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RESEARCH ARTICLE

# Differential expression of sarcoplasmic proteins in four heterogeneous ovine skeletal muscles

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Fiber-type distribution is known to vary widely within and between muscles according to differences in muscle functions. 2-DE and MALDI-MS were used to investigate the molecular basis of muscle fiber type-related variability. We compared four lamb skeletal muscles with heterogeneous fiber-type composition that are relatively rich in fast-twitch fiber types, *i.e.*, the semimembranosus, *vastus medialis*, *longissimus dorsi*, and *tensor fasciae latae* (TL). Our results clearly showed that none of the glycolytic metabolism enzymes detected, including TL which was most strongly glycolytic, made intermuscular differentiation possible. Muscle differentiation was based on the differential expression of proteins involved in oxidative metabolism, including not only citric acid cycle enzymes but also other classes of proteins with functions related to oxidative metabolism, oxidative stress, and probably to higher protein turnover. Detected proteins were involved in transport (carbonate dehydratase, myoglobin, fatty acid-binding protein), repair of misfolding damage (heat shock protein (HSP) 60 kDa, HSP-27 kDa, alpha-crystallin beta subunit, DJ1, stress-induced phosphoprotein), detoxification or degradation of impaired proteins (GST-Pi, aldehyde dehydrogenase, peroxiredoxin, ubiquitin), and protein synthesis (tRNA-synthetase). The fractionating method led to the detection of proteins involved in different functions related to oxidative metabolism that have not previously been shown concomitancy.

**Keywords:**
Muscle-fiber types / Oxidative stress regulators / Sarcoplasmic proteins / Sheep / Two-dimensional gel electrophoresis

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**Abbreviations: 20-αHSD,** 20-alpha-hydroxysteroid dehydrogenase; **A1AT,** alpha-1-antitrypsin; **HSP,** heat shock protein; **LD,** *longissimus dorsi*; **MHC,** myosin heavy chain; **ROS,** reactive oxygen species; **SM,** semimembranosus; **TL,** *tensor fasciae latae*; **VM,** *vastus medialis*

## 1 Introduction

The technological and sensorial qualities of meat are to a great extent influenced by the biochemical characteristics of muscle [1]. The degree to which these characteristics vary is governed by the functional diversity of the muscles, which is expressed by their fiber types. The fiber-type composition of most skeletal muscles is heterogeneous: fiber-type distribution is known to vary widely both between and within mus-

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cles depending on the muscle location and function [2]. This leads to a significant variability in the sensorial properties of meat derived from different animal muscles. In addition, fiber-type composition may vary within a species according to breed [3], physiological maturity [4], or physical activity [5].

Skeletal fibers are classified as either slow-twitch or fast-twitch fibers depending on their function properties. They are commonly distinguished by myosin ATPase activity and polymorphism of the myosin heavy chain (MHC) [6, 7]. Slow-twitch fibers express type I MHC isoform, whereas fast-twitch fibers may express three different isoforms, *i.e.* types IIa, IIb, or IIx. Fiber types with different contractile equipment present different metabolic pathways for producing contractile energy. Slow-twitch fibers have a predominantly oxidative metabolism, while fast-twitch fibers rich in IIb or IIx MHC isoforms have a predominantly glycolytic metabolism and fibers rich in the IIa MHC isoform share both oxidative and glycolytic metabolism.

Until recently, traditional biochemical or histochemical approaches to studying the properties of muscle fiber types were based on only a few proteins, and therefore did not take into account the full complexity and multiplicity of biochemical mechanisms. Recently developed proteomics tools (2-DE followed by protein identification using MS) appear capable of providing a more detailed characterization of the protein expression profiles of different muscle types. The majority of studies to date were performed on the whole proteome, including sarcoplasmic and myofibrillar fractions. Most of the proteins characterized were found to be metabolic enzymes involved in ATP synthesis and contractile proteins which are the most abundant protein in the muscles [8, 9]. Other classes of proteins such as transcription factor and signal transduction factors are present at low levels and are therefore not detected. Fractionating methods, such as those developed for the specific study of sarcoplasmic proteome containing only 30% of total proteins of muscle cells, appear suitable for the detection of less-expressed proteins [10, 11].

The overall objective of the present study was to characterize the protein expression of four different ovine skeletal muscles. For this purpose, differential proteome analysis was performed on sarcoplasmic fraction. The muscles chosen all present heterogeneous fiber-type composition and are relatively rich in fast-twitch fiber types.

## 2 Materials and methods

### 2.1 Animals and muscle samples

We used crossbred lambs originating from an F2 crossing between Romanov ewes and Belgian Texel rams. Seventeen lambs ($N = 8$ males, 9 females) were slaughtered at a weight set at 33 kg for females and 39 kg for males to match the commercial carcass weight. After slaughter, the carcasses were dressed according to the commercial practices. The four muscles studied were the *longissimus dorsi* (LD), which is the main back muscle, and the *vastus medialis* (VM), semimembranosus (SM), and *tensor fasciae latae* (TL) which are found in the proximal segment of the hind legs. These heterogeneous muscles were chosen according to the contractile and metabolic type. LD and SM muscles were chosen as fast-twitch red muscle while the TL is fast-twitch white and the VM presents the highest proportion of slow-twitch red fibers of all the four muscles. The samples were typed by histochemical analysis to confirm these patterns.

Muscle samples were taken within 30 min after following slaughter from the center of the muscle slices. Slices were cut transversally to fiber direction in the midbelly muscle region of the VM, SM, and TL muscles. The LD muscle slice was sampled between the last thoracic vertebra and the first lumbar vertebra. Samples intended for histochemical typing ($N = 17$) were cooled with isopenthane frozen in liquid nitrogen and stored at −80°C until experimental use. Five randomly chosen electrophoresis samples (three males and two females) were frozen in liquid nitrogen, and then reduced to a fine powder under liquid nitrogen using a mortar and a mechanical pestle, and stored at −80°C until protein extraction.

### 2.2 Histochemistry

Transverse muscle cryo-sections (10 μm) were prepared as described by Sayd *et al.* [3]. One section was stained using the myosin ATPase method following preincubation at pH 4.45 in order to define the three fiber types I, IIa, and IIb. The next section was stained for succinate dehydrogenase to divide the IIb type into either a more oxidative (IIbox) or less oxidative (IIbno) subgroup. Typing was realized on an average of 300 fibers *per* muscle.

### 2.3 Sarcoplasmic protein extraction

The 2-DE gels were run on five SM, LD, and TL muscles and four VM muscles. Gels were produced in triplicate. The sarcoplasmic fraction was obtained using a method adapted from the subcellular fractionation technique described by Pietrzak *et al.* [12]. The extraction buffer consisted of 50 mM KCl, 4 mM $MgCl_2$, 20 mM Tris, 2 mM EDTA, 1% w/v DTT, and 5 mM Pefabloc at pH 7. Muscle samples (150 mg) were added to 1.5 mL of extraction buffer in a microcentrifuge tube containing a glass bead. Homogenization was performed in a ball mill (Retsch MM2 agitator, Retsch, Haan, Germany) for 1 h at 4°C. Extracts were centrifuged at $10\,000 \times g$ for 15 min at 6°C and the supernatant was collected. Samples were frozen in liquid nitrogen and stored at −80°C.

### 2.4 2-DE

IPG IEF was carried out in a Protean IEF cell (BioRad, Hercules, CA), using a 17-cm, pH 5–8 BioRad ReadyStrip. The strips were protein-loaded for analytical (110 μg) or preparative electrophoresis gels (300 μg) by adding an appropri-

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ate volume of extract to a buffer consisting of 7 M urea, 2 M thiourea, 2% w/v CHAPS, 5 mM Pefabloc, 0.2% w/v DTT, and 0.2% carrier ampholytes. The strips were rehydrated overnight. For the subsequent IEF, voltage was increased gradually to 8000 V to reach a total of 80 000 V·h. The strips were then immediately frozen and stored at −20°C until further use. Prior to SDS-PAGE, strips were equilibrated for 15 min followed by 20 min in a solution of 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 50 mM Tris-HCl successively supplemented with 1% w/v DTT or 2.5% w/v iodoacetamide and Bromophenol blue as a dye. SDS-PAGE was performed in a Protean IIxi cell (BioRad) on 12% polyacrylamide gels at 15 mA *per* gel, until the dye track reached the end of the gels. Analytical and preparative gels were silver-stained following the protocol described by Yan *et al.* [13].

### 2.5 Image analysis

Gel images were acquired through a GS-800 densitometer and analyzed using PDQuest software (BioRad). After automated detection and matching, highly saturated or illdefined spots were manually removed, and crossgel matching was checked and corrected when necessary. Spots were normalized by expressing relative quantity of each spot as the ratio of individual spot quantity to the total quantity of valid spots, expressed in ppm. For one sample and one spot, the mean of the three values (for gels run in triplicate) was calculated.

### 2.6 Statistics

Statistical analysis was performed using SAS software (SAS Institute, Cary, NC, USA). The ANOVA procedure was used to test for the significance ($p<0.05$) of the statistical fixed effect of muscle on fiber-type percentage. Where significant effects were found, Tukey comparison tests were used to identify differences between pairs of muscles at the 5% significance level. Concerning 2-DE, because data were unbalanced (N = 4 in VM; N = 5 in LD, SM, and TL muscles), the GLM procedure was used to test for the significance ($p<0.05$) of the statistical fixed effect of muscle on the averaged spot quantities. Where significant effects were found, Tukey–Kramer comparison tests for unbalanced data were used to identify differences between pairs of muscles at the 5% significance level.

### 2.7 Identification of spots of interest by MALDI-TOF-MS

Spots were excised from gels using pipette tips. Gel pieces were placed in a 1.5 mL Eppendorf tube and destained for 2 min with a solution containing 30 mM KFe and 100 mM sodium thiosulfate, and the gel pieces were washed three times for 10 min in purified water (*e.g.* 18.2 Ω). The spots were then washed with 100 μL of 25 mM $NH_4HCO_3$/5% ACN for 30 min, followed by 100 μL of 25 mM $NH_4HCO_3$/ACN v/v twice for 30 min, and dehydrated in ACN. Gel spots were completely dried using a SpeedVac before trypsin digestion. The dried gel volume was evaluated, and three volumes of trypsin (V5111; Promega, Madison, WI, USA) 10 ng/μL in 25 mM $NH_4HCO_3$ were added. Digestion was performed at 37°C for 5 h. The gel pieces were centrifuged and 8–12 μL of ACN (depending on gel volume) was added to extract the peptides. This mixture was sonicated for 5 min and centrifuged. For MALDI-TOF-MS analysis, 1 μL of supernatant was loaded directly onto the MALDI target. The matrix solution (5 mg/mL CHCA in 50% ACN/0.1% TFA) was immediately added and allowed to dry at room temperature. A Voyager DE-Pro model MALDI-TOF mass spectrometer (PerSeptive BioSystems, Farmingham, MA, USA) was used in positive-ion reflector mode for PMF. External calibration was performed with a standard peptide solution (Proteomix, LaserBio Labs, Sophia-Antipolis, France). Internal calibration was performed using peptides resulting from autodigestion of porcine trypsin with protonated masses of 842.509, 1045.564, and 2211.104. Peptide mass fingerprints were compared to mammalian databases (NCBI and Swiss-Prot) using MASCOT and ProFound software (accessed at http://www.matrixscience.com and http://prowl.rockefeller.edu, respectively). The search criteria used were one missing trypsin cleavage site, partial methionine oxidation, partial carbamidomethylation of cysteine, and a mass deviation lower than 30 ppm. We required at least five matched peptides *per* protein for identification and used ProFound and MASCOT probabilistic scores, accuracy of the experimental-to-theoretical p*I* and molecular weight (MW).

## 3 Results and discussion

### 3.1 Histochemistry

Results of the histochemical analysis are presented in Table 1. In comparison with VM, the TL, SM, and LD muscles were predominantly fast-twitch (IIb). Among this group, the TL presented the highest rate of white fast-twitch fibers (IIb). The LD muscle was characterized by a higher rate of fast-twitch red IIa fibers, while the SM showed a higher rate of slow-twitch red fibers (I). The VM had higher rate of slow-twitch red fibers and a predominantly oxidative metabolism (I, IIbox).

### 3.2 Differences in protein profiles according to muscle

Up to 500 spots were detected *per* gel (Fig. 1). After removal of saturated zones and poorly reproducible zones, about 300 spots were successfully matched across the whole set of images. The number of gel-detected proteins from sarcoplasmic fractions was comparable to the number of proteins evidenced on the whole muscle proteome [14], thus indicating that the fractionating method allowed us to evidence less-expressed proteins.

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**Table 1.** Effect of muscle type on fiber-type percentage

| Fiber type percentage[a)] | Muscles[b] | | | |
|---|---|---|---|---|
| | LD | SM | TL | VM |
| I | $10 \pm 4^{c}$ | $13.6 \pm 4.5^{d}$ | $7.3 \pm 3.7^{c}$ | $24.4 \pm 4.4^{e}$ |
| IIa | $22.8 \pm 7.7^{c}$ | $18.5 \pm 7.9^{c}$ | $18.9 \pm 5.7^{c}$ | $17.9 \pm 3.3^{c}$ |
| IIb | $67.2 \pm 7.8^{c}$ | $68.9 \pm 9^{c}$ | $73.8 \pm 7.6^{d}$ | $57.7 \pm 4.9^{e}$ |
| IIbox | $27.9 \pm 9.6^{c}$ | $24.8 \pm 10.5^{c}$ | $28.9 \pm 10.2^{c}$ | $34.5 \pm 6.5^{c}$ |
| IIbno | $39.3 \pm 7.7^{c}$ | $43.1 \pm 13.9^{c}$ | $44.8 \pm 8.1^{c}$ | $23.2 \pm 3^{d}$ |

a) I, fiber type I; IIa, fiber type IIa; IIb, fiber type IIb; IIbox, fiber type IIb, more oxidative; IIbno, fiber type IIb, less oxidative. IIbox and IIbno are subdivisions of IIb.

b) $N = 17$ *per* muscle.

c-e) On a same line, means with different superscripts differ ($p<0.05$), if two means have the same superscript they are not significantly different.

Seventy-seven spots were differentially expressed between muscles. We managed to identify 47 proteins (Tables 2 and 3, Figs. 1 and 2). This relatively high fail-rate in identification was in part due to the fact that the ovine genome is not fully sequenced. Furthermore, although the highly sensitive silver staining method, we used, allowed precision detection of protein expression, there was often an insufficient quantity of protein to enable identification by MALDI-MS, even when pooling excised spots from different gels. Based on apparent MW lower than theoretical MW, some of the spots identified were protein fragments. All these fragments were derived from glycolytic enzymes (pyruvate kinase, lactate dehydrogenase, creatine kinase, and glycogen phosphorylase). Five of these fragments were overexpressed in SM muscle and one was overexpressed in TF muscle. Previous studies using 2-DE also reported protein fragments in muscles sampled very early after slaughter [15, 14, 10]. It was supposed that the presence of fragments which are often glycolytic enzymes was due to early proteolysis after slaughter, probably *intra vitam* proteolysis. Based on the observed equivalent MW to theoretical MW, the other 41 proteins were full-length proteins. These full-length proteins are grouped according to their functions as described below.

### 3.2.1 Glycolytic metabolism

Sixteen spots were identified as glycolytic enzymes, six of which were fragments. Some full-length proteins, *i.e.* pyruvate kinase, creatine kinase, enolase, and triosephosphate isomerase were redundant, and probably corresponded to isoforms of the protein. In the case of pyruvate kinase, three spots (isoforms) were specifically overexpressed in VM, TL, and SM muscles. Two isoforms of triosephosphate isomerase were up-regulated in LD muscle. This protein is involved in shunting the main glycolysis pathway. Thus, the increased triosephosphate isomerase expression in LD muscle can be interpreted by underactivation of the main glycolysis pathway. The LD muscle was also characterized by a lower expression of two isoforms of creatine kinase which is an enzyme essential to a rapid replenishment of cellular ATP stocks. Okumura *et al.* [9] found creatine kinase was more strongly expressed in more glycolytic muscles. One enolase isoform was also down-regulated in LD muscle, while the two other isoforms of enolase and fructose-1,6-biphosphatase were up-regulated in SM muscle. In comparison with other muscles, SM seems to present an accelerated glycolytic metabolism and, in contrary to the LD, also seems to be less glycolytic. Interestingly, VM muscle, which has the highest rate of slow oxidative fiber types, did not express specific down-regulation of glycolytic enzymes. The TL muscle, which has the highest rate of IIb fiber type, did not show specific up-regulation of glycolytic enzymes.

### 3.2.2 Oxidative metabolism

Six spots related to oxidative metabolism were identified as enzymes involved in the citric acid cycle (NADH dehydrogenase 24 kDa subunit, succinate-CoA ligase, ubiquinol-cytochrome *c* reductase, dihydrolipoamide *S*-succinyltransferase, and malate dehydrogenase). Malate dehydrogenase was expressed under two isoforms which were not specific to muscle type. Most of these proteins were up-regulated in VM muscle, which had the highest rate of slow-twitch oxidative fibers, and down-regulated in TF muscle, which had the highest rate of fast-twitch glycolytic fibers. The other two muscles presented intermediate profiles.

### 3.2.3 Contractile-associated proteins

Parvalbumin expression may be related to muscle contraction. This calcium-binding protein is highly expressed in mammalian fast-twitch muscle fibers. By acting as a $Ca^{2+}$ buffer, its overexpression does not affect the rapid contraction phase but leads to a significant increase in the rate of relaxation [16]. It was overexpressed in TL, which is the most abundant in fast-twitch fibers.

Actin alpha 1 which is the monomeric form of actin filaments was more expressed in VM, the most oxidative muscle. This overexpression may be related to a differential extractability of this structural protein in VM muscle or may indicate an increased need of material to promote myofibril assembly, related to a higher protein turnover.

### 3.2.4 Oxidative metabolism-related proteins

Proteins were identified presenting a different oxidative metabolism-associated. The three transport proteins carbonate dehydratase II, also known as carbonic anhydrase, which catalyzes the reversible hydration of carbon dioxide and facilitates its transport, the intracellular oxygen-binding protein myoglobin, and the heart-type cytoplasmic fatty acid-binding protein were all overexpressed in VM muscle and underexpressed in TL muscle. Other muscles showed inter-

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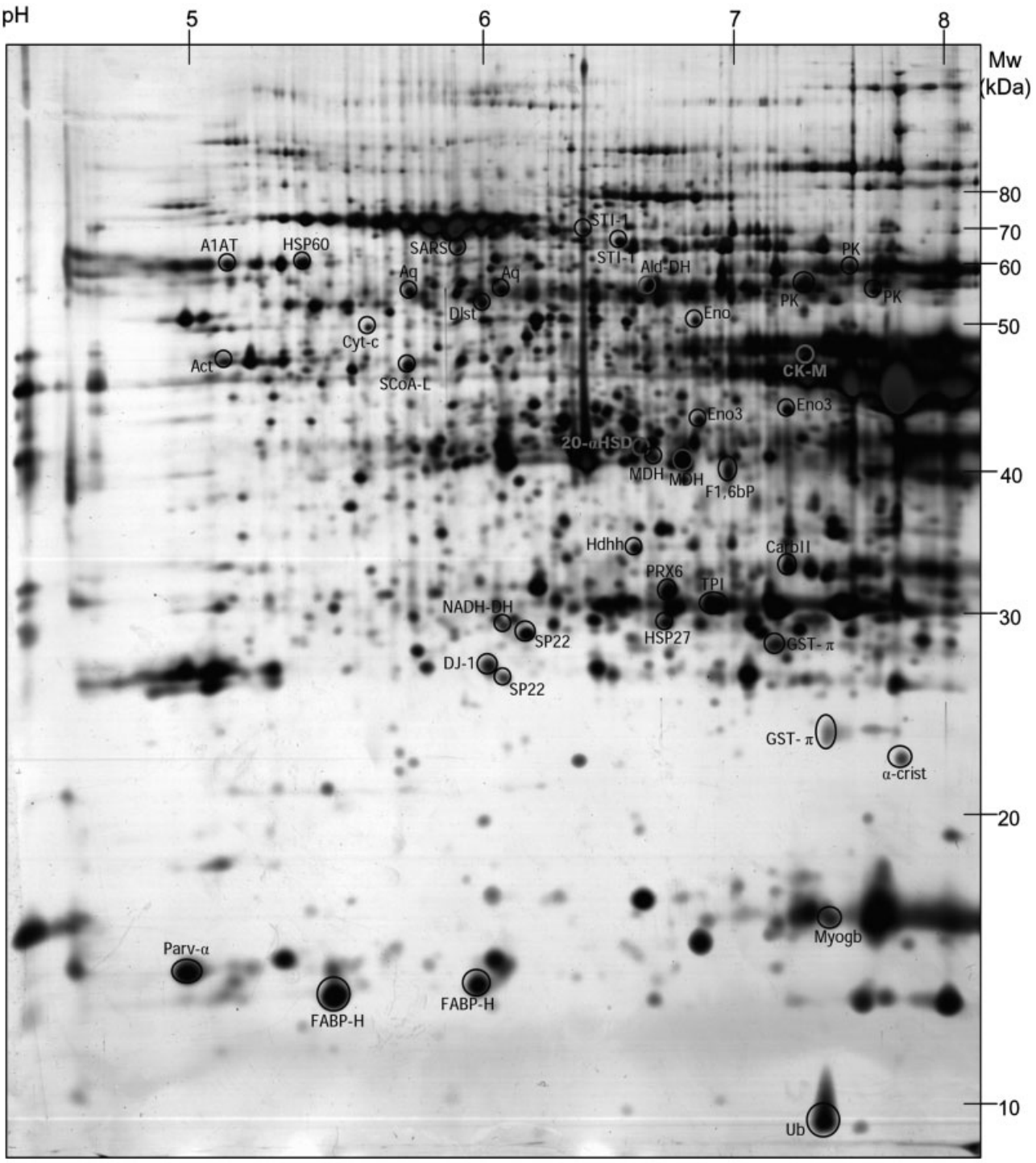


**Figure 1.** Representative 2-D gel map of muscle sarcoplasmic proteins. The differentially expressed proteins in the four muscles are encircled (spots corresponding to fragments are not shown). Abbreviations: 20-αHSD, 20-alpha-hydroxysteroid dehydrogenase; A1AT, α-1-antitrypsin; Ald-DH, aldehyde dehydrogenase; Aq, antiquitin 1; CarbII, carbonate dehydratase II; CK-M, creatine kinase M; Cyt-c, ubiquinol-cytochrome *c* reductase; DJ-1, DJ-1 protein; Eno, enolase; Eno3, enolase 3; FABP-H, fatty acid-binding protein; GST-π, glutathione *S*-transferase class Pi; Hdhh, haloacid dehalogenase-like hydrolase domain containing 2; HSP-27, heat shock protein 27 kDa; HSP-60, heat shock protein 60 kDa; MDH, malate dehydrogenase; Myogb, myoglobin; NADH-DH, NADH dehydrogenase, 24 kDa subunit; Parv-α, parv-albumin α; PK, pyruvate kinase; PRX6, peroxiredoxin 6; SARS, seryl-aminoacyl-tRNA synthetase 1; SCoA-L, succinate-CoA ligase; SP22, substrate protein of mitochondrial ATP-dependant proteinase; STI-1, stress-induced phosphoprotein 1; TPI, triosephosphate isomerase 1; Ub, ubiquitin; α-crist, α-crystallin, beta subunit.

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**Table 2.** Proteins differentially expressed between the four muscles

| SSP[a)] | Protein ID[b)] | Accession number[b)] | MW kDa/p*I* observed[c)] | MW kDa/p*I* theoretical[c)] | % Coverage[d)] | Matched peptide[d)] | $p<0.05$[e)] | Species[d)] |
|---|---|---|---|---|---|---|---|---|
| 7530R | Creatine kinase M | gi\|4838363 | 46/7.25 | 43/6.63 | 53 | 21 | <0.0001 | *Bos taurus* |
| 7202R | Creatine kinase M **fgt** | gi\|4838363 | 29/7.1 | 43/6.63 | 27 | 10 | <0.0001 | *B. taurus* |
| 7409R | Enolase 3 (a) | gi\|7331113 | 45/7.3 | 47/7.58 | 46 | 24 | <0.0001 | *Ovis aries* |
| 6407R | Enolase 3 (b) | gi\|56206033/gi\|57164373 | 45/6.8 | 69/5.80 | 31 | 16 | <0.0001 | *Mus musculus* |
| 6607R | Enolase alpha | gi\|57086919/gi\|27806645 | 50/6.9 | 47/6.44 | 23 | 9 | <0.0001 | *Homo sapiens* |
| 6307R | Fructose-1,6-bisphosphatase 2 | gi\|2154755 | 39/7.0 | 37/6.84 | 13 | 5 | <0.0001 | *H. sapiens* |
| 6203R | Glycogen phosphorylase (a) **fgt** | gi\|2352268/gi\|57163939 | 35/6.9 | 97/6.65 | 16 | 13 | <0.0001 | *O. aries* |
| 6207R | Glycogen phosphorylase (b) **fgt** | gi\|2352268/gi\|57163939 | 35/7.0 | 97/6.65 | 12 | 13 | <0.0001 | *O. aries* |
| 6005R | Lactate dehydrogenase A **fgt** | gi\|217590 | 13/6.9 | 37/8.44 | 24 | 8 | <0.0001 | *O. aries* |
| 7617R | Pyruvate kinase | gi\|1177221 | 56/7.3 | 58/7.2-7.96 | 40 | 22 | <0.0001 | *O. cuniculus* |
| 8702R | Pyruvate kinase, isoform M1 (a) | gi\|89089 | 60/7.5 | 58/7.2-7.96 | 35 | 19 | <0.0001 | *Felis catus* |
| 8609R | Pyruvate kinase, isoform M1(b) | gi\|2117873/gi\|33286422 | 55/7.8 | 58/7.2-7.96 | 39 | 20 | <0.0001 | *H. sapiens* |
| 5515R | Pyruvate kinase 3, isoform 2 **fgt** | gi\|33286422 | 47/6.5 | 58/7.2-7.96 | 27 | 9 | <0.0001 | *H. sapiens* |
| 5437R | Pyruvate kinase 3, isoform 1 **fgt** | gi\|31416989 | 43/6.5 | 58/7.2-7.96 | 23 | 11 | <0.0001 | *H. sapiens* |
| 6205R | Triosephosphate isomerase 1 (a) | gi\|59858493 | 30/6.87 | 27/6.45 | 70 | 16 | <0.0001 | *B. taurus* |
| 6206R | Triosephosphate isomerase 1 (b) | gi\|59858494 | 30/6.93 | 27/6.45 | 82 | 18 | <0.0001 | *B. taurus* |
| 3614R | Dlst protein, dihydrolipoamide *S*-succinyltransferase | gi\|23271834 | 53/6.15 | 22/6.43 | 40 | 8 | <0.0001 | *M. musculus* |
| 6302R | Malate dehydrogenase (a) | gi\|164543 | 40/6.7 | 36/6.91 | 31 | 9 | <0.0001 | *B. taurus* |
| 5308R | Malate dehydrogenase (b) | gi\|61856478 | 40.5/6.6 | 33/6.2 | 32 | 9 | <0.0001 | *B. taurus* |
| 3208R | NADH dehydrogenase 24 kDa subunit | gi\|1364245 | 29/6.1 | 23.5/5.7 | 27 | 5 | <0.0001 | *B. taurus* |
| 3501R | Succinate-CoA ligase | gi\|55957255/gi\|55957259 | 46.5/5.8 | 48/6.65 | 31 | 12 | <0.0001 | *H. sapiens* |
| 2611R | Ubiquinol-cytochrome *c* reductase | gi\|27807137 | 50/6.7 | 53/5.94 | 43 | 15 | <0.0001 | *B. taurus* |
| 1509R | Actin, skeletal muscle | gi\|71611 | 47/5.1 | 42/5.31 | 37 | 13 | <0.0001 | *O. cuniculus* |
| 1008R | Parvalbumin α | gi\|131103 | 12/4.9 | 12/4.98 | 48 | 8 | <0.0001 | *O. aries* |
| 7206R | Carbonate dehydratase II | gi\|68289 | 32/7.4 | 29/6.41 | 50 | 7 | <0.0001 | *O. aries* |
| 2007 | FABP-H (a) | Q8HY78/gi\|26000692 | 11.5/5.5 | 15/6.11 | 49 | 7 | <0.0001 | *Canis Familiaris* |
| 3002R | FABP-H (b) | Q8HY78/gi\|26000691 | 11.5/6.0 | 15/6.11 | 35 | 6 | <0.0001 | *O. aries* |
| 7009R | Myoglobin | gi\|70563 | 15/7.3 | 17/6.94 | 51 | 7 | <0.0001 | *O. aries* |
| 3106R | DJ-1 protein | gi\|50513593/gi\|59858513 | 25/6.0 | 20/6.84 | 47 | 6 | <0.0001 | *B. taurus* |
| 5206R | HSP-27 kDa | gi\|50979116 | 29/6.7 | 23/6.0-6.2 | 44 | 9 | <0.0001 | *B. taurus* |
| 2702R | HSP-60 kDa | gi\|1334284 | 60/5.4 | 61/5.71 | 27 | 8 | <0.0001 | *Rattus norvegicus* |
| 5701R | Stress-induced phosphoprotein1 (a) | gi\|13277819 | 63/6.45 | 63/6.40 | 34 | 21 | <0.0001 | *M. musculus* |
| 5706R | Stress-induced-phosphoprotein1 (b) | gi\|57099701 | 62/6.55 | 63/6.40 | 23 | 13 | <0.0001 | *C. familiaris* |
| 8107 | α-Crystallin, beta subunit | A39608 | 21/7.85 | 20/6.76 | 32 | 7 | <0.0001 | *M. musculus* |
| 5617R | Aldehyde dehydrogenase | gi\|57526379 | 55/6.75 | 55/6.37 | 36 | 14 | <0.0001 | *O. aries* |
| 3619R | Antiquitin 1 (a) | gi\|25108887 | 55/6.2 | 55/6.24 | 21 | 10 | <0.0001 | *H. sapiens* |
| 3601R | Antiquitin 1 (b) | gi\|25108887 | 55/5.8 | 55/6.24 | 22 | 13 | <0.0001 | *H. sapiens* |
| 7220R | GST Pi (a) | gi\|404 | 27/7.2 | 24/6.89 | 48 | 7 | <0.0001 | *B. taurus* |
| 7118R | GST Pi (b) | gi\|6013379 | 22/7.5 | 24/7.65 | 44 | 5 | <0.0001 | *C. hircus* |
| 5305R | Haloacid dehalogenase-like hydrolase | gi\|14149777 | 35/6.6 | 28.5/5.84 | 41 | 10 | <0.0001 | *H. sapiens* |
| 5207R | Peroxiredoxin 6 | gi\|27807167/NP_777068 | 31/6.7 | 25/6.00 | 73 | 17 | <0.0001 | *B. taurus* |
| 3107R | SP22 (a) | gi\|627764 | 26/6.05 | 22/5.72 | 22 | 4 | <0.0001 | *B. taurus* |
| 4202R | SP22 (b) | gi\|627764 | 28/6.15 | 22/5.72 | 37 | 6 | <0.0001 | *B. taurus* |
| 7006R | Ubiquitin | gi\|51701909 | 9/7.5 | 8.6/6.56 | 19 | 7 | <0.0001 | *O. aries* |
| 3710R | Seryl-aminoacyl-tRNA synthetase 1 | gi\|14250361/gi\|33468931 | 62/6.0 | 61/7.13 | 33 | 14 | <0.0001 | *M. musculus* |
| 1720R | A1AT | gi\|57526646 | 60/5.0 | 46/5.83 | 26 | 11 | <0.0001 | *O. aries* |
| 5307R | 20-αHSD | gi\|2201516/gi\|265404 | 40.5/6.55 | 37.3/5.90 | 53 | 11 | <0.0001 | *B. taurus* |

a) Spot label.
b) Protein names and accession numbers were taken from the NCBI database.
c) MW and p*I*, both theoretical (recorded in the NCBI database) and estimated (calculated from the spot position on the gel).
d) Number of peptides that matched the protein sequence, total percentage of sequence coverage, and the species for which sequence was matched.
e) *p*-Value from GLM analysis, significant level is 5%.
**Fgt:** protein fragment.

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**Table 3.** Muscle effect on protein expression

| SSP[a)] | Protein ID[b)] | Muscles[c)] | | | |
|---|---|---|---|---|---|
| | | LD | SM | TL | VM |
| Glycolytic metabolism | | | | | |
| 7530R | Creatine kinase M | 1100 ± 189[d] | 2127 ± 268[e] | 2526 ± 373[e] | 2757 ± 255[e] |
| 7202R | Creatine kinase M **fgt** | 3269 ± 1237[d] | 5337 ± 886[e] | 2640 ± 769[d] | 3304 ± 617[d] |
| 7409R | Enolase 3 (a) | 791 ± 299[d] | 1609 ± 257[e] | 608 ± 258[d] | 563 ± 267[d] |
| 6407R | Enolase 3 (b) | 1047 ± 248[d] | 1688 ± 270[e] | 1020 ± 284[d] | 547 ± 156[d] |
| 6607R | Enolase alpha | 189 ± 22[d] | 271 ± 32[e] | 325 ± 52[e] | 294 ± 47[e] |
| 6307R | Fructose-1,6-bisphosphatase 2 | 1974 ± 180[d] | 2547 ± 217[e] | 1390 ± 233[d] | 1625 ± 142[d] |
| 6203R | Glycogen phosphorylase (a) **fgt** | 361 ± 174[d] | 940 ± 160[e] | 410 ± 158[d] | 318 ± 113[d] |
| 6207R | Glycogen phosphorylase (b) **fgt** | 457 ± 231[d] | 1128 ± 168[e] | 463 ± 197[d] | 405 ± 128[d] |
| 6005R | Lactate dehydrogenase A **fgt** | 5139 ± 457[d] | 6101 ± 433[e] | 4403 ± 507[d] | 4938 ± 352[d] |
| 7617R | Pyruvate kinase | 1139 ± 175[d] | 1660 ± 173[d] | 1684 ± 59[d] | 3056 ± 461[e] |
| 8702R | Pyruvate kinase, isoform M1 (a) | 393 ± 106[d] | 676 ± 210[d] | 1106 ± 138[d] | 458 ± 115[e] |
| 8609R | Pyruvate kinase, isoform M1(b) | 313 ± 84[d] | 964 ± 212[e] | 418 ± 106[d] | 320 ± 125[d] |
| 5515R | Pyruvate kinase 3, isoform 2 **fgt** | 238 ± 34[d] | 236 ± 38[d] | 349 ± 29[e] | 192 ± 26[d] |
| 5437R | Pyruvate kinase 3, isoform 1 **fgt** | 625 ± 253[d] | 1165 ± 274[e] | 387 ± 151[d] | 331 ± 168[d] |
| 6205R | Triosephosphate isomerase 1 (a) | 5613 ± 518[d] | 4088 ± 647[e] | 3796 ± 528[e] | 3593 ± 467[e] |
| 6206R | Triosephosphate isomerase 1 (b) | 7128 ± 117[d] | 4560 ± 734[e] | 4825 ± 364[e] | 5120 ± 650[e] |
| Oxidative metabolism | | | | | |
| 3614R | Dlst protein, dihydrolipoamide S-succinyltransferase | 865 ± 92[d] | 891 ± 143[d] | 517 ± 51[e] | 1120 ± 140[d] |
| 6302R | Malate dehydrogenase (a) | 3878 ± 486[d] | 5061 ± 538[d] | 4676 ± 277[d] | 6185 ± 321[e] |
| 5308R | Malate dehydrogenase (b) | 336 ± 36[d] | 770 ± 83[e] | 451 ± 79[d] | 817 ± 83[e] |
| 3208R | NADH dehydrogenase 24 kDa subunit | 119 ± 19[d] | 147 ± 36[d] | 198 ± 33[d] | 428 ± 57[e] |
| 3501R | Succinate-CoA ligase | 1155 ± 405[d] | 1895 ± 375[d] | 693 ± 192[e] | 1279 ± 208[d] |
| 2611R | Ubiquinol-cytochrome *c* reductase | 243 ± 31[d] | 198 ± 37[d] | 115 ± 17[e] | 270 ± 41[d] |
| Contractile apparatus associated protein | | | | | |
| 1509R | Actin, skeletal muscle | 137 ± 76[d] | 141 ± 57[d] | 209 ± 72[d] | 1103 ± 724[e] |
| 1008R | Parvalbumin α | 119 ± 20[d] | 457 ± 118[e] | 10835 ± 1567[f] | 4951 ± 915[g] |
| Transport | | | | | |
| 7206R | Carbonate dehydratase II | 3892 ± 537[d] | 4652 ± 543[d] | 2598 ± 609[e] | 4000 ± 836[d] |
| 2007 | FABP-H (a) | 4716 ± 452[d] | 9376 ± 1221[e] | 3522 ± 506[d] | 10394 ± 470[e] |
| 3002R | FABP-H (b) | 673 ± 166[d] | 2407 ± 416[e] | 629 ± 170[d] | 4395 ± 637[f] |
| 7009R | Myoglobin | 194 ± 49[d] | 778 ± 157[e] | 381 ± 117[f] | 1135 ± 75[g] |
| Stress regulator | | | | | |
| 3106R | DJ-1 protein | 704 ± 50[d] | 925 ± 66[d] | 1021 ± 104[d] | 1308 ± 173[e] |
| 5206R | HSP-27 kDa | 2062 ± 856[d] | 1263 ± 449[d] | 236 ± 105[e] | 2006 ± 874[d] |
| 2702R | HSP-60 kDa | 820 ± 99[d] | 974 ± 192[d] | 862 ± 153[d] | 1590 ± 159[e] |
| 5701R | Stress-induced phosphoprotein 1 (a) | 202 ± 39[d] | 354 ± 66[e] | 408 ± 11[e] | 462 ± 9[e] |
| 5706R | Stress-induced phosphoprotein 1 (b) | 475 ± 57[d] | 607 ± 121[d] | 523 ± 74[d] | 837 ± 80[e] |
| 8107 | α-Crystallin, beta subunit | 1010 ± 600[d] | 1291 ± 690[d] | 73 ± 7[e] | 1539 ± 947[d] |
| Detoxification | | | | | |
| 5617R | Aldehyde dehydrogenase | 788 ± 106[d] | 893 ± 103[d] | 1155 ± 98[d] | 1777 ± 89[e] |
| 3619R | Antiquitin 1 (a) | 601 ± 107[d] | 877 ± 110[e] | 954 ± 106[e] | 1129 ± 65[e] |
| 3601R | Antiquitin 1(b) | 520 ± 67[d] | 664 ± 51[e] | 801 ± 43[f] | 1020 ± 57[g] |
| 7220R | GST Pi (a) | 361 ± 87[d] | 444 ± 61[d] | 690 ± 166[d] | 1200 ± 67[e] |
| 7118R | GST Pi (b) | 54 ± 24[d] | 114 ± 38[d] | 125 ± 46[d] | 326 ± 90[e] |
| 5305R | Haloacid dehalogenase-like hydrolase | 230 ± 28[d] | 404 ± 69[e] | 352 ± 28[e] | 414 ± 67[e] |
| 5207R | Peroxiredoxin 6 | 2084 ± 214[d] | 3624 ± 256[e] | 3525 ± 473[e] | 4177 ± 170[e] |
| 3107R | SP22 (a) | 303 ± 62[d] | 339 ± 56[d] | 151 ± 34[d] | 546 ± 140[e] |
| 4202R | SP22 (b) | 2000 ± 226[d] | 2070 ± 104[d] | 783 ± 124[e] | 2095 ± 280[d] |
| 7006R | Ubiquitin | 218 ± 30[d] | 350 ± 53[e] | 380 ± 63[e] | 468 ± 16[e] |

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**Table 3.** Continued

| SSP[a)] | Protein ID[b)] | Muscles[c)] | | | |
|---|---|---|---|---|---|
| | | LD | SM | TL | VM |
| Protein synthesis | | | | | |
| 3710R | Seryl-aminoacyl-tRNA synthetase 1 | 106 ± 12[d] | 170 ± 19[e] | 215 ± 48[e] | 246 ± 29[e] |
| Miscellaneous | | | | | |
| 1720R | A1AT | 879 ± 263[d] | 1312 ± 274[d] | 2638 ± 327[e] | 1519 ± 205[d] |
| 5307R | 20 alpha-hydroxysteroid dehydrogenase | 763 ± 151[d] | 1216 ± 122[e] | 380 ± 71[f] | 1505 ± 221[g] |

a) Spot label.
b) Protein names were taken from the NCBI database.
c) Means and SEM of the LD ($N = 5$), SM ($N = 5$), TL ($N = 5$), VM ($N = 4$).
d-g) On a same line, means with different superscripts differ ($p<0.05$), if two means have the same superscript they are not significantly different.
**Fgt:** protein fragment.

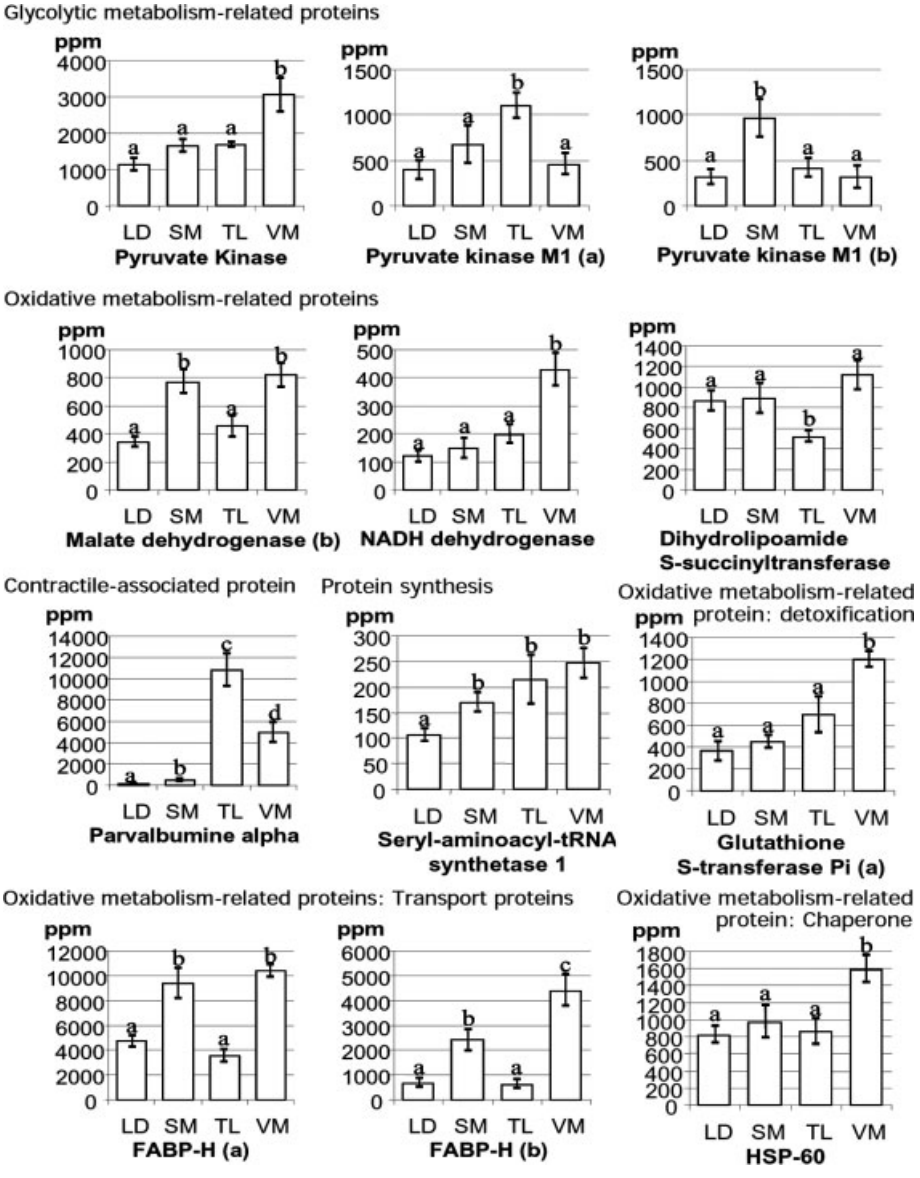


**Figure 2.** Expression profiles of the most representative proteins differentially expressed between muscles. Each bar represents the mean spot volume values (ppm) from five animals in LD, SM, TL, and VM muscles. Letters a, b, c, and d indicate differences between muscles significant at $p<0.05$, if two means have the same letter they are not significantly different.

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mediate patterns. The fatty acid-binding protein plays a role in the uptake of fatty acids by skeletal muscles. It is most abundant in oxidative muscles [17] where fatty acids contribute significantly to oxidative metabolism.

Besides these transport proteins, the majority of the oxidative metabolism-related proteins detected may play a role in the regulation of stress. Some presented a detoxification function. The GST class Pi catalyzes the conjugation of glutathione to potentially toxic compounds such as nitric oxide species (NOS) and reactive oxygen species (ROS) [18]. Aldehyde dehydrogenase and antiquitin (of the aldehyde dehydrogenase family) are required for the clearance of potentially toxic aldehydes derived from lipid oxidation. Peroxiredoxin has antioxidant activity and also presents signal transduction functions. There is little information on the substrate protein of mitochondrial ATP-dependent proteinase SP-22 and haloacid dehalogenase-like hydrolase domain containing 2. ATP-dependent proteinase SP-22 was detected in two isoforms and classified as an antioxidant protein. Haloacid dehalogenase-like hydrolase domain containing 2 was classified as a stress-regulating and defense protein [19]. Proteins involved in cell detoxification were up-regulated in the most oxidative muscle (VM). Oxidative metabolism is known to produce ROS. These, generally very small molecules, are highly reactive due to the presence of unpaired valence shell electrons. Natural ROS byproducts of the normal metabolism of oxygen can damage proteins in cells which are normally able to neutralize them. Interestingly, more glycolytic muscles such as the TL and SM showed high levels of certain detoxifying proteins (peroxiredoxin, antiquitin), thus indicating that they were also subjected to toxic compounds.

The following proteins are chaperones whose function is to repair protein misfolding damage and stabilize protein assembly. The HSP-60 kDa stabilizes proteins and facilitates protein folding and assembly in the mitochondrial matrix. Its expression is increased in skeletal muscle after a prolonged endurance training leading to an increased metabolic demand associated with oxidative fiber-type muscle [20]. The DJ1 enables protein refolding and may also be involved in degradation pathways [21]. The HSP-27 has been reported to play a central role in the structural and functional organization of the 3-D intermediate filament and the actin microfilament system [22]. Neufer and Benjamin [23] reported that the pool of immunologically detected HSP-27 was larger in oxidative fibers and increased in response to an increased oxidative metabolism. The α-crystallin beta subunit is most abundant in tissues with high oxidative capacity, including the heart and type I skeletal muscle fibers [23]. We also detected two isoforms of stress-induced phosphoprotein 1, which mediates the association of the molecular chaperones HSC-70 and HSP-90. In the detoxification protein group, chaperone proteins were up-regulated in the most oxidative muscle (VM), thus confirming that oxidative metabolism is a source of protein denaturation. Except for HSP-27 and α-crystallin, the chaperone proteins displayed relatively high expression in the most glycolytic TL muscle, suggesting that some chaperone proteins are also required in glycolytic muscles.

When they are not repaired, misfolded proteins are degraded by the ubiquitin-proteasome system. Ubiquitin monomers are sequentially attached to the target proteins. These ubiquitin-marked proteins are then digested by the proteasome. Ubiquitin was up-regulated in the most oxidative VM muscle as well as the most glycolytic SM and TL muscles but down-regulated in LD. Finally, we also identified seryl-aminoacyl-tRNA synthetase 1, which showed a similar pattern of expression to ubiquitin and which is involved in protein synthesis. Similarly to ubiquitin and the monomeric form of actin, it may be related to a higher protein turnover.

### 3.2.5 Miscellaneous

In addition, alpha-1-antitrypsin (A1AT) and 20-alpha-hydroxysteroid dehydrogenase (20-αHSD) were detected. A1AT is a member of the serine protease inhibitor protein family (serpins) whose main target is the elastase [24]. Graziadei [25] reported another function for A1AT, because it presents a great affinity to the Transferrin (Tf) membrane receptor, meaning it competes directly with Tf for the receptor site. A1AT was overexpressed in TL muscle.

20-αHSD catalyzes the reaction of progesterone to the inactive form 20 α-hydroxyprogesterone. The ovarian hormone estrogen is known to increase metabolic capacity, particularly fatty acid oxidation. Conversely, progesterone negates this effect. Inactivation of progesterone by 20-αHSD may therefore have consequences on metabolism [26]. The 20-αHSD presented differential expression in the four muscles studied. It was overexpressed in the more oxidative VM and down-regulated in the most glycolytic TL, while SM and LD muscles showed intermediate profiles.

To summarize, protein expression was most clearly differentiated in the VM, where 17 proteins (of which 16 were overexpressed) distinguished it from the other three muscles. The VM showed the highest abundance of enzymes involved in oxidative metabolism and oxidative stress-related proteins. Enzymes involved in the glycolytic pathway were not down-regulated. The muscle showing the least differentiated protein expression was the SM, in which nine spots were differentially expressed. The SM showed intermediate expression patterns for enzymes involved in both glycolytic and oxidative metabolisms. Its main feature was that it presented the highest levels of protein fragments. The TL muscle was characterized by the lowest expression of oxidative pathway enzymes. It should be underlined that except for HSP-27 and α-crystallin, the proteins classified as "oxidative metabolism-related proteins" displayed rela-

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tively high expression, suggesting that most stress proteins were not specific to oxidative metabolism. The LD muscle expressed low abundance of glycolytic enzymes and an intermediate abundance of oxidative enzymes, and was characterized by a relatively low abundance of "stress-related proteins."

## 4 Concluding remarks

This study compared the expression of sarcoplasmic proteins in four different muscles (VM, SM, LD, and TL) which were all relatively rich in fast-contracting fiber types, as is the case in most muscles of farm animals. The TL was fast-glycolytic, the VM was slow-oxidative, and the SM and LD were intermediates. The main finding of the study was that glycolytic metabolism enzymes did not allow muscle differentiation, even in the most glycolytic muscle (TL). Intermuscular differentiation was based on the differential expression of proteins involved in oxidative metabolism, not only enzymes of the citric acid cycle but also other classes of proteins whose functions can be related to oxidative metabolism. The fractionating method led to the detection of many proteins involved in stress regulation, signal transduction, and transcription. These observations suggest that more oxidative muscle fibers are submitted to more stressful conditions and probably present a higher protein turnover. We have shown that proteins involved in cell detoxification, repair of misfolding damage, degradation of impaired proteins, and protein synthesis were more strongly expressed in oxidative muscles.

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