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Proteomic investigation of the effects of weight loss in the gastrocnemius muscle of wild and NZW rabbits via 2D-electrophoresis and MALDI-TOF MS

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Summary

The study of changes within the key agents regulating metabolism during genetic upgrading because of selection can contribute to an improved understanding of genomic and physiological relationships. This may lead to increased efficiencies in animal production. These changes, regarding energy and protein metabolic saving mechanisms, can be highlighted during food restriction periods. In this study, a 20% weight reduction was induced in two rabbit breeds: New Zealand white, a selected meat producer (*Oryctolagus cuniculus cuniculus*), and Iberian wild rabbit (*Oryctolagus cuniculus algirus*), with the aim of determining differential protein expression in the gastrocnemius muscle within control (*ad libitum*) and restricted diet experimental animal groups, using techniques of two-dimensional gel electrophoresis and peptide mass fingerprinting. Results show that L-lactate dehydrogenase, adenylate kinase, β enolase and α enolase, fructose bisphosphate aldolase A and glyceraldehyde 3-phosphate dehydrogenase, which are enzymes involved in energy metabolism, are differentially expressed in restricted diet experimental animal groups. These enzymes are available to be further tested as relevant biomarkers of weight loss and putative objects of manipulation as a selection tool towards increasing tolerance to weight loss. Similar reasoning could be applied to 2D gel electrophoresis spots corresponding to the important structural proteins tropomyosin β chain and troponin I. Finally, a spot identified as mitochondrial import stimulation factor seems of special interest as a marker of undernutrition, and it may be the object of further studies aiming to better understand its physiological role.

Keywords biomarkers of weight loss, energy metabolism enzymes, muscle, proteome, rabbit.

Introduction

Livestock breed selection aims at improving productivities mainly concerning meat, milk and egg production. Studying changes within the key metabolism-regulating agents during genetic upgrading due to of breed selection can contribute to an improved understanding of the genomic and physiological relationships, leading to increased efficiencies in animal production.

Food restriction and seasonal weight loss are the major constraints towards animal production in tropical regions (Almeida *et al.* 2006a, 2007; Almeida & Cardoso 2008a,b). The study of the metabolic changes because of food restriction, which highlight energy and protein metabolic saving mechanisms, can be a useful approach to identify the physiological pathways relevant in breed selection, and to identify genetic biomarkers that could be used for the selection of breeds and varieties with metabolic pathways more capable of energy and nitrogen retention, thus increasing productivity. These mechanisms hasten the whole process of selection and development and ultimately lead to relevant contributions to the improvement of animal husbandry itself, and therefore their general use as a tool in animal selection. This is envisaged to be of special interest in the 21st century (Fadiel *et al.* 2005).

The use of molecular tools in domestic animal selection has been done essentially at the level of genomics; i.e. the study of genomes and the genetic background of several

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animal species with special reference to cattle, swine and poultry. Recently, the use of proteomics; i.e. the study of the proteome (or of the expressed proteins) under certain conditions, has allowed a much greater insight into metabolic mechanisms of a large range of physiological systems. In fact, in domestic animals, proteomics was recently considered to be of significant promise to the development of animal science to meet the challenges of agriculture in the 21st century (Lippolis & Reinhadt 2008). Examples of proteomics studies in farm animals include aspects of disease and immunity (Hughes *et al.* 2007), milk traits (Daniels *et al.* 2006), reproduction (Strzezek *et al.* 2005) and muscle and meat characteristics (Hollung *et al.* 2007).

Amid other physiological aspects (van Harten *et al.* 2003a,b), we have previously studied the effect of weight loss on myofibrillar protein profiles of *Rattus norvegicus* (Almeida *et al.* 2002, 2006b) and domestic goats, *Capra hircus* (Almeida *et al.* 2004, 2008), with particular emphasis on major expressed structural proteins, such as actin or myosin heavy chains (MHC). Recently, the advent of mass spectrometry techniques, with special reference to the PMF (peptide mass fingerprinting) strategy (Hjernø 2007), has allowed a much broader approach to protein identification, revealing biochemical pathways involved in a great number of biological conditions, particularly in well-characterized mammal species such as humans, rats or mice, and to a lesser extent in rabbits (*Oryctolagus cuniculus*).

The goal of this study was to determine differential protein expression in the gastrocnemius muscle of two rabbit breeds subjected to food restriction (FR): wild rabbits (not selected to increased meat productivity) and the well-known meat producer New Zealand white (the most common domesticated breed). This study used techniques of two-dimensional gel electrophoresis and mass spectrometry. The use of the rabbit as a mammalian model will allow the extrapolation of the results to other conventional farm animal species such as poultry, swine, cattle or sheep.

Material and methods

Animals and experimental design

Two breeds of rabbit were purchased from registered breeders at an age of 10–12 weeks. All animals used in the trial were male. The New Zealand white (*Oryctolagus cuniculus cuniculus*) and the Iberian wild rabbit (*Oryctolagus cuniculus algirus*) genotypes were used. The latter breed descends from caught wild animals kept in captivity and that are generally used for the repopulation of reserves. After an acclimatization period of 30 days, all animals were found to be healthy, free from any disease, parasitic or locomotion disorders, and were thus considered to be applicable for use in the trial. Each breed was divided randomly into two weight matched groups: New Zealand Control (NZC; $n = 4$) and Restricted (NZR; $n = 4$), Wild Rabbit Control (WRC; $n = 3$) and Restricted (WRR; $n = 3$). The control groups were fed *ad libitum* and the restricted groups were gradually underfed until their weight was about 80% of their initial weight, 30 days later. This was achieved with a 70% reduction of their *ad libitum* daily ration. The individual feed intake of the four experimental groups was as follows: NZC 190.78 ± 28.5 g; NZR 68.31 ± 5.7 g; WRC 63.13 ± 4.7 g and WRR 38.41 ± 2.0 g. All experimental animals were fed on standard rabbit commercial pellets (Biona 701, Saprogal, Portugal) with the following composition: 14.9% crude protein, 2.7% crude fat, 14% crude fibre and 13% ash. Animals were kept in individual cages (3300 cm^2) using cylindrical ceramic shelter structures as environmental enrichment following recommendations on the care and use of laboratory animals (Baumans & Brain 2006). Animals were allowed free access to water, and bioterium environmental conditions were as follows: temperature (20 ± 2 °C), humidity (70 ± 5%) and kept on a 12-h light/12-h dark cycle. Animal activity was monitored daily by researchers based on individual *in loco* observations of the animals and was found to be very similar for all experimental groups, except during feeding time when restricted animals tended to display a more agitated behaviour.

Control groups were euthanized after 30 days whilst restricted animals were sacrificed once a 20% decrease from the initial live weight was attained. Experimental animals were anaesthetized and euthanized with 4% isoflurane (IsoFlo®, Veterinaria Esteve). Muscle samples (gastrocnemius) were subsequently collected using a scalpel. The effect of post-mortem alterations for cattle (Jia *et al.* 2007) and of freezing temperatures on protein degradation has been described in species such as the pig (Lametsch & Bendixen 2001; Lametsch *et al.* 2002) and cod (Kjærsgård *et al.* 2006). To avoid degradation and clustering of proteins, all muscle samples were snap-frozen in liquid nitrogen immediately upon excision and subsequently stored at −80 °C until extraction, following suggestions by Bouley *et al.* (2004) and Jia *et al.* (2007).

Both European Union (European Legislation n°86/609/CEE) and Portuguese regulations and guidelines on care, use and handling of laboratory animal experimentation were followed. The experiment was monitored by competent veterinary authorities and approved by the Scientific Council of the Faculty of Veterinary Medicine (Lisboa). Authors A.M Almeida, S. van Harten and L.A. Cardoso are holders of a FELASA (Federation of European Laboratory Animal Science Associations) grade C certificate, which enables designing and conducting laboratory animal experimentation in the European Union.

Protein extraction and quantification

Total protein was extracted following the methods described by Bouley *et al.* (2004). In brief, approximately 40 mg of

frozen gastrocnemius muscle was homogenized in 1 ml of extraction buffer (8.3 M Urea; 2 M Thiourea; 1% (w/v) DTT and 2% (w/v) CHAPS), using an Ultraturrax T8 homogenizer (IKA Werke, Staufen, Germany). Subsequently, samples were vigorously shaken for 30 min at 4 °C and centrifuged for 10 min at 10 000 × ***g***.

Protein extracts were kept at −80 °C until use. Protein concentrations for each extract were determined using the 2D Quant kit (GE Lifesciences) following manufacturer's instructions.

Two-dimensional gel electrophoresis

Isoelectric focusing (IEF) followed by SDS-PAGE was used to separate proteins. Samples with 600 μg of protein were diluted to 470 μl in 3-[(3-cholamidopropyl)dimethylammonium]-1-propane sulfonate (CHAPS) (2%, w/v), urea (8 M), IPG buffer pH 3–10 (0.5%, v/v) and dithiothreitol (DTT) (0.02 mM), and were then used for IEF. Electrophoresis was performed according to Görg *et al.* (2000) in the IPGphor system (Amersham Biosciences), using immobiline drystrips of 24 cm with a linear pH gradient from 3 to 10 (Amersham Biosciences). The program consisted of a sequence of six steps starting with 30 V for 12 h followed by 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, a voltage gradient of 30 min until 8000 V and a final step at 8000 V until reaching 50 000 V/h. After IEF, the strips were stored at −80 °C until further use. Before SDS-PAGE, IEF strips were equilibrated in two steps using 10 ml of Tris-HCl (pH 8.8; 50 mM) with urea (6 M), glycerol (30%, v/v) and SDS (2%, w/v) (Görg *et al.* 2000). Dithiothreitol at 10 mg/ml was used in the first and iodoacetamide at 25 mg/ml in the second equilibration steps. Each equilibration step lasted 15 min under slow agitation (Görg *et al.* 2000). SDS-PAGE was performed in 12% (w/v) acrylamide slab gels (20 cm × 25 cm × 1 mm) in a Hoeffer DALT system (Amersham Biosciences). Second dimension gels were run at 125 V in an overnight (14 h) run.

Gels were stained using colloidal Coomasie blue, according to methodology described by Neuhoff *et al.* (1988). Gels were stained for 48 h and subsequently washed three times in double distilled water. Gels were stored at 4 °C in a 20% (w/v) ammonium sulphate solution until image acquisition and band excision. In this experiment, one gel was run per muscle sample per animal in the experimental group.

Digitalization, image and statistical analysis

Digital images of the gels were acquired in an Imagemaster digital scanner and analysed with the IMAGEMASTER PLATINUM software v.5.0 (Amersham Biosciences). Coomassie stained spots were quantified on the basis of their normalized volume: the percentage of the volume of each spot was determined in comparison with total volume for all matched spots, following methodology previously described (Gardini *et al.* 2006). A protein was considered to be present in one experimental group when it was visible in at least 50% of individuals. All spots present in at least three experimental groups were considered for spot excision and identification. Spots found in one or two experimental groups were only considered when the average normalized volume of such spot was over 0.3.

Statistical differences concerning protein expression levels among groups were analysed by one-way ANOVA. The level of significance was set at $P < 0.05$.

Protein identification

Methodology for protein identification through peptide mass fingerprinting has been thoroughly described in previous works conducted in our laboratory (Lamy *et al.* 2008). In summary, protein spots were excised from gels using a scalpel and proteins subjected to in-gel digestion with trypsin (Promega, Madison, WI, USA) according to methodology initially described by Pandey *et al.* (2000) and Gobom *et al.* (1999).

Spots were excised, destained, reduced with dithiothreitol, alkylated with iodoacetamide, and dried in a speedvac. Gel pieces were rehydrated with digestion buffer (50 mM NH_4HCO_3) containing trypsin (6.7 ng/μl) (Promega) and incubated overnight at 37 °C. The buffered peptides were acidified with formic acid, desalted and concentrated using C8 microcolumns (POROS R2, Applied Biosystems). The peptides were eluted with matrix solution that contained 10 mg/ml α-cyano-4- hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid. The mixture was allowed to air-dry (dried droplet method). Mass spectra were obtained with a Voyager-DE STR (Applied Biosystems) MALDI-TOF mass spectrometer in the positive ion reflectron mode. External calibration was made by using a mixture of standard peptides (Pepmix 1, LaserBiolabs). Spectra were processed and analysed with MOVERZ software (Genomic Solutions Bioinformatics). PEAKERAZOR software (GPMAW, General Protein/Mass Analysis for Windows, Lighthouse Data, Odense, Denmark; http://www.gpmaw.com) was used to remove contaminant m/z peaks and for internal calibration. Monoisotopic peptide masses were used to search for protein identification by using MASCOT software (Matrix Science, London, UK; http://www.matrixscience.com). Database searches were performed against MSDB (a non-identical protein sequence database maintained by the Proteomics Department at the Hammersmith Campus of Imperial College, London; http://csc-fserve.hh.med.ic.ac.uk/msdb.html). The following criteria were used to perform the search: (1) mass accuracy of 50–100 ppm; (2) one missed cleavage in peptide masses; and (3) carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications respectively. Criteria used for protein identification in the MASCOT software were (1) significant homology scores achieved in Mascot; (2) sequence coverage values; and (3) similarity between the protein molecular mass calculated

from the gel and for the identified protein. To ensure accuracy and repeatability of identifications obtained by peptide mass fingerprinting, the process was conducted in triplicate for each spot. To assess functions and roles of proteins identified, the website http://www.expasy.org was used.

Results

Animals and weight loss

Animals in the restricted groups lost approximately 20% of the initial body weight. Weight evolution of restricted fed groups is presented in Table S1.

Two dimensional electrophoresis

Examples of two-dimensional electrophoresis (2D) gels obtained in this experiment are set out in Fig. 1. The figure demonstrates that similar amounts of protein were loaded per gel and that electrophoresis was carried out successfully, allowing the separation of proteins according to their pI and molecular mass. The location of the majority of the spots subsequently referred to in this study is also presented in Fig. 1.

Differential expression analysis

The identification of spots detected in only two experimental groups, but lacking statistical significant difference, is presented in Table S2. Only five spots with significant difference between the experimental groups were found: one unidentified (131), actin chain A (156), myosin heavy chains (722 and 740) and finally lactate dehydrogenase chain A (595), which was only found in the restricted fed experimental groups. Relative volumes of spots found in only one experimental group are presented in Table S3. Twenty-one spots were found under such circumstances, including structural and contractile apparatus proteins, such as several actin chains, myosin heavy chains and tropomyosin. Important metabolic proteins like glyceraldehyde-3-phosphate dehy-

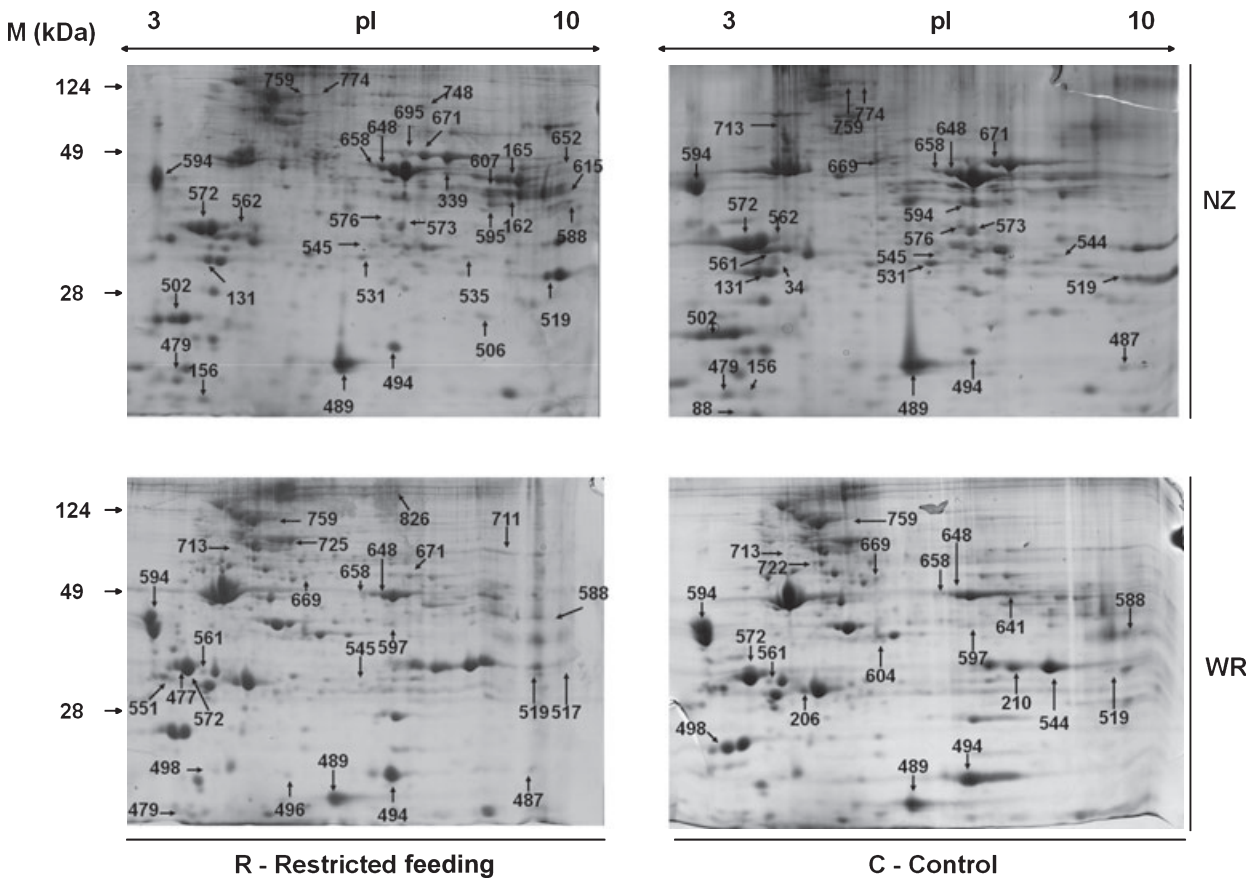

Figure 1 Two-Dimensional gel electrophoresis. Examples of 2D gels obtained for the four experimental groups. Results for the New Zealand white (NZ) breed are presented in the upper row whilst wild rabbit (WR) results are depicted in the lower row. Restricted fed (R) and control (C) animals are presented in the left and right 2D gels, respectively. Molecular mass marker (M) positions in the gel and isoelectric points (pI) are also presented. Isoelectric focusing separation was carried out in 24 cm immobiline dryStrips between pI 3-10. Large format gels were stained with colloidal Coomassie G-250. Arrows and numbers indicate excised spots used for protein identification.

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drogenase, fructose-bisphosphate aldolase or creatine kinase, as well as mitochondrial import stimulation factor from *Rattus norvegicus* (laboratory rat), and four unidentified proteins were also found.

Relative volumes of spots found in at least two experimental groups and showing statistical significant difference between at least two groups are presented in Table 1. These include proteins with an important role in structural and contractile apparatus, as well as relevant metabolism proteins. Fig. 2 highlights three of the latter mentioned spots as an example, depicting an image of the differences in average spot intensities between groups and an image of such spots in the gels. Figure 3 depicts a graphic visualization of some of the most relevant expression profiles for energy metabolism enzymes, with special reference to those involved in the glycolytic pathway, as well as structural proteins.

Protein identification

Data obtained from spectra such as the one exemplified in Figure S1 were used for peptide mass fingerprinting.

An analysis of the spots identified by PMF is summarized in Table 2 (cell structure and contractile apparatus proteins) and Table S4 (metabolism and other proteins). Data include the number of peptides searched in the databases per number of peptides matched with the identified sequence, the putative protein theoretical molecular masses and isoelectric point (pI) of the protein, the percentage of sequence coverage, and the identification score. For the search criteria used, protein scores higher than 68 or 59 (searches in the 'all mammalia' and 'other mammalia' databases, respectively) were considered significant ($P < 0.05$). Tables 2 and S4 also present the species from

Table 1 Normalized volume of spots detected in at least two experimental groups showing differential expression.

Spot reference and identification	NZC	NZR	WRC	WRR
479 myosin heavy chain	0.33^{a} (0.001)	0.34^{a} (0.001)	–	0.14^{b} (0.007)
487 unidentified Protein	0.11^{a} (0.003)	–	0.29^{b} (3.2E-05)	0.11^{a} (0.001)
489 actin f-actin, chain V	4.37^{a} (0.730)	3.04^{a} (0.110)	1.89^{b} (0.280)	1.61^{b} (0.030)
494 unidentified Protein	0.92^{a} (1.110)	0.30^{a} (0.070)	3.39^{b} (0.089)	0.17^{a} (0.001)
496 actin f-actin, chain V	–	–	0.13^{a} (0.001)	0.05^{b} (0.000)
498 actin, chain A – rabbit	0.36^{a} (0.001)	0.29^{a} (0.001)		0.08^{b} (0.001)
502 myosin regulatory light chain 2, skeletal muscle isoform type	0.97^{a} (0.17)	0.86^{a} (0.01)	1.51^{b} (0.107)	1.57^{b} (0.07)
506 creatine kinase	0.18^{a} (0.00)	0.14^{a} (5.78E-06)	–	0.08^{b} (0.00)
517 troponin I	–	0.41^{a} (0.005)	–	0.19^{b} (0.001)
519 adenylate kinase 1	0.21^{a} (0.01)	0.63^{b} (0.001)	0.23^{a} (0.01)	0.53^{b} (0.04)
531 creatine kinase	0.33^{a} (0.006)	0.14^{b} (0.001)	0.13^{b} (0.004)	0.13^{b} (0.001)
544 - triose-phosphate isomerase	0.12^{a} (0.032)	–	3.05^{b} (0.11)	0.08^{a} (0.001)
545 triose-phosphate isomerase	0.13^{a} (4.21E-06)	0.14^{a} (0.001)	0.06^{b} (8E-06)	0.07^{b} (0.001)
561 actin f-actin, chain V	0.59^{a} (0.002)	0.55^{a} (0.05)	0.10^{b} (0.002)	0.07^{b} (0.001)
572 actin f-actin, chain V	8.2^{a} (1.34)	6.32^{a} (0.20)	3.85^{b} (0.55)	3.31^{b} (0.014)
573 Fructose-bisphosphate aldolase C	0.56^{a} (0.001)	0.38^{b} (0.003)	–	0.20^{c} (0.001)
576 creatine kinase (fragments)	0.11^{a} (0.0001)	0.07^{a} (0.0001)	–	0.04^{b} (2.65E-06)
588 unidentified Protein	0.14^{a} (0.006)		0.55^{b} (0.007)	0.13^{a} (0.0001)
594 tropomyosin beta chain,	1.21^{a} (0.01)	$1.97^{a,b}$ (0.23)	2.38^{b} (0.007)	2.56^{c} (0.06)
596 creatine kinase chain M	0.26^{a} (0.0003)	0.29^{a} (0.0002)	–	0.16^{b} (6.13E-06)
597 creatine kinase chain M	0.70^{a} (6.16E-05)	0.48^{b} (0.003)	0.19^{c} (0.01)	0.29^{c} (0.02)
604 unidentified Protein	–	–	0.37a (0.0003)	0.05b (0.0005)
615 glyceraldehyde-3-phosphate dehydrogenase	–	–	0.39^{a} (0.03)	0.81^{b} (0.0002)
648 creatine kinase chain M	1.19^{a} (0.004)	1.76^{a} (0.15)	0.50^{b} (0.07)	0.88^{b} (0.08)
658 creatine kinase chain M	0.20^{a} (0.001)	0.21^{a} (6.59 E-05)	0.13^{b} (0.0001)	0.14^{b} (0.0004)
669 pyruvate kinase	0.30^{a} (0.03)	0.27^{a} (0.004)	0.13^{b} (0.0003)	0.14^{b} (0.04)
671 phosphopyruvate hydratase beta	0.47^{a} (0.11)	0.96^{b} (0.03)	0.36^{a} (0.08)	0.51^{a} (0.10)
711 phosphopyruvate hydratase alpha	0.40^{a} (0.09)	0.25^{a} (0.002)	0.05^{b} (0.001)	0.10^{b} (0.002)
713 actin f-actin, chain V	0.22^{a} (0.0005)	–	0.06^{b} (0.0004)	0.05^{b} (0.0001)
759 skeletal myosin heavy chain	0.29^{a} (0.002)	0.37^{a} (0.005)	0.08^{b} (0.0001)	0.15^{b} (0.002)
774 myosin heavy chain	0.14^{a} (0.0004)	0.30^{b} (8.8E-06)	–	0.27^{b} (0.008)
826 glycogen phosphorylase b	–	–	0.07^{a} (0.001)	0.35^{b} (0.004)

Results are shown in arbitrary units – Normalized Spot Volumes; Values of means and variances are shown between parentheses. Rows with different subscripts indicate statistical significance ($P < 0.05$). NZC (New Zealand white control); NZR (New Zealand white restricted); WRC (wild rabbit control); WRR (wild rabbit restricted).

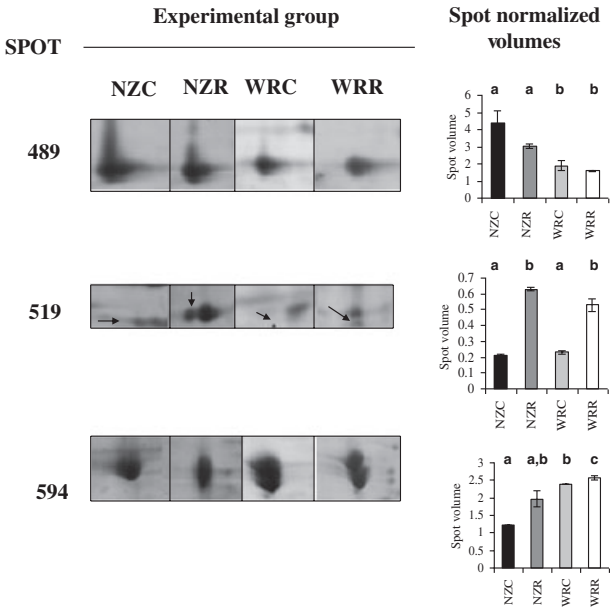

Figure 2 Gel images of spots 489, 519 and 594 in the four experimental groups. For each of the experimental groups, the fraction of the gel depicting the spot is presented. Graphics show normalized spot volumes. Error bars indicate variances. WRR (wild rabbit restricted); WRC (wild rabbit control); NZR (New Zealand white restricted) and NZC (New Zealand white control). Spot 489 was identified as rabbit actin f-actin, chain V, spot 519 as human adenylate kinase and spot 594 as rabbit tropomyosin beta chain. Bars with different superscripts (a,b,c) indicate significant statistical difference ($P < 0.05$).

which the identified protein was found to have the highest homology.

Discussion

Weight loss and atrophy-related pathological conditions have been shown to significantly affect muscle structure and fibre composition in a broad range of species, from laboratory rodents (Goldspink & Ward 1979; Mattson *et al.* 2004; Guderley *et al.* 2008) to production animals like the sheep (Daniel *et al.* 2007). In the latter, calorie restriction resulted in an increase in the numbers of type II fibres in the semitendinosus muscle, whilst in rats and hamsters AT-Pase-high fibres showed a significant decrease as a consequence of starvation (Goldspink & Ward 1979). Although the process is not entirely understood, it is generally accepted that starvation leads to a reduction in fibre size, with fibres IIB being more vulnerable to atrophy, as recently reviewed (Matsakas & Patel 2009). Despite previous studies in rats (Almeida *et al.* 2002) and goats (Almeida *et al.* 2004) pointing to maintenance of the expression of major myofibrillar proteins as a consequence of malnutrition, little information seems to be available on how it influences the above-mentioned characteristics or protein expression.

As different breeds of a particular domestic animal species show different levels of adaptation to weight loss, it is of paramount importance to relate the physiological and biochemical aspects underlying such tolerance for both understanding these processes and ultimately manipulating them for future selection purposes. In this work we have used a proteomic approach to the problem using breeds with different tolerance levels to weight loss (a domestic rabbit breed and its wild type counterpart), and two-dimensional gel electrophoresis and protein identification by peptide mass fingerprinting. The combination of these two methodologies allows a much broader comprehension of the physiological aspects of adaptation to weight loss, as a significant number of proteins are putatively identifiable, with particular relevance to metabolic proteins. The vast majority of the protein spots identified in this study are either energy metabolism-related or structural or contractile apparatus-related; however, other differentially expressed spots have a less obvious connection to these processes.

Energy metabolism proteins–non glycolytic

L-lactate dehydrogenase A chain (EC 1.1.1.27 or LDH-A, in this study corresponding to spot 595) was found only in the experimental groups subjected to weight loss (Table S2). This enzyme is widely distributed in animals and plants and catalyses the inter-conversion of pyruvate to lactate and of NAD^+ to NADH. This enzyme is considered an indicator of muscle breakdown, such as that caused by damage in rabbit myocardium subjected to ischemia (White *et al.* 2005). As the spot identified was only found in experimental groups that had been subjected to weight loss, it can be inferred that the animals in both groups subjected to nutritional stress had clearly initiated a process of muscle catabolism.

Creatine kinase (CK) chain M was detected in multiple isoforms in the four experimental groups (spots 506, 531, 576, 596, 597, 648 and 658; Table 1). This spot was also found in the WRR group (spot 607; Table S3). As can be inferred from the spot with the higher spot relative intensity for CK (spot 648), it seems that this protein is expressed at two different levels. In fact, both wild rabbit groups show relative intensities of about half those recorded for both New Zealand wild rabbit groups, a tendency found in other spots. This enzyme catalyses the conversion of creatine to phosphocreatine, a nitrogenous organic acid that serves as an energy reservoir for the rapid regeneration of cellular ATP stocks (Hamelin *et al.* 2006). This could explain the results obtained for the spots identified as CK between the two breeds: breeds with higher protein deposition, such as the New Zealand white rabbit, would tend to show higher expression of CK than unselected breeds like wild rabbits. Similar results were obtained for double-muscled Texel sheep by comparison with the Romanov breed (Hamelin *et al.* 2006). It was, however, surprising that the relative intensities of CK spots of the control groups were generally

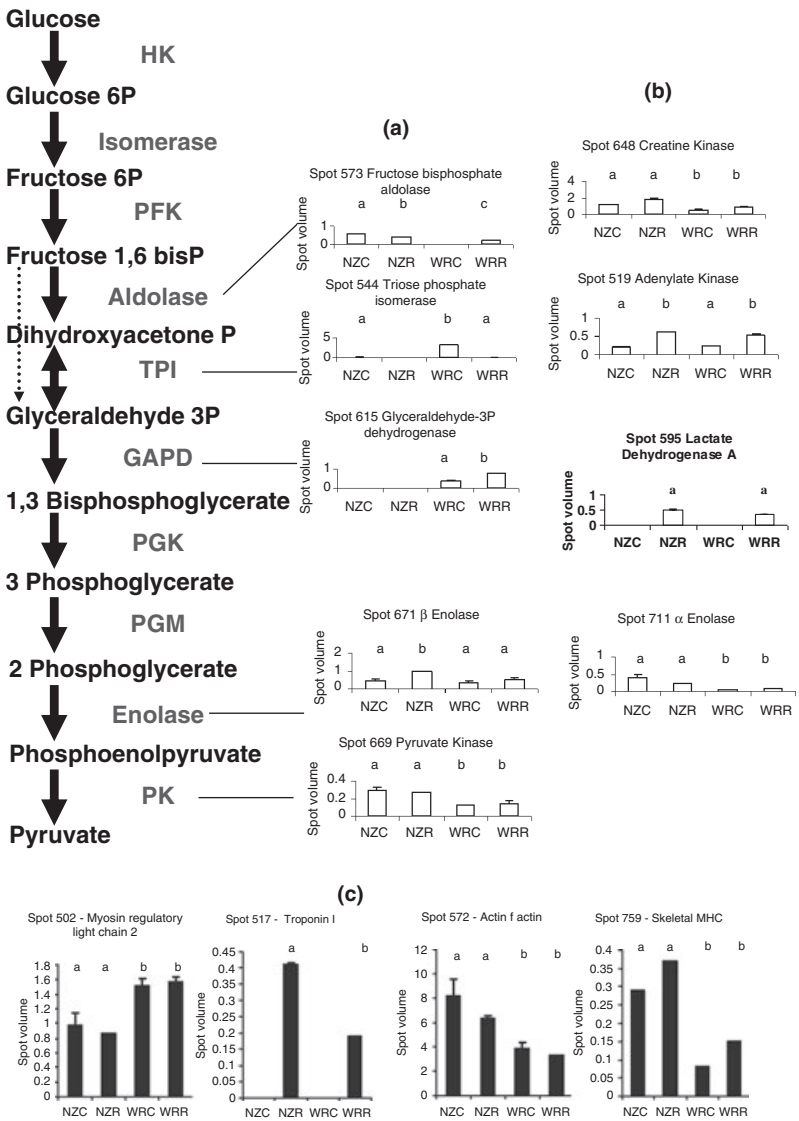

Figure 3 Expression profiles of some of the most relevant energy metabolism and structural proteins found to have significant differences. a – expression profiles of enzymes involved in glycolysis and in the context of that pathway; b – expression profiles of other energy metabolism enzymes; c – expression profiles of structural proteins. Graphics show normalized spot volumes. Error bars indicate variances. WRR (wild rabbit restricted); WRC (wild rabbit control); NZR (New Zealand white restricted) and NZC (New Zealand white control). (a,b,c) Bars with different superscript indicate significant statistical difference ($P < 0.05$). Commonly accepted abbreviations for enzymes in the glycolytic pathway were used: HK (hexokinase); PFK (phosphofructokinase); TPI (triose phosphate isomerase); GAPD (glyceraldehyde-3-phosphate dehidrogenase); PGK (phosphoglycerate kinase); PGM (phosphoglycerate mutase) and PK (pyruvate kinase).

similar to those recorded for the correspondent restricted group. Sun *et al.* (2006) observed that CK expression dropped immediately after muscle atrophy was induced through sciatic nerve injury. Such reduction was followed by a subsequent increase until initial levels were attained. It is therefore likely that under the conditions of our experiment CK levels had dropped after the onset of food restriction and if the experiment had been extended, the initial values would be attained.

Adenylate kinase (AK) is a phosphotransferase enzyme that catalyses the interconversion of adenine nucleotides, and plays an important role in cellular energy homeostasis. Expression of this enzyme has been shown to increase as a consequence of several physiological conditions associated with muscular atrophy, such as ageing in the rat (Doran *et al.* 2008), muscular dystrophy in mice (Ge *et al.* 2003) or temperature drop and hibernation in the *Cynomys leucurus* prairie dog (English & Storey 2000). Analogously, in situations that lead to muscle development, such as the anabolic effects of clenbuterol, AK expression tends to decrease (Burniston *et al.* 2007). Such results are in accordance with the findings presented in our work that show a 3 fold increase in the expression of the spot identified as this enzyme in underfed animals (Table 1, spot 519). It can therefore be concluded that AK is likely to be a valid biomarker for weight loss. These results are of particular importance as the expression seems to increase regardless of the genotype.

Glycogen phosphorylase b plays a central role in the mobilization of carbohydrate reserves in a wide variety of organisms and tissues (Newgard *et al.* 1989) through glycogenolysis. In our trial, a tendency towards a higher expression of the enzyme in wild rabbits was noted [spot 826, Table 1, has a 5-fold expression in the WRR group when compared with WRC for this spot. Another form of the enzyme (spot 341, Table S3) was found in the NZC group].

Table 2 Identification of cell structure and contractile apparatus proteins.

Spot	Peptide search/match	Coverage (%)	M(Da)/pI	Score/Significance level	MSDB database reference; Protein name and species
Cell structure proteins					
156	15/7	14	40878/5.48	70/68	1DB0A actin, chain A - rabbit
206	37/15	40	40878/5.48	124/68	1DB0A actin, chain A - rabbit
212	18/7	29	40878/5.48	76/59	1DB0A actin, chain A - rabbit
269	26/16	30	41640/5.16	125/68	1ALMV actin f-actin, chain V - rabbit
339	48/9	28	41640/5.16	67/59	1ALMV actin f-actin, chain V - rabbit
489	42/9	18	41640/5.16	63/59	1ALMV actin f-actin, chain V - rabbit
496	37/12	34	41640/5.16	118/68	1ALMV actin f-actin, chain V - rabbit
498	45/10	26	40878/5.48	80/59	1DB0A actin, chain A - rabbit
561	34/15	36	41640/5.16	141/68	1ALMV actin f-actin, chain V - rabbit
572	28/13	25	41640/5.16	109/59	1ALMV actin f-actin, chain V - rabbit
713	41/11	34	41640/5.16	90/68	1ALMV actin f-actin, chain V - rabbit
Contractile apparatus proteins					
77	33/11	27	32718/4.69	103/59	TMRBA tropomyosin alpha chain, cardiac and skeletal muscle - rabbit
479	63/21	28	103804/5.27	110/68	Q63939_9MURI myosin heavy chain *Rattus* sp.
502	47/13	49	18997/4.82	111/68	MLRS_RABIT myosin regulatory light chain 2, skeletal muscle isoform type 2 (G2) (DTNB) (MLC-2) rabbit.
517	29/9	31	21241/8.86	74/68	TNNI2_RABIT troponin I, fast skeletal muscle (troponin I, fast-twitch isoform).- rabbit
594	30/14	33	32931/4.66	140/68	TMRBB tropomyosin beta chain, skeletal muscle - rabbit
722	40/14	20	103804/5.27	77/68	Q63939_9MURI myosin heavy chain *Rattus* sp
740	49/19	25	103804/5.27	113/68	Q63939_9MURI myosin heavy chain - *Rattus* sp.
759	46/22	13	223841/5.62	80/59	A59293 skeletal myosin heavy chain - rabbit
774	72/25	31	103804/5.27	131/68	Q63939_9MURI myosin heavy chain.- *Rattus* sp.

M – Molecular Mass; pI – Isolectric point. Protein Score is -10*Log(P), where *P* is the probability that the observed match is a random event. Protein scores larger than either 59 or 68 are considered significant ($P < 0.05$).

Energy metabolism proteins – glycolytic

Protein from spot 573 was identified as fructose bisphosphate aldolase C, an enzyme that catalyses the reaction where fructose 1,6-bisphosphate is broken down into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The C isoform of the enzyme is extremely similar to the A isoform that is more commonly found in muscle tissue (St-Jean *et al.* 2005), In our assay, spot 573 showed that the enzyme had four distinct levels of expression (0.56 for NZC, 0.38 for NZR, absence/non-detection in WRC and 0.20 for the WRR), and additionally another form (spot 165) of relative intensity higher than 0.4 was found for the NZR (Table S3), that, if (putatively) added, would represent higher expression in the NZR group. This pattern of expression seems to indicate a tendency towards higher expression of spots identified as fructose bisphosphate aldolase A in New Zealand white animals when compared with their wild counterparts (0.56 vs. absence/non-detection) and also higher levels of expression in restricted fed animals. Expression of the A form of the enzyme was shown to decrease in animals subjected to muscle growth by the action of growth promoters, such as β_2-adrenegic receptor agonist clenbuterol (Burniston *et al.* 2007). Conversely, an increase in the A form of this enzyme occurs during old age-induced muscle mass loss (Doran *et al.* 2008), which seems to concur with the results obtained in our study, indicating that the enzyme is a valid biomarker of weight loss.

Triose phosphate isomerase (TPI) is an enzyme that catalyses the reversible inter-conversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate, and is considered to be an important housekeeping enzyme (Ationu *et al.* 1999). TPI forms have shown inconsistent patterns of expression as a result of muscle hypertrophy in sheep (Hamelin *et al.* 2006). In our experiment, spots identified as TPI (Table 1, 544 and 545) also showed an irregular pattern of expression, with no correlation with weight loss observed, with the exception of isoform 544.

Glyceraldehyde 3-phosphate dehydrogenase (GAPD) is an ubiquitous glycolitic enzyme that catalyses the sixth step of glycolysis, the oxidative phosphorylation of GAP (glyceraldehyde-3-phosphate) to 1,3-bisphosphoglycerate (BPG), promoting the reduction of NAD^+. In addition to its relevant role in glycolysis, GAPD can act as a DNA binding protein and has important functions in DNA repair (Applequist *et al.* 1995) and in the initiation of apoptosis. In our experiment, spots identified as the GAPD isoform (615) were only found in wild rabbits, with the restricted animals showing a two-fold expression increase when compared

with wild rabbit controls. In addition, other forms of the enzyme were also detected in the NZR group (spot 161, Table S3) and the WRR group (spots 616 and 620). As expression tends therefore to be higher in restricted fed animals, this enzyme is a potentially good weight loss biomarker. Although present in the NZC, it is relevant to notice that expression of the multiple forms of the enzyme in the wild rabbits tend to be higher than in the domesticated breed, a situation that is different to that of all other glycolytic enzymes described.

A spot identified as α Enolase (711) showed higher expression in New Zealand white animals (with similar results for fed and underfed groups), while wild rabbits showed levels of expression of about 20 to 30% of those recorded in the selected breed. Interestingly, spot identified as β Enolase (Table 1, spot 671) showed similar levels for all experimental groups, except the NZR where a 2 fold increase was detected. Enolase (phosphopyruvate hydratase) is a glycolytic enzyme involved in the dehydration of 2-phosphoglycerate to phosphoenolpyruvate (Donoghue *et al.* 2005). Expression levels of several enolase isoforms decrease during chronic low frequency muscle stimulation (Donoghue *et al.* 2005), ageing muscle (Doran *et al.* 2008) and in situations of weight loss followed by compensatory growth of pigs (Lametsch *et al.* 2006). In bovine muscles subjected to post-mortem alterations (Jia *et al.* 2007), in cases of ovine muscle hypertrophy (Hamelin *et al.* 2006) and animals treated with clenbuterol (Burniston *et al.* 2007), enolase levels have been shown to increase. These references seem to demonstrate that lower levels of enolases are logically implicated in a reduced capacity of glycolytic substrate phosphorylation (Donoghue *et al.* 2005) and higher levels of the enzyme are involved in a higher rate of glycolysis to support and maintain ATP production (Jia *et al.* 2007). It is interesting, however, to point out that both α enolase and β enolase expression seem to decrease after a very high initial increase as a consequence of muscle atrophy (Sun *et al.* 2006). It could therefore be plausible that the levels obtained for α enolase in the restricted fed groups (similar to controls) might be a consequence of a descent in protein expression at a later stage of muscle atrophy, induced by weight loss. If the same reasoning is applied to β enolase, it could be speculated that higher expression levels recorded in the NZR group might be because of the fact that these animals are in an earlier stage of the weight loss process when compared with WRR. However, if weight loss hypothetically progresses, it could be expected that β enolase expression would drop to the levels recorded in control animals. It could hence be suggested that both forms of enolase could be used as markers of weight loss metabolism studies, especially in situations of mild forms of weight loss.

Pyruvate kinase (PK) is an enzyme that catalyses the hydrolysis of phosphenol-pyruvate in the second ATP-generating step of the glycolytic pathway, and is of particular importance in energy production as it yields almost 50% of total ATP produced in this metabolic pathway (Zanella *et al.* 2007). The activity of the enzyme was shown to decrease in rainbow trout (*Oncorhynchus mykiss*) as a consequence of food deprivation, it increased upon re-feeding (Polakof *et al.* 2007), and its expression was shown to increase in cases of muscle hypertrophy, such as double muscled Belgian Texel sheep (Hamelin *et al.* 2006) and pathological cardiac hypertrophy of rabbit myocardium (Schott *et al.* 2008). In contrast, PK expression levels decreased (up to 11-fold) in aged atrophied rat skeletal muscle, and it is considered to be a relevant marker of sarcopenia (Doran *et al.* 2008). In this assay, the spot identified as PK (669) showed differential expression, as the selected New Zealand breed had around a 2-fold increase expression compared with the wild rabbits. This situation seems to be in accordance with data published by Hamelin *et al.* (2006). However, no significant differences were found between groups within the same breeds, which seems to indicate that weight loss does not affect PK expression.

The glycolytic pathway is generally considered to be a well-described process with particular relevance to the energy metabolism. It could therefore be expected that, in the context of such an integrated biochemical process, changes in the expression of enzymes involved in the early stages of glycolysis would have repercussions in the expression profiles of downstream enzymes. Consequently, the trend of enzyme expression would likely be similar between experimental groups for all enzymes. Studies on gene expression and enzyme activities in rats subjected to α-glucosidase (Kotler *et al.* 1984) and on obese and normal rats subjected to weight loss (Pérez *et al.* 1998), and cod (*Gadus mohrua*) (Martínez *et al.* 2003) and rainbow trout (Polakof *et al.* 2007) subjected to several nutritional status seem to either confirm or contradict such theory. However, all previously mentioned studies were hindered by the fact that only some of the enzymes were studied. Proteomic and mass spectrometry-based methodologies provide a more thorough view of protein expression as in theory all major changes in the proteome and therefore glycolytic enzyme expression levels are tracked. Proteomic experiments seem to support the theory that not all glycolytic enzymes respond in the same fashion to particular physiological stimuli, including nutritional changes, as most of the references mentioned above indicate. As shown in Fig. 3a, results obtained in our study seem to be in accordance with those previously described. Several roles, other than catalysing glycolysis reactions, have been proposed in recent years (see review by Kim & Dang 2005), particularly transcriptional regulation (GAPD, enolase) and apoptosis (enolase), which possibly explain the results obtained here.

Cell structure and contractile apparatus proteins

Actin was detected in multiple isoform spots in our study. Results indicate that similar expression levels are detected in

control and restricted animals, although expression levels tend to be higher in the New Zealand white group (e.g. spots 572 or 489, Table 1). Six isoforms of Myosin heavy chain, another major contractile protein, were detected in our study. The spots identified as this protein seem to maintain a similar pattern, as expression levels are similar between restricted animals and control animals, although expression in the New Zealand white group is higher than in wild rabbits. This situation could probably be explained by a lower muscular development in the latter. The fact that these major proteins maintain their relative importance and proportions is most likely related to maintenance of the physiological function and contractibility of the muscle as previously demonstrated in our earlier studies (Almeida *et al.* 2002, 2004, 2006b).

Myosin regulatory light chain (MLC; spot 502, Table 1) was found to have differential expression between breeds, although no differences were recorded for between nutritional treatments within the same breed. Bouley *et al.* (2005) detected a 3-fold increase in the expression of myosin light chain in double-muscled bovines (muscle hypertrophy bovine such as the Blanc Bleu Belge breed) when compared with control animals. Lametsch *et al.* (2006) reported similar results for MLC expression in pigs subjected to compensatory growth. Such results seem to indicate that higher levels of muscle deposition, such as those observed in double-muscled animals, correspond to a substantial increase in the expression of MLC. These results seem to contradict ours, as the breed with lower muscle mass deposition showed higher levels of expression of the protein, perhaps as a physiological reaction to overcome the low muscle mass deposition.

Troponin I is part of the troponin complex, with known functions in muscle contractibility. In our study, spots identified as this enzyme were only detected in the underfed groups (spot 517, Table 1), with the NZR showing a two-fold expression when compared with WRR. These results seem to indicate that this protein could be used as a valid biomarker of undernutrition.

Tropomyosin, in association with actin, is a protein of relevance to muscle contraction. We have identified two different chains: tropomyosin β chain (spot 594) and tropomyosin α chain (spots 77 and 758). The spot identified as tropomyosin β chain was found in all experimental groups, with a tendency to have higher expression levels in wild rabbits. In addition, restrictively fed wild animals show higher expression levels than controls. Spots identified as tropomyosin α chain were found in groups NZC and WRR with levels of expression above 0.4. These results seem to indicate that tropomyosin β chain can be used as a marker of weight loss.

Other proteins

Two other proteins that do not belong to the categories previously described were identified as being differentially expressed in the WRR group: the serum albumin precursor AAB58347 (spot 725) from the rabbit and the mitochondrial import factor (spot 551) from the laboratory rat.

AAB58347 is a precursor of serum albumin, a well-known protein involved in the regulation of the colloidal osmotic pressure of blood, with a good binding capacity for water, Ca^{2+}, Na^{+}, K^{+}, fatty acids and hormones.

The characterization of the mitochondrial import stimulation factor L subunit or MSF L (also known as 14-3-3ϵ Protein epsilon) was relatively recent, and information about its role and functions is still rather scarce. It is a protein that belongs to the 14-3-3 family, is present at high levels in the pineal gland, and functions as an adapter protein implicated in the regulation of a large spectrum of both general and specialized signalling pathways. It binds to a large number of partners, usually by recognition of phosphoserine or phosphothreonine motifs (http://www.expasy.org/uniprot/P62260 accessed April the 8th, 2009). The 14-3-3 protein family has been localized in several tissues, but is predominantly localized to the nervous system. However, different isoforms show distinct patterns of spatial, temporal and sub-cellular distribution, for example the ϵ isoform is found in the synaptosomal membranes (Satoh *et al.* 2004). According to these authors, the protein family has been associated with neurodegenerative processes like Creutzfeld-Jacob disease or multiple sclerosis, and it is considered as a relevant marker for such diseases that have characteristics of a generalized loss in body condition or muscle atrophy. The 14-3-3ϵ form has also been found to have an important role in stress response and apoptosis regulation through association with relevant stress proteins like heat shock transcription factor 1 (HSF1) and extracellular signal regulated protein kinase (ERK) in human cell cultures (Wang *et al.* 2004). Such role in stress response might explain why this protein was over-expressed in the WRR group that was the most severely affected by weight loss, as previously explained. Interestingly, and in accordance with what was previously described, another 14-3-3 isoform (14-3-3γ) has been found in porcine muscle as a consequence of post-mortem alterations (Lametsch *et al.* 2006), hence confirming the importance of the protein in apoptosis regulation. To the best of our knowledge, this is the first time that 14-3-3ϵ protein was identified in muscle tissue. However, an analysis of the sequences shows that both isoforms are highly similar in the rat. It is hence plausible that the two isoforms share several roles that are still to be reported or that the identification system is not accurate to the point of distinction between the two isoforms, despite the high coverage attained (47%). This last aspect is of particular relevance, as no information seems to be available regarding the sequences of these protein isoforms in the rabbit or their differences to the rat sequences available in the databases. It can finally be added that, after more

thorough studies, this protein (and isoforms) is likely to be a candidate for a biomarker of weight loss.

Hypothesis, concluding remarks, and future perspectives

To the best of our knowledge, this work constitutes the first proteomics-based approach to the study of protein expression profiles at the muscle level in underfed animals using large format electrophoresis gels. The study is also enriched by comparing two genotypes of rabbits with different tolerance levels to weight loss. Results indicate that differences between breeds are extremely relevant and are most likely a consequence of the domestication process that promoted animals of larger sizes and higher muscle deposition. Such results might be extrapolated to more conventional production animals such as pigs or ruminants. We were able to identify and detect differential expression levels of several spots identified as proteins of relevant metabolic, physiological and structural roles, as well as others less familiar such as mitochondrial import factor. This study also indicates that several muscle proteins have a putative role as valid biomarkers for weight loss andmalnutrition, (lactate dehyrogenase, adenylate kinase aldolase, enolase, troponin and tropomyosin and the mitochondrial stimulation factor) and may be an important tool for studying breed adaptation to weight loss. To further investigate their possible future role as biomarkers, validation studies will need to be conducted. To have a broader view on the physiological processes underlying weight loss and the establishment of proteins for use as biomarkers, especially related to energy metabolism, it would be interesting to conduct a similar experiment using other tissues, with special reference to hepatic tissue. To have additional information on both traditional energy metabolism and other roles of glycolytic enzymes, it would be interesting to conduct a proteomics approach at the level of the mitochondria, which are organelles of major importance to energy metabolism.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1 Example of a mass spectrum obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) for spot 576.

Table S1 Rabbit live weights.

Table S2 Normalized volume of spots detected in only two experimental groups and lacking statistical significance.

Table S3 Normalized volume of spots detected in only one experimental group.

Table S4 Identification of proteins involved in metabolism and other roles.

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