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ORIGINAL ARTICLE

The effect of weight loss on protein profiles of gastrocnemius muscle in rabbits: a study using 1D electrophoresis and peptide mass fingerprinting

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Summary

The study of physiological changes occurring during selection contributes to an improved understanding of relationships leading to efficiencies in animal production. To investigate the effects of food restriction in gastrocnemius muscle protein expression, 20% weight reduction was induced in New Zealand White (meat producing) and wild rabbits, using one-dimensional gel electrophoresis and peptide mass fingerprinting. Lower expression levels of myosin heavy chains were found in the Wild Rabbits Restricted Group, while myosin light chain and α-crystallin proteins were not detected in restricted groups. Glyceraldeyde-3-phosphate dehydrogenase and glycogen phosphorylase expression levels were similar for all experimental groups. Phosphopyruvate hydratase β was not detected in the wild rabbit restricted diet group. Pyruvate kinase levels were 50% lower in the New Zealand Restricted group. LIM protein detection was absent in the control New Zealand group. Results also show relevance of actin in preserving muscle structure in depressed food availability, the sensitivity of both myosin light chain and α-crystallin protein to restricted feed and the role of PK in the resistance of New Zealand rabbits to food restriction.

Introduction

Livestock breed selection aims at improving productivities mainly concerning meat, milk and egg production. The study of physiological changes occurring as a consequence of selective breeding can contribute to an improved understanding regarding genomic relationships, leading to increased efficiencies in animal production.

The study of the metabolic reaction to food restriction (FR), highlighting energy and protein sparing mechanisms, can be a useful approach in defining physiological pathways relating functional genetic consequences of selection. Identification of markers useful for selective breeding for metabolic pathways more capable of energy and nitrogen retention may lead to increased stock productivity and is envisaged to be of capital interest in the 21st century (Fadiel et al., 2005).

The use of molecular tools in domestic animal selection has been carried out essentially at the level of genomics, the study of genomes and the genetic background of several animal species with special reference to cattle, swine and poultry. Recently, the use of proteomics, the study of the expressed proteins (proteome), has allowed a greater insight on the molecular mechanisms at the protein level in a large range of physiological systems in domestic animals. These studies include immune mechanisms (Hughes et al., 2007), milk traits (Daniels et al.,

2006), reproduction aspects (Strzezek et al., 2005) or muscle and meat characteristics (Hollung et al., 2007).

One dimensional gel electrophoresis of muscle protein extracts has been widely used in muscle physiology and meat science for the past two decades as shown in rabbits (Prates et al., 2001), pigs (Gunawan et al., 2008), goats (Gadiyaram et al., 2008), cattle and camels (Soltanizadeh et al., 2008). Amid other physiological aspects (van Harten et al., 2003a,b), we had previously studied the effect of weight loss on myofibrillar protein profiles of *Rattus norvegicus* (Almeida et al., 2002; Almeida et al., 2006a) and domestic goats, *Capra hircus* (Almeida et al., 2004), with particular emphasis on major expressed structural proteins such as actin or myosin heavy chains (MHC) using this particular technique. Recently, the advent of mass spectrometry techniques, with special reference to PMF – peptide mass fingerprinting (Hjernø, 2007) has allowed a broader approach to protein identification of particular importance in well characterized mammal species, such as humans, rat, mice or rabbit. Studies conducted in our group, using one-dimensional gel electrophoresis coupled with PMF techniques, have contributed to the study of the proteome of saliva in mice (da Costa et al., 2008) and small ruminants (Lamy et al., 2008). Such studies demonstrate the importance of the use of these methods and also their applicability to varied physiological studies, such as protein expression profiles under weight loss conditions.

The gastrocnemius muscle is considered to be an excellent model to study muscle plasticity and characteristics under several stresses (Alford et al., 1987) and additionally is of easy access rendering subsequent manipulations easier (Almeida et al., 2006a); it is therefore frequently used in both skeletal muscle physiology driven studies and in economically relevant meat science experiments. The goal of this study is therefore to determine the differential protein expression in the gastrocnemius muscle of two rabbit breeds subjected to FR: wild rabbits, not selected to increased meat productivity and the most common domesticated breed; the New Zealand White, a well known meat producer, using techniques of one-dimensional gel electrophoresis and PMF.

Materials and methods

Animals and experimental design

Two breeds of rabbits 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 rabbits (*Oryctolagus cuniculus algirus*) genotypes were used. The latter refers to rabbits that descend from wild caught animals, kept in captivity and generally used for the repopulation of reserves. After an adaptation period of 30 days, all animals were considered to be healthy, free from any disease, parasitic or locomotion disorders and considered to be apt to be used 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 approximately 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, Vila Chã de Ourique, 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 with environmental enrichment and were allowed free access to water.

Control groups were euthanized after 30 days, whilst restricted animals were sacrificed once a 20% decrease of the initial live weight was attained. Experimental animals were anaesthetized and euthanized with 4% isoflurane (IsoFlo®; Veterinaria Esteve, Bologna, Italy). Muscle samples (gastrocnemius) were collected, snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. Carcass characteristics were obtained following standard methodologies previously described (Almeida et al., 2006b).

Both European Union (European Legislation no. 86/609/CEE) and Portuguese regulations and guidelines on care, use and handling of laboratory animal experimentation were followed. Experiment was monitored by competent veterinary authorities and approved by the Scientific Council of the Faculty of Veterinary Medicine (Lisboa, Portugal). 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.

Insulin and glucose quantifications

Blood was obtained from experimental animals under anaesthesia at the end of the experiment. Serum was separated by centrifugation at 850 *g* (4 °C) for 15 min using a Universal 32R centrifuge (Hettich, Tuttlingen, Germany) and stored in aliquots at −80 °C until further analysis.

Serum insulin concentrations were determined by solid-phase radioimmunoassay using commercial kits of insulin labelled with the isotope I^{125} (Coat-A-Count; Diagnostic Product Corporation, Los Angeles, CA, USA) following the protocols provided by the manufacturer. All samples were assayed in duplicate.

Glucose was determined in plasma using an enzymatic-colorimetric kit from Spinreact (Sant Esteve de Bas, Spain) following the protocols provided by the manufacturer. All samples were assayed in duplicate.

Protein extraction and quantification

Total protein was extracted following the method described by Bouley et al. (2004). Briefly, 40 mg of frozen gastrocnemius muscle was homogenized in 1 ml of extraction buffer (8.3 M Urea; 2 M Thiourea; 1% DTT and 2% CHAPS), using an Ultraturrax T8 homogenizer (IKA Werke, Staufen, Germany). Subsequently, they 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 concentration for each extract was determined using the PlusOne 2D Quant kit (GE Lifesciences, Uppsala, Sweden) following manufacturer's instructions.

One-dimensional gel electrophoresis

A total of 20 μg of protein was separated by 12.5% acrylamide, sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) methodology (Laemmli, 1970) using the Bio-Rad (Hercules, CA, USA) minigel systems. Molecular mass markers (Prestained SDS-PAGE markers #161-0318 from Bio-Rad) were run simultaneously with the samples in each gel to calibrate the molecular masses of the protein sample bands.

Gels were stained using Colloidal Coomasie Blue (Merck, Darmstadt, Germany), 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 preserved at 4 °C in a 20% (w/v) ammonium sulphate solution until image acquiring and band excision.

Digitalization, image and statistical analysis

Digital images of the gels were acquired in an Imagemaster digital scanner (Amersham Biosciences Europe GmbH, Freiburg, Germany). Images were analysed using an adaptation of procedures previously described (Almeida et al., 2002, 2004). The LabImage 1D 2006 software (Kapelan technologies, Halle, Germany) was used. It provides, for each individual band, the relative intensity by comparison to the full intensity of the respective lane. The use of relative intensities allows the correction of putative minor errors that might occur during sample handling or quantification. A protein was considered to be present in one experimental group when visible in at least 50% of individuals.

Statistical differences concerning protein expression levels among groups were analysed by two-factorial ANOVA 2×2 (two breeds vs. two conditions). The level of significance was set at $p < 0.05$. Whenever a significant difference was detected, a *post-hoc* comparison test (Tukey test) was performed. Whenever protein expression level was absent, a one-way ANOVA was performed, comparing the results separately.

Protein identification

Methodology for protein identification through PMF has been thoroughly described in works conducted in our laboratory (Lamy et al., 2008). Summarily, protein bands were excised from gels using a scalpel and proteins subjected to digestion with trypsin (Promega, Madison, WI, USA) according to the methodology initially described (Gobom et al., 1999; Perkins et al., 1999 and Pandey et al., 2000).

Stained bands were excised, washed, 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 (Promega) and incubated overnight at 37 °C. The buffered peptides were acidified with formic acid, desalted and concentrated with C8 micro-columns (POROS R2; Applied Biosystems, Foster City, CA, USA). 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, Sophia-Antipolis, France). Spectra were processed and analysed with MoverZ software (Genomic Solutions Bioinformatics, Ann Arbor, MI, USA). 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). Searches were restricted to mammal entries in the MSDB database.

The following criteria were used to perform the search: (i) mass accuracy of 50–100 ppm; (ii) one missed cleavage in peptide masses; and (iii) 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 (i) significant homology scores achieved in MASCOT; (ii) sequence coverage values; and (iii) similarity between the protein molecular mass calculated from the gel and for the identified protein. To ensure that only one protein was present per band, the Search Unmatched tool from MASCOT software was used. The tool submits to identification through MASCOT the unmatched peaks from the first identification. If no significant identification was achieved through Search Unmatched tool, it was considered that the identified protein was the only protein present in the analysed band.

Results

Animals in the restricted groups lost approximately 20% of the initial body weight. Weight evolution of restricted fed groups is presented in Table 1, as well as major carcass composition characteristics. Insulin and glucose concentrations in the serum are presented in Fig. 1.

Sodium dodecyl sulphate polyacrylamide gel results of the total protein extracts are presented in Fig. 2. A total of 18 different bands (P33, P34, P35, P35a, P36, P37, P38, P38a, P38b, P39, P39a, P39b, P310, P310a, P311, P312, P313 and P314) were found at different molecular weights in the 14 samples loaded. From the above-mentioned proteins, we were capable of successfully identify P33, P34, P35, P37, P38, P38b, P39, P311, P312 and P314, representing a degree of success of 56% in protein identification. Figure 3 depicts all 18 proteins found in this trial, highlighting positively identified ones.

From band in-gel digestion with trypsin and subsequent analysis through PMF, spectra such as the one presented in Fig. 4 were obtained.

Data obtained from PMF analysis of the bands positively identified with significant MASCOT scores are summarized in Table 2. Data include the number of peptides searched in the databases per number of peptide matched, the putative protein theoretical molecular weight and the iso-electric point (pI) and the percentage of coverage. Table presents also the score of the identification, where protein score is $-10*\mathrm{Log}(P)$ and P is the probability that the observed match is a random event. For the searched criteria, used protein scores >68 were considered significant ($p < 0.05$). Table 2 presents the protein name and species from which the protein to be identified was found to have the highest resemblance.

Table 1 Rabbit live weight evolution and carcass composition

	NZC	NZR	WRC	WRR
Live weight day 0 (g)	$3817.29^{a} \pm 110.31$	$4296.79^{a} \pm 57.26$	$840.96^{b} \pm 52.83$	$860.54^{b} \pm 21.03$
Live weight day 30 (g)	$4322.70^{a} \pm 79.45$	$3539.25^{b} \pm 84.35$	$1002.06^{c} \pm 52.83$	$678.46^{d} \pm 16.99$
Growth/decrease (g)	$505.46^{a} \pm 47.56$	$-757.55^{b} \pm 46.22$	$161.30^{c} \pm 38.10$	$-182.08^{d} \pm 13.90$
Live weight day 30 (% LW day 0)	116.85	82.30	105.37	78.93
Carcass yield (% live weight)	$58.56^{a} \pm 0.71$	$54.28^{b} \pm 0.45$	$56.31^{a,b} \pm 1.24$	$48.11^{c} \pm 1.76$
Dry matter (%)	$36.40^{a} \pm 1.18$	$30.88^{b} \pm 1.01$	$32.04^{a,b} \pm 1.57$	$27.28^{b} \pm 0.38$
Crude protein (% DM)	$53.62^{a} \pm 2.63$	$69.97^{b} \pm 5.50$	$69.79^{b} \pm 2.70$	$87.56^{c} \pm 1.76$
Crude fat (% DM)	$46.38^{a} \pm 2.63$	$30.03^{b} \pm 5.50$	$30.21^{b} \pm 2.70$	$12.44^{c} \pm 1.76$

NZC, New Zealand White control; NZR, New Zealand White restricted; WRC, wild rabbit control; WRR, wild rabbit restricted; DM, dry matter.
Values are mean ± SEM.
[a,b]Values within the same line that do not share the same superscript letter are significantly different ($p < 0.05$).

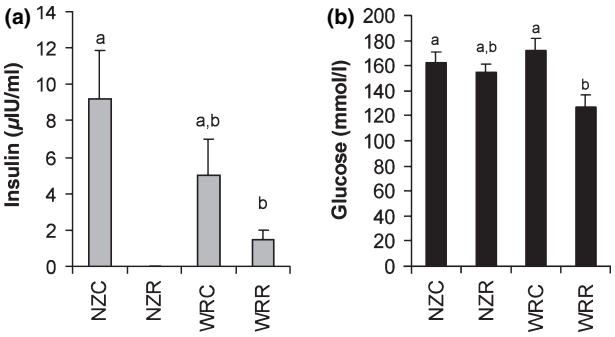

Fig. 1 Insulin and glucose serum levels in the four experimental groups. (a) Insulin (mIU/ml) and (b) glucose (mmol/l). Bars with different superscripts (a,b) indicate significant statistical differences ($p < 0.05$). WRR (wild rabbit restricted); WRC (wild rabbit control); NZR (New Zealand White restricted) and NZC (New Zealand White control). Error bars indicate standard error of mean.

Significant scores were attained for all proteins identified ranging from 72 to 133 for a significance score of 68, required as minimal for MASCOT searches conducted in the MSDB database restricted to mammals.

Proteins considered to be positively identified were compared through statistical analysis for differential expression analysis. Data are presented in Table 3. Proteins bands that showed to have different expression in at least one of the four experimental groups are indicated (P33, P37, P39, P311, P312, P314 and P35) as well as the three remaining proteins that showed to have the same level of expression (P34, P38 and P38b). Results for pyruvate kinase (P35) and β-enolase are highlighted in a graphic presented in Fig. 5.

Discussion

Seasonal weight loss is one of the most serious limitations to animal production in tropical and Mediterranean environments. 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 the process and ultimately manipulate it for hypothetical future selection purposes. In this work, we have conducted a proteomic approach to the problem using two different breeds: a domestic rabbit breed and their wild type counterpart, using one-dimensional gel SDS electrophoresis and protein identification by PMF. These two techniques allow a much broader comprehension of the physiological aspects of adaptation to weight loss than other methodologies previously used in our laboratory, regarding simple myofibrillar protein profiling (Almeida et al., 2002, 2004) as more proteins are putatively identifiable, with particular relevance proteins involved in important metabolic pathways.

The NZ breed presented a decrease in the insulin levels when subjected to FR, which reached values below the detection level in the restricted group, while no influence of FR was detected in the WR breed. The results relatively to the NZ breed agree with a study effectuated in rabbits, in which insulin levels were lower in food restricted animals than in *ad libitum* (Rommers et al., 2004). Similar results were obtained by Brecchia et al. (2006) also in fasted rabbits. Our results of the NZ breed relatively to the low values of insulin may reflect on our glucose results. One of the effects of insulin is to lower blood glucose levels. With non-existent insulin levels, blood glucose values tend to be elevated as observed in our study. The clear reduction in our results of insulin might be because of the reduced availability of carbohydrates. Results for insulin and with particular reference to glucose provide indications on the physiological status of the experimental animals in what concerns weight loss. Similar information can

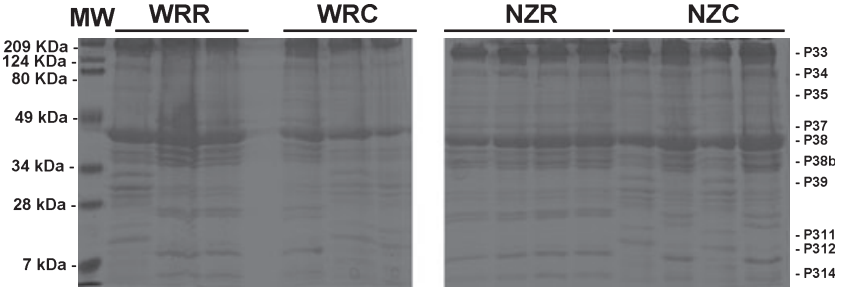

Fig. 2 Electrophoresis of total protein extracts from the gastrocnemius muscle of the experimental groups: WRR (wild rabbits restricted); WRC (wild rabbit control); NZR (New Zealand White restricted) and NZC (New Zealand White control). Molecular Weight Markers are also presented (MW), as well as proteins identified. Staining performed with Coomassie colloidal blue and 20 μg of protein extract was loaded per lane.

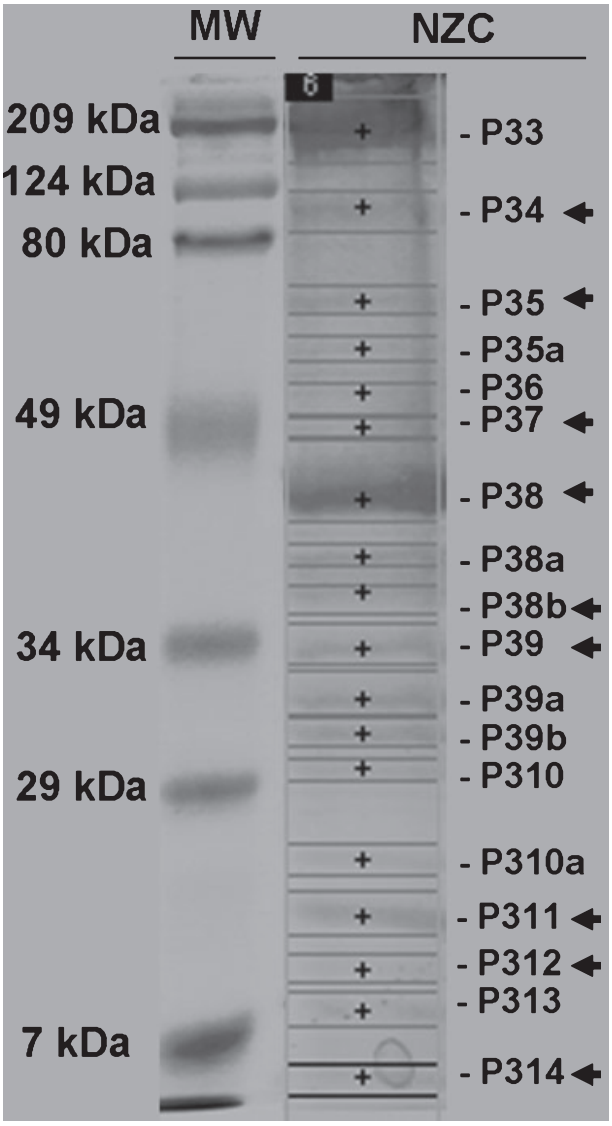

Fig. 3 One Dimension Electrophoresis of protein extract from the gastrocnemius muscle of a New Zealand White control (NZC) animal – proteins found in the sample. Both unidentified and identified proteins are presented. The latter are highlighted with a black arrow. Each band has a perimeter selected defining the region of the lane from which relative areas were calculated in the LabImage software. Molecular weight markers (MW) are also presented. Staining performed with Coomassie colloidal blue and 20 μg of protein extract was loaded.

be obtained from the live weight loss recording and the carcass composition data presented.

Protein P38 was identified as being actin (chain V) of *Oryctolagus cuniculus*. Actin is one of the most important proteins in muscle as it plays a vital role in muscle contractibility. As expected, relative intensities obtained in our study (approximately 20) indicate that actin is one of the most abundant proteins in muscle extract. Similar levels for actins relative intensities were found between the four experimental groups indicating that this major protein maintains its relative importance to maintain subsequently the physiological function as demonstrated in our earlier studies (Almeida et al., 2002, 2004, 2006a).

Like actin, MHC (identified in our study as P33) is also an extremely important protein with a relevant and well-known role in muscle contraction. Interestingly, our results show that all groups had similar levels of MHC, with the exception of the WRR group that showed values of approximately 75% of those obtained for the other groups. These results are an indication that weight loss had noticeable effect on the WRR group. In fact, the expression of structural proteins with a relevant role in muscle contraction is severely affected. Under normal feeding conditions, wild rabbits tend to have less accumulation of fat depots and muscle deposition when in relation to fed normally New Zealand White rabbits. It is therefore likely that under situations of weight loss, WRR animals would more likely loose weight at a faster pace and hence the catabolism and biosynthesis of major structural proteins like MHC would be affected at an earlier stage.

Two forms of myosin light chain (MLC; P312 and P314) were found to have differential expression levels between control and restricted groups of the two breeds. In fact, MLC bands were not detected in the restricted groups, possibly indicating a very low level of expression to be detected using small amounts of protein loaded. Bouley et al. (2005) detected a threefold increase in the expression of MLC 3 skeletal muscle isoform (reference P314), in doubled-muscled bovines (muscle hypertrophy bovine such as the *Blanc Bleu Belge* breed) when in relation to 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 be in accordance with ours as, analogously, it could be inferred that when an animal is losing weight and hence depleting muscle mass, the expression of MLC will necessarily be lower. As MLC has an important role in the structure of the muscle fibre (Zubay, 1998; Lametsch et al., 2006), it can be concluded that a decrease in MLC expression in restricted animals would strongly affect muscle function with

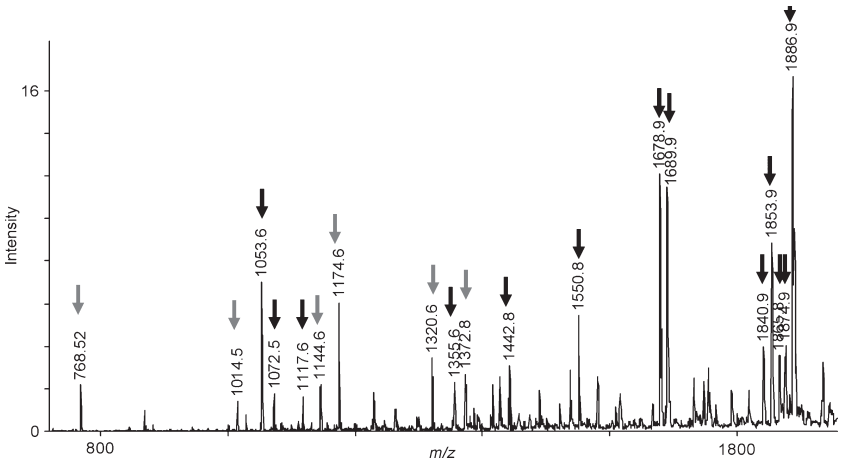

Fig. 4 Mass Spectrum obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) technique for band P34. The monoisotopic mass of each individual peak considered is marked and used for protein identification by peptide mass fingerprinting using the mascot program. Peaks submitted are marked with an arrow (peptides Searched), black for matched and grey for unmatched peptides. As shown in Table 2, protein P34 was identified as 2GPB glycogen phosphorylase from rabbit.

Table 2 Protein identification

Protein reference in the study	Peptide search /match	Theoretical molecular weight (kDa)/pI	Coverage (%)	Score*	Identified protein and MSDB database reference	Species
P33	21/16	223/5.6	9	124	Myosin heavy chain (A59293)	*Oryctolagus cuniculus*
P34	19/13	96/6.5	17	133	Gycogen phosphorylase (2GPB)	*Oryctolagus cuniculus*
P35	33/13	57/7.7	29	105	Pyruvate kinase chain A (1A49A)	*Oryctolagus cuniculus*
P37	33/8	47/7.7	29	72	Phosphopyruvate hydratase β (or β-enolase) (A37210)	*Oryctolagus cuniculus*
P38	21/10	41/5.1	37	121	Actin, chain V (1ALMV)	*Oryctolagus cuniculus*
P38b	32/9	35/6.9	24	72	Glycerladehyde-3-phosphate dehydrogenase (DEPGG3)	*Sus scrofa*
P39	23/8	33/8.7	37	86	LIM protein (gil4894849) (AAD3624)	*Rattus* sp.
P311	40/8	20/6.7	36	86	α-Crystalin chain B (A53871)	*Oryctolagus cuniculus*
P312	18/9	17/4.5	38	94	Myosin light chain slow skeletal muscle (A61567)	*Oryctolagus cuniculus*
P314	26/8	16/4.6	41	72	Myosin light chain 3, skeletal muscle isoform (MLC3F)	*Oryctolagus cuniculus*

*Protein score is −10*Log(*P*), where *P* is the probability that the observed match is a random event. Protein scores >68 are significant ($p < 0.05$).

special reference to contractility. No differences were recorded between rabbit breeds, a suggestion that under such levels of weight loss and regarding MLC levels of expression, both breeds are equally affected by weight loss. Finally, it can be inferred that MLC forms can be considered as markers of weight loss.

Protein P38b was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). According to Cowan-Jacob et al. (2003), GAPDH is an ubiquitous glycolitic enzyme that catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate using the cofactor NAD^+. Additionally, to its relevant role in glycolysis, GAPDH can act as a DNA binding protein and has important functions in DNA repair (Applequist et al., 1995). In our experiment, GAPDH relative intensities were similar for all experimental groups indicating that at the gastrocnemius muscle level and under the conditions of the experiment there are no changes visible in this step of glycolysis.

B-enolase (phosphopyruvate hydratase β) is a glycolytic enzyme involved in the dehydration of 2-phosphoglycerate to phosphoenolpyruvate (Donoghue et al., 2005). Levels of expression of the

Table 3 Protein expression level comparison

Protein	NZC	NZR	WRC	WRR
P33 (myosin heavy chain)	24.92[a] ± 1.86	25.79[a] ± 2.32	28.19[a] ± 2.02	19.65[b] ± 1.33
P34 (2GPB glycogen phosphorylase)	7.71 ± 0.51	7.83 ± 0.49	6.53 ± 1.16	7.39 ± 0.68
P35 (pyruvate kinase chain A)	4.80[a] ± 0.37	2.45[b] ± 0.20	3.82[ab] ± 0.21	4.21[a] ± 0.50
P37 (phosphopyruvate hydratase β or β-enolase)	4.59[a] ± 1.07	4.96[a] ± 0.37	3.88[a] ± 1.17	Not detected
P38 (1ALMV actin, chain V)	20.06 ± 1.16	22.66 ± 0.22	19.17 ± 0.48	19.54 ± 1.23
P38b (glyceraldehyde-3-phosphate dehydrogenase)	4.05 ± 0.63	5.88 ± 0.47	4.77 ± 0.48	5.83 ± 0.33
P39 (LIM protein – gi|4894849)	Not detected	4.81[a] ± 0.35	3.74[a] ± 0.05	6.06[a] ± 0.98
P311 (α-crystallin chain B)	2.00[a] ± 0.32	Not detected	2.20[a] ± 0.53	Not detected
P312 (myosin light chain slow skeletal muscle)	2.47[a] ± 0.79	Not detected	3.76[a] ± 0.13	Not detected
P314 (myosin light chain 3, skeletal muscle isoform)	1.48[a] ± 0.09	Not detected	2.54[a] ± 0.36	Not detected

WRR, wild rabbit restricted; WRC, wild rabbit control; NZR, New Zealand White restricted; NZC, New Zealand White control.
Relative intensities results are shown in arbitrary units – values are mean ± SEM.
[a,b]Rows with different superscripts indicate statistical differences ($p < 0.05$).

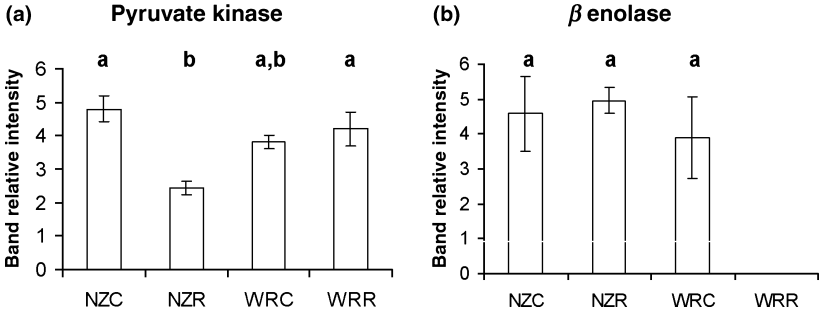

Fig. 5 Expression of pyruvate kinase (a) and β-enolase (b) in the four experimental groups. Bars with different superscripts (a,b) indicate significant statistical differences ($p < 0.05$). WRR (wild rabbit restricted); WRC (wild rabbit control); NZR (New Zealand White restricted) and NZC (New Zealand White control). Error bars indicate standard error of mean.

protein were demonstrated to decrease in several physiological situations namely 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) and in cases of ovine muscle hypertrophy (Hamelin et al., 2006), β-enolase levels increased. These references seem to demonstrate that lower levels of β-enolase 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). Similarly, pyruvate kinase chain A (PK – labelled as P35 in our study) is an enzyme that catalyses the reaction 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), hydrolizing phosphoenolpyruvate. This reaction has the most negative free energy among the phosphorylated of the central pathways (Zubay, 1998). In agreement with the previously stated, the activity of the enzyme was shown to decrease in rainbow trout (*Oncorhynchus mykiss*) as a consequence of food deprivation and increased upon re-feeding (Polakof et al., 2007) and its expression shown to increase in cases of muscle hypertrophy such as double muscled Belgian *Texel* sheep (Hamelin et al., 2006) and pathological cardiac hypertrophy of the rabbit's myocardium (Schott et al., 2008). Oppositely, PK expression levels decreased (up to 11-fold) in aged atrophied rat skeletal muscle and is considered a relevant marker of sarcopenia (Doran et al., 2008).

Results found in the literature and previously stated indicate that the expression of both PK and β-enolase would be down-regulated in the restricted fed groups in both wild and domesticated rabbits. Our results showed similar β-enolase levels of expression in the NZC, NZR and WRC groups, while no expression was detected in WRR rabbits. This implies a likely higher sensitivity of wild rabbits to weight loss. In fact, as previously stated and because of a lower deposition of fat and muscle under normal feeding circumstances, wild rabbits are more likely prone to muscle catabolism at a more initial phase of weight loss. Whilst, for the same stage of weight loss, NZR animals would still be degrading body reserves and not important structural proteins, hence explaining our results for β-enolase. Such results seem therefore to be in accordance with our results obtained for MHC. Conversely, PK results are contrary to what would be expectable, as significantly lower levels expression were indeed detected in NZR animals. These interpretations must however also take into consideration the equilibrium composition of the three carbon phosphorylated acids the 'third metabolic pool': glycerate-3-phosphate, glycerate-2-phosphate and phosphoenolpyruvate, which are approximately 80%, 13% and 7% respectively (Zubay, 1998). Additionally, the glycolytic proteins identified in this study represent a mere fraction (30%) of all enzymes involved in this metabolic pathway (Zubay, 1998). It seems therefore to be of interest to conduct an approach that would enable us to evaluate expression levels of the remaining enzymes to obtain a more complete picture of the process. Such approach would imply working with higher amounts of protein (for instance large format gels) or protein separation through two-dimensional gel electrophoresis.

Protein P34 was identified as glycogen phosphorylase. This enzyme plays a central role in the mobilization of carbohydrate reserves in a wide variety of organisms and tissues (Newgard et al., 1989) through glycogenolysis. Interestingly and similarly to GAPDH, results obtained in this experiment for Glycogen Phosphorylase were statistically similar for all experimental groups with relative intensities of approximately seven. It would have been expected that as both enzymes are involved in the breakdown of body reserves, relative intensities would be higher in WRR and NZR groups by comparison with the control groups. As such differences were not noted and it could be concluded that glycogenolysis are stable processes in the study conditions. Muscle is a relatively stable structure and depletion of body reserves at the muscle level occurs at higher FR stages when in relation to other more relevant tissues, notably the liver. It would therefore be interesting to conduct a similar study using other organs and tissues with special relevance to hepatic tissue.

Similarly to MLC, no expression was recorded in underfed groups regarding α-crystallin chain B, also known as αB-crystallin (P311). According to Clark and Muchowski (2000), members of the family of small heat shock proteins contain a core domain named α-crystallin, composed of two subunits (αA-Crystallin and αB-Crystallin) that form large complexes and function as molecular chaperones. It is abundantly expressed in muscle tissues where it also functions as a molecular chaperone and is an important regulator of apoptosis (Kamradt et al., 2002). In accordance with the above stated, results by Jia et al. (2006) and Park et al. (2007) regarding protein expression during carcass maturation in bovine and pig muscle, respectively, demonstrate a major increase in the expression of αB-crystallin, as well as other heat shock proteins. Such results were however obtained in muscle samples excised from carcasses a few hours after slaughter. After experimental sciatic nerve injury, Sun et al. (2006) determined a significantly important down-regulation in αB-crystallin expression, which could be related to progressive muscle atrophy such as the one induced by the experimental conditions. Such results are in accordance with those recorded in our experiment but are apparently contrary to the role attributed to αB-crystallin by Kamradt et al. (2002) that would suggest an increase in the expression of this protein when muscles or organisms are subjected to stresses. An explanation might be found in the work of White et al. (2005) that studied the proteomics of ischemia/reperfusion injury in rabbit myocardium muscle. These authors detected an increase in the expression of αB-crystallin in the initial phases after muscle injury followed by an expression decrease below control levels in latter stages. Such decrease is related with the removal of αB-crystallin from the cell caused by a translocation of the protein to the Z line of the myocardium from the cytosol (White et al., 2005). It could therefore be expected that in the initial phases of weight loss, over-expression of αB-crystallin might have occurred followed by a significant subsequent decrease in the latter stages of undernutrition. Our results demonstrate the role of αB-crystallin as a relevant marker of weight loss.

Contrary to all proteins previously mentioned, LIM protein (P39) is a relatively new family of

proteins. The term LIM derives from the first letter of each of the three genes from which the motif was first discovered. Their function is still unknown; however roles in facilitating protein-protein interactions, transcription regulation and especially driving muscle hypertrophy were proposed (Morgan et al., 1995). Results regarding gene transcription profiling in the muscle of *Duroc* (high growth and muscle deposition breed) and *Taoyuan* (low growth and muscle deposition breed) pigs seem to confirm such results (Lin and Hsu, 2005) as higher levels of LIM expression were detected in the first breed. Such results are contradictory to ours as we have not detected any expression in NZC rabbits and all other groups had similar expression levels. Because ofthe relatively novel nature of this family of proteins, information does not abound, especially regarding possible existence of forms or fractions of the protein, therefore an attempt to explain the results obtained would be very speculative.

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. Such results might be extrapolated to production animals such as pigs or ruminants. The study leads to interesting results as we were capable of identifying and detecting differential expression levels of several proteins with relevant metabolic/physiological (enolase, PK) and structural (MHC, MLC, actin) roles, as well as others less familiar such as LIM protein and α-crystallin chain B. This study also indicates that several muscle proteins can be used as valid markers for weight loss and undernutrition, hence an important role as tool in studying breed adaptation to weight loss. The study is also enriched by the possibility of comparing two different genotypes of rabbits that show apparent different tolerance levels to weight loss. Regarding this last aspect, it is to highlight the following: MLC and α-crystallin where not detected in the restricted groups of both breeds; phosphopyruvate hydratase β was not detected in the wild rabbit restricted diet group; pyruvate kinase levels were 50% lower in the NZR group and LIM protein detection was absent in the control New Zealand group. These results can be considered as a consequence of the selection process that has led to a higher tolerance of New Zealand rabbits to weight loss.

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