, this family of enzymes plays a key role in the regulation of the assembly and organization of cell cytoskeleton and more particularly, the assembly of filamentous actin (Bishop and Hall, 2000; Di Ciano-Oliveira et al., 2006). Among this family, it was reported that RhoA is involved in stress fiber assembly and lamellipodia and filopodia formation (Hall, 1998; Bishop and Hall, 2000; Riddick et al., 2008). A schematic bibliographic synthesis of the mechanisms of stress fiber assembly is shown Figure 6. From this, it can be observed that several proteins were differentially expressed in our experiment. All these proteins were down-expressed in RT test muscles. A hypothesis could be that, in our experiment, myofibrils were reinforced by these mechanisms of fiber stress formation in the RT test group. Consequently, these proteins were less extractible, explaining the apparent down-expression. To our knowledge, this is the first time that such cellular events are reported in striated skeletal muscles in vivo after a stress test.

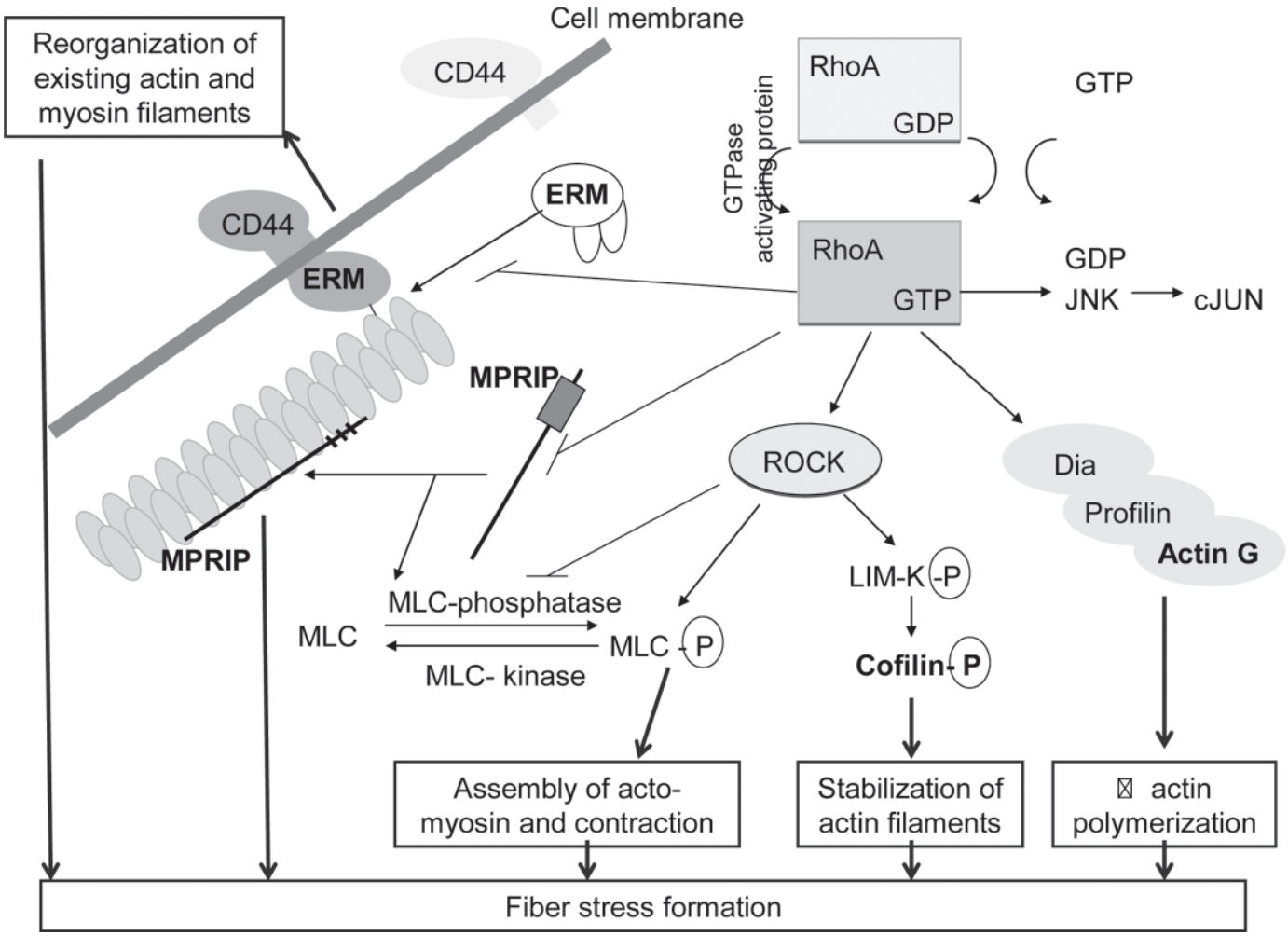

Figure 6. Bibliographic reconstruction of the mechanisms of stress fiber formation (adapted from Hirao et al., 1996; Hall, 1998; Bishop and Hall, 2000; Riddick et al., 2008). Proteins appearing in bold refer to proteins differentially expressed in our experiment. ERM, ezrin moesin radixin; MPRIP, myosin phosphatase rho A interacting protein; Dia, diaphanous homolog; LIM-K, LIM-kinase; MLC, myosin light chain; GDP, guanosine diphosphate; GTP, guanosine triphosphate; JNK, c-jun N-terminal kinase; ROCK, Rho-associated coiled coil kinase.

However, the implications on muscle physiology remain unknown and deserve further investigation.

In conclusion, the use of generic approaches to assess muscle responses to stress allowed pointing out transcripts, proteins and metabolites that were differentially expressed between control and treated chickens. The integration of these cellular components indicates that the cellular mechanisms of carbohydrate metabolism and cytoskeleton stabilization were affected by stressors in muscle tissue. We reported that the under-expression of transcripts and proteins could be due to exhaustion of the muscle during the RT test. Several proteins and transcripts involved in stress fiber formation were differentially expressed but further studies are required to understand the implication of such events and the physiological consequences on muscle biology.

LITERATURE CITED

Almon, R. R., D. D. DuBois, Z. Yao, E. P. Hoffman, S. Ghimbovschi, and W. J. Jusko. 2007. Microarray analysis of the temporal response of skeletal muscle to methylprednisolone: Comparative analysis of two dosing regimens. Physiol. Genomics 30:282–299.

Barrientos, T., D. Frank, K. Kuwahara, S. Bezprozvannaya, G. C. T. Pipe, R. Bassel-Duby, J. A. Richadson, H. A. Katus, E. N. Olson, and N. Frey. 2007. Two novel members of the ABLIM protein family, ABLIM-2 and -3, associate with STARS and directly bind F-actin. J. Biol. Chem. 282:8393–8403.

Bassett, J. M. 1970. Metabolic effects of catecholamines in sheep. Aust. J. Biol. Sci. 23:903–914.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc., B 57:289–300.

Bergmeyer, H. U. 1974. Pages 1127, 1196, 1238, 1464 in Methods in Enzymatic Analysis. G. H. Bourne, ed. Academic Press, New York, NY.

Bishop, A. L., and A. Hall. 2000. Rho GTPases and their effector proteins. Biochem. J. 348:241–255.

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

Carrasco, G. A., and L. D. Van de Kar. 2003. Neuroendocrine pharmacology of stress. Eur. J. Pharmacol. 463:235–272.

Chen, G., T. G. Gharib, C. C. Huang, J. M. G. Taylor, D. E. Misek, S. L. R. Kardia, T. J. Giordanno, M. D. Iannettoni, M. B. Orringer, S. M. Hanash, and D. G. Beer. 2002. Discordant protein and mRNA expressions in lung adenocarcinomas. Mol. Cell. Proteomics 1:304–313.

Cherepanova, O., A. Oriova, V. E. Galkin, P. F. Van der Ven, D. O. Furst, J. P. Jin, and E. H. Egelman. 2006. Xin-repeats and nebulin-like repeats bind to F-actin in a similar manner. J. Mol. Biol. 356:714–723.

Cízková, D., T. Soukup, and J. Mokry. 2009. Expression of nestin, desmin and vimentin in intact and regenerating muscle spindles of rat hind limb skeletal muscles. Histochem. Cell Biol. 131:197–206.

Coderre, L., A. K. Srivastava, and J. L. Chiasson. 1991. Role of glucocorticoid in the regulation of glycogen metabolism in skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 260:927–932.

Dallman, M. F., A. M. Strack, S. F. Akana, M. J. Bradbury, E. S. Hanson, K. A. Scribner, and M. Smith. 1993. Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. Front. Neuroendocrinol. 14:303–347.

Dalrymple, R. H., and R. Hamm. 1973. A method for the extraction of glycogen and metabolites from a single sample. J. Food Technol. 8:439–444.

Debut, M., C. Berri, C. Arnould, D. Guemene, V. Sante-Lhoutellier, N. Sellier, E. Baeza, N. Jehl, Y. Jego, C. Beaumont, and E. Le Bihan-Duval. 2005. Behavioural and physiological responses of three chicken breeds to pre-slaughter shackling and acute heat stress. Br. Poult. Sci. 46:527–535.

Debut, M., C. Berri, E. Baeza, N. Sellier, C. Arnould, D. Guemene, N. Jehl, B. Boutten, Y. Jego, C. Beaumont, and E. Le Bihan-Duval. 2003. Variation of chicken technological meat quality in relation to genotype and preslaughter stress conditions. Poult. Sci. 82:1829–1838.

de Nobel, H., L. Lawrie, S. Brul, F. Klis, M. Davis, H. Alloush, and P. Coote. 2001. Parallel and comparative analysis of the proteome and transcriptome of sorbic acid stressed *Saccharomyces cerevisiae*. Yeast 18:1413–1428.

Désert, C., M. Duclos, P. Blavy, F. Lecerf, F. Moreews, C. Klopp, M. Aubry, F. Herault, P. Le Roy, C. Berri, M. Douaire, C. Diot, and S. Lagarrigue. 2008. Transcriptome profiling of the feeding-to-fasting transition in chicken liver. BMC Genomics 9:611.

Di Ciano-Oliveira, C., A. C. P. Thirone, K. Szaszi, and A. Kapus. 2006. Osmotic stress and the cytoskeleton: The R(h)ole of Rho GTPases. Acta Physiol. (Oxf.) 187:257–272.

Ekstrand, A., C. Schalin-Jantti, M. Lofman, M. Parkkonen, E. Widen, A. Franssila-Kallunki, C. Saloranta, V. Koivisto, and L. Groop. 1996. The effect of (steroid) immunosuppression on skeletal muscle glycogen metabolism in patients after kidney transplantation. Transplantation 61:889–893.

El Rammouz, R., F. Letisse, S. Durand, J. C. Portais, Z. Wadih-Moussa, and X. Fernandez. 2010. Analysis of skeletal muscle metabolome—Evaluation of extraction methods for targeted metabolite quantification using liquid chromatography tandem mass spectrometry (LC-MS/MS). Anal. Biochem. 398:169–177.

Etches, R. J. 1976. A radioimmunoassay for corticosterone and its application to the measurement of stress in poultry. Steroids 28:763–773.

Fernandez, X., and E. Tornberg. 1991. A review of the causes of variation in muscle glycogen content and ultimate pH in pigs. J. Muscle Foods 2:209–235.

Frolow, J., and C. L. Milligan. 2004. Hormonal regulation of glycogen metabolism in white muscle slices from rainbow trout (*Oncorhynchus mykiss* Walbaum). Am. J. Physiol. Regul. Integr. Comp. Physiol. 287:R1344–R1353.

Görg, A., W. Weiss, and M. J. Dunn. 2004. Current two-dimensional electrophoresis technology for proteomics. Proteomics 4:3665–3685.

Guay, J., H. Lambert, G. Gingras-Breton, J. N. Lavoie, J. Huot, and J. Landry. 1997. Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J. Cell Sci. 110:357–368.

Hall, A. 1998. Rho GTPases and the actin cytoskeleton. Science 279:509–514.

Halter, J. B., J. C. Beard, and D. Jr. Porte. 1984. Islet function and stress hyperglycemia: Plasma glucose and epinephrine interaction. Am. J. Physiol. 247:E47–E52.

Hawke, T. J., D. J. Atkinson, S. B. Kanatous, P. F. M. Van der Ven, S. C. Goetsch, and D. J. Garry. 2007. Xin, an actin binding protein, is expressed within muscle satellite cells and newly regenerated skeletal muscle fibers. Am. J. Physiol. Cell Physiol. 293:C1636–C1644.

Hazard, D., M. Couty, S. Richard, and D. Guémené. 2008. Intensity and duration of corticosterone response to stressful situations in Japanese quail divergently selected for tonic immobility. Gen. Comp. Endocrinol. 155:288–297.

Henriksen, J. E., F. Alford, A. Vaag, A. Handberg, and H. Beck-Nielsen. 1999. Intracellular skeletal muscle glucose metabolism is differentially altered by dexamethasone treatment of normoglycemic relatives of type 2 diabetic patients. Metabolism 48:1128–1135.

Hirao, M., N. Sato, T. Kondo, S. Yonemura, M. Monden, T. Sasaki, Y. Takai, S. Tsukita, and S. Tsukita. 1996. Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. J. Cell Biol. 135:37–51.

Ivaska, J., H.-M. Pallari, J. Nevo, and J. E. Eriksson. 2007. Novel functions of vimentin in cell adhesion, migration, and signaling. Exp. Cell Res. 313:2050–2062.

Jaffrézic, F., D. J. de Koning, P. J. Boettcher, A. Bonnet, B. Buitenhuis, R. Closset, S. Dejean, C. Delmas, J. C. Detilleux, P. Dovc, M. Duval, J. L. Foulley, J. Hedegaard, H. Hornshoj, I. Hulsegge, L. Janss, K. Jensen, L. Jiang, M. Lavric, K. A. Le Cao, M. S. Lund, R. Malinverni, G. Marot, H. Nie, W. Petzl, M. H. Pool, C. Robert-Granie, M. San Cristobal, E. M. van Schothorst, H. J. Schuberth, P. Sorensen, A. Stella, G. Tosser-Klopp, D. Waddington, M. Watson, W. Yang, H. Zerbe, and H. M. Seyfert. 2007. Analysis of the real EADGENE data set: Comparison of methods and guidelines for data normalisation and selection of differentially expressed genes (open access publication). Genet. Sel. Evol. 39:633–650.

Jung-Ching Lin, J., E. A. Gustafson-Wagner, H. W. Sinn, S. Choi, S. M. Jaacks, D. Z. Wang, S. Evans, and L. J. Lin-Chun. 2005. Structure, expression and function of a novel intercalated disc protein Xin. J. Med. Sci. 25:215–222.

Kannan, G., J. L. Heath, C. J. Wabeck, and J. A. Mench. 1997. Shackling of broilers: Effects on stress responses and breast meat quality. Br. Poult. Sci. 38:323–332.

Kim, I. F., E. Mohammadi, and R. C. Huang. 1999. Isolation and characterization of IPP, a novel human gene encoding an actin-binding, kelch-like protein. Gene 228:73–83.

Kvetnanský, R., K. Pacak, K. Fukuhara, E. Viskupic, B. Hiremagalur, B. Nankova, D. S. Goldstein, E. L. Sabban, and I. J. Kopin. 1995. Sympathoadrenal system in stress. Interaction with the hypothalamic-pituitary-adrenocortical system. Ann. N. Y. Acad. Sci. 771:131–158.

Laville, E., T. Sayd, C. Terlouw, S. Blinet, J. Pinguet, M. Fillaut, J. Glénisson, and P. Chérel. 2009. Differences in pig muscle proteome according to HAL genotype: Implications for meat quality defects. J. Agric. Food Chem. 57:4913–4923.

Liu, J., M. Damon, N. Guitton, I. Guisle, P. Ecolan, A. Vincent, P. Cherel, and F. Gondret. 2009. Differentially-expressed genes in pig Longissimus muscles with contrasting levels of fat, as identified by combined transcriptomic, reverse transcription PCR, and proteomic analyses. J. Agric. Food Chem. 57:3808–3817.

Luciani, F., A. Molinari, F. Lozupone, A. Calcabrini, L. Lugini, A. Stringaro, P. Puddu, G. Arancia, M. Cianfriglia, and S. Fais. 2002. P-Glycoprotein-actin association through ERM family proteins: A role in P-glycoprotein function in human cells of lymphoid origin. Blood 99:641–648.

Mashego, M. R., W. M. Van Gulik, J. L. Vinke, and J. J. Heijnen. 2003. Critical evaluation of sampling techniques for residual

glucose determination in carbon-limited chemostat culture of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 83:395–399.

Meunier, B., J. Bouley, I. Piec, C. Bernard, B. Picard, and J. F. Hocquette. 2005. Data analysis methods for detection of differential protein expression in two-dimensional gel electrophoresis. Anal. Biochem. 340:226–230.

Molloy, M. P., B. R. Herbert, B. J. Wals, M. I. Tyler, M. Traini, J.-C. Sanchez, D. F. Hochstrasser, K. L. Wuilliams, and A. A. Gooley. 1998. Extraction of membrane proteins by differential solubilization for separation using two-dimensional gel electrophoresis. Electrophoresis 19:837–844.

Moncek, F., R. Kvetnansky, and D. Jezova. 2001. Differential responses to stress stimuli of Lewis and Fischer rats at the pituitary and adrenocortical level. Endocr. Regul. 35:35–41.

Mormède, P., S. Andanson, B. Auperin, B. Beerda, D. Guémené, J. Malmkvist, X. Manteca, G. Manteuffel, P. Prunet, C. G. van Reenen, S. Richard, and I. Veissier. 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92:317–339.

Odorizzi, G. 2006. The multiple personalities of Alix. J. Cell Sci. 119:3025–3032.

Okar, D. A., A. J. Lange, À. Manzano, A. Navarro-Sabatè, L. Riera, and R. Bartrons. 2001. PFK-2/FBPase-2: Maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem. Sci. 26:30–35.

Ono, S. 2003. Regulation of actin filament dynamics by actin polymerizing factor/cofili, and actin-interacting protein 1: New blades for twisted filaments. Biochemistry 42:13363–13370.

Pan, S., R. Wang, X. Zhou, G. He, J. Koomen, R. Kobayashi, L. Sun, J. Corvera, G. E. Gallick, and J. Kuang. 2006. Involvement of the conserved adaptor protein Alix in actin cytoskeleton assembly. J. Biol. Chem. 281:34640–34650.

Ramos, A., and P. Mormède. 1998. Stress and emotionality: A multidimensional and genetic approach. Neurosci. Biobehav. Rev. 22:33–57.

Riddick, N., K. Ohtani, and H. K. Surks. 2008. Targeting by myosin phosphatise-RhoA interacting protein mediates RhoA/ROCK regulation of myosin phosphatase. J. Cell. Biochem. 103:1158–1170.

Sapolsky, R. M., L. M. Romero, and A. U. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21:55–89.

Sayd, T., M. Morzel, C. Chambon, M. Franck, P. Figwer, C. Larzul, P. Le Roy, G. Monin, P. Cherel, and E. Laville. 2006. Proteome analysis of the sarcoplasmic fraction of pig Semimembranosus muscle: Implications on meat color development. J. Agric. Food Chem. 54:2732–2737.

Scheurink, A. J., and A. B. Steffens. 1990. Central and peripheral control of sympathoadrenal activity and energy metabolism in rats. Physiol. Behav. 48:909–920.

Selye, H. 1936. A syndrome produced by diverse nocuous agents. Nature 138:32.

Smith, M. A., S. Makino, S. Y. Kim, and R. Kvetnansky. 1995. Stress increases brain-derived neurotropic factor messenger-ribonucleic-acid in the hypothalamus and pituitary. Endocrinology 136:3743–3750.

Smyth, G. K. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3:Article3.

Smyth, G. K. 2005. Limma: Linear models for microarray data. Pages 397–420 in Bioinformatics and Computational Biology Solutions using R and Bioconductor. R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, and W. Huber, ed. Springer, New York, NY.

Smyth, G. K., and T. Speed. 2003. Normalization of cDNA microarray data. Methods 31:265–273.

Van Dam, J. C., M. R. Eman, J. Frank, H. C. Lange, G. W. K. Van Dedem, and J. J. Heijnen. 2002. Analysis of glycolytic intermediates in *Saccharomyces cerevisiae* using anion exchange chromatography and electrospray ionisation with tandem mass spectrometric detection . Anal. Chim. Acta 460:209–218.

Vandenborne, K., B. De Groef, S. M. Geelissen, E. R. Kuhn, V. M. Darras, and S. Van der Geyten. 2005. Corticosterone-induced negative feedback mechanisms within the hypothalamo-pituitary-adrenal axis of the chicken. J. Endocrinol. 185:383–391.

VanHouten, J. N., H. L. Asch, and B. B. Asch. 2001. Cloning and characterization of ectopically expressed transcripts for the actin-binding protein MIPP in mouse mammary carcinomas. Oncogene 20:5366–5372.

Viguerie, N., K. Clement, P. Barbe, M. Courtine, A. Benis, D. Larrouy, B. Hanczar, V. Pelloux, C. Poitou, Y. Khalfallah, G. S. Barsh, C. Thalamas, J.-D. Zucker, and D. Langin. 2004. In vivo epinephrine-mediated regulation of gene expression in human skeletal muscle. J. Clin. Endocrinol. Metab. 89:2000–2014.

Witczak, C. A., C. G. Sharoff, and L. J. Goodyear. 2008. AMP-activated protein kinase in skeletal muscle: From structure and localization to its role as a master regulator of cellular metabolism. Cell. Mol. Life Sci. 65:3737–3755.

Witt, C. C., S. H. Witt, S. Lerche, D. Labeit, W. Back, and S. Labeit. 2008. Cooperative control of striated muscle mass and metabolism by MuRF1 and MuRF2. EMBO J. 27:350–360.

Wong, D. L. 2006. Epinephrine biosynthesis: Hormonal and neural control during stress. Cell. Mol. Neurobiol. 26:891–900.

Yahara, I., H. Aizawa, K. Moriyama, K. Iida, N. Yonezawa, E. Nishida, H. Hatanaka, and F. Inagaki. 1996. A role of cofilin/destrin reorganization of actin cytoskeleton in response to stresses and cell stimuli. Cell Struct. Funct. 21:421–424.