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3 # Tackling proteome changes in the *longissimus thoracis*
4 # bovine muscle in response to pre-slaughter stress

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25 ABSTRACT

26 Pre-slaughter stress has adverse effects on meat quality that can lead to the
27 occurrence of Dark Firm Dry (DFD) meat in cattle. This study explores the previously
28 uncharacterized proteome changes linked to pre-slaughter stress in the *longissimus*
29 *thoracis* (LT) bovine muscle. Differential proteome profiles of DFD and normal (non-
30 DFD) LT meat samples from male calves of Rubia Gallega breed were assessed by 2-
31 DE coupled to MS analysis (LC-MS/MS and MALDI TOF/TOF MS). A total of seven
32 structural-contractile proteins (three different myosin light chain isoforms, two fast
33 skeletal myosin light chain 2 isoforms, troponin C type 2 and cofilin-2) and three
34 metabolism enzymes (triosephosphate isomerase, ATP synthase and beta-galactoside
35 alpha-2,6-sialyltransferase) were found to have statistically significant differential
36 abundance in sample groups. In addition, 2-DE in combination with the
37 phosphoprotein-specific fluorescent dye Pro-Q DPS revealed that highly
38 phosphorylated fast skeletal myosin regulatory light chain 2 isoforms underwent the
39 most intense relative change in muscle conversion to DFD meat. Therefore, they appear
40 to be the most sensitive biomarkers of stress just prior to slaughter in Rubia Gallega.
41 Overall, these findings will facilitate a more integrative understanding of the
42 biochemical processes associated with stress in cattle muscle and their effects in meat
43 quality.

44

45 *Keywords*: Pre-slaughter stress biomarkers, *Bos taurus*, DFD meat, *Longissimus*
46 *thoracis*, Proteome, Relative change measure

47 1. **Introduction**

48 Pre-slaughter stress (PSS) is a very complex trait conditioned by a variety of
49 endogenous (e.g. sex, genetics and age) and exogenous animal factors linked to pre-
50 slaughter transport and handling activities [1,2]. Exogenous animal stressors include
51 practices of loading and unloading, human presence, changes in social structure through
52 separation and mixing with strange animals, feed and water deprivation during
53 transportation, lairage in slaughter house and exposure to new and unfamiliar
54 environment. PSS has serious adverse effects on animal welfare [3,4] and causes
55 detrimental effects to the biochemical processes that occur during the transformation of
56 muscle into meat [5,6]. Thus, exposing cattle to PSS can lead to the formation of Dark
57 Firm Dry (DFD) meat due to depletion of muscle glycogen reserves and the
58 accumulation of lactic acid that alters the normal process of post-mortem acidification
59 of meat, affecting decisive factors of final meat quality such as tenderness, juiciness,
60 color and flavor [5-7].

61 DFD meat occurs when the ultimate pH post-mortem measured after 12-48
62 hours is higher than 6.0 [8]. The elevated pH is associated with relatively little
63 denaturation of proteins, water is tightly bound and little or no exudates are expelled
64 [6]. Consequently, this type of meat has poor processing characteristics, darker color
65 [9], great variations in tenderness and high water-holding capacity [10] and high
66 potential of spoilage at an early age [11] compared to normal meat. It can also be
67 inferior in flavor and consumer acceptability [12]. The incidence of DFD in meat varies
68 markedly over different countries, often with rates of about 10%, and is the cause of
69 large economic losses [5,8,13,14].

70 A number of biochemical parameters, indicators of perimortem (plasma and/or
71 urinary levels of cortisol, adrenaline, noradrenaline, catecholamines, etc.) and post-
72 mortem muscle metabolism (pH, lactate, glycogen, etc.), are traditionally used to
73 evaluate the stress status of animals at slaughter [15-17]. However, the occurrence of
74 DFD meat provides an excellent opportunity to obtain insight into the structural and
75 regulatory proteins that directly affect muscle in response to PSS and their influence on
76 meat quality. The use of proteomics has opened new and promising avenues for tackling
77 control quality and product safety in the meat industry [18-20]. Reference map of beef
78 muscle proteome and its differentiation over different scenarios have already been
79 established on the basis of 2-DE coupled with MS analyses [21-27]. Intensive proteomic
80 and functional analyses have been particularly addressed to assess the biochemical
81 mechanisms underlying tenderization processes of meat, one of the most important
82 factors contributing to meat quality [19,22,25,26,28-31]. To our knowledge, no previous
83 systematic research has been addressed to evaluate proteome changes due to stress with
84 adverse effects on meat quality in cattle.
85 This study is a first attempt to unravel stress-dependent proteome changes in
86 beef muscle. Specifically, we have studied proteome changes in the *longissimus*
87 *thoracis* muscle in response to PSS from male calves of the Rubia Gallega breed. This
88 study opens the way towards a better understanding of the molecular mechanisms
89 linked to stress in cattle and their accompanying effects on meat quality.

90 **2. Materials and methods**

91 2.1. Animals, sample preparation and experimental design

92 Proteome changes linked to PSS in cattle were studied in animals that
93 experienced the usual practices in the Spanish beef industry. The occurrence of DFD

94 meat was used as indicator of animals affected by PSS. A total of four biological
95 replicates of DFD and control (normal or non-DFD) meats from male calves of Rubia
96 Gallega breed (Spain) were used in this study. Meat samples were selected according to
97 discriminatory meat quality parameters between DFD and normal meat, in a total of 76
98 male calves. Therefore, the incidence of DFD meat in our study was 5.3%. Animals with
99 a mean age of 10 months were transported from family farms to the abattoir the day
100 before slaughter, stunned with a captive bolt, slaughtered and dressed according to
101 European Union regulations (Council Directive 93/119/EEC), in an accredited abattoir
102 (Lugo, Spain). Control samples were obtained from the same farm and slaughtered the
103 same day as DFD samples in order to homogenize the experimental conditions over
104 sample groups. Carcasses were chilled for 24 h in a refrigerated chamber at 2 °C and
105 relative humidity of 98%. At this point, the *longissimus thoracis* (LT) muscle was
106 excised from the left half of each carcass. Two 2.5 cm thick steak taken at the fifth rib
107 were packed under vacuum conditions and transported under refrigerated conditions.
108 The first steak was used for pH and color determination and proteome analysis; the
109 second one to assess water holding capacity and textural tests. Samples for proteome
110 analysis were lyophilized under optimal conditions as previously described [32] and
111 subsequently frozen at -80 °C until required, whereas samples for water holding
112 capacity and texture determinations were analyzed immediately.
113 2.2. Determination of meat quality parameters
114 The pH of the samples was measured using a digital portable pH-meter (Hanna
115 Instruments, Eibar) equipped with a penetration probe. A portable colorimeter (Konica
116 Minolta CM-600d, Osaka) with the following machine settings (pulsed xenon arc lamp,
117 angle of 0° viewing angle geometry, standard illuminant D65 and aperture size of 8

118 mm) was used to measure the meat color in the CIELAB space [lightness (L*), redness
119 (a*) and yellowness (b*)]. Three measurements were performed for each sample in
120 homogeneous and representative areas, free of intramuscular fat. The water holding
121 capacity (WHC) was measured as cooking loss. Steaks were cooked placing vacuum
122 package bags in a water bath with automatic temperature control (JP Selecta, Precisdg,
123 Barcelona) until they reached an internal temperature of 70 °C, controlled by
124 thermocouples type K (Comark, PK23M, Norwich), and connected to a data logger
125 (Comark Dilligence EVG, N3014, Norwich). After cooking, samples were cooled in a
126 circulatory water bath set at 18 °C for a period of 30 min and the percentage cooking
127 loss was recorded. All samples were cut or compressed perpendicular to the muscle
128 fiber direction at a crosshead speed of 3.33 and 1 mm s^{-1} for Warner- Bratzler (WB) and
129 textural profile analysis (TPA) tests, respectively. A texture analyzer (TA-XT2, Stable
130 Micro Systems, Godalming) was used in both tests. Four pieces of meat of 1 × 1 × 2.5
131 cm (height × width × length) were cut completely using a WB shear blade with a
132 triangular slot cutting edge (1 mm thickness). Maximum shear force [33] was assessed
133 by the higher peak of the force-time curve, which represents the maximum resistance of
134 the sample to the cut. Four pieces of meat of 1 × 1 × 1 cm (height × width × length)
135 were removed for TPA test according to methodology proposed by Bourne [34].
136 Hardness was measured by compressing to 80% with a probe of 19.85 cm^2 of surface
137 contact. Hardness was determined as the maximal force of the first compression of the
138 meat piece.
139 2.3. Protein extraction and quantification
140 Lyophilized beef powder (50 mg) was resuspended in 1.5 mL of lysis buffer (7
141 M urea; 2 M thiourea; 4% CHAPS; 10 mM DTT; and 2% Pharmalyte^{TM} pH 3-10, GE

142 Healthcare, Uppsala) for 2 h at 25 ºC. An aliquot of 250 µL was lysed using a Sonifier
143 250 (Branson, Danbury) by cycling. During this process, the sample vial was kept in an
144 ice-water bath to prevent significant heating in the sample during sonication. Protein
145 purification and extraction from crude cell lysates was carried out with the Clean-Up kit
146 (GE Healthcare) as described in manufacturer´s indications [35]. The proteins were then
147 resuspended in 250 µL of lysis buffer. Protein quantification was assessed for each
148 extraction using the CB-X protein assay kit (G-Biosciences, St. Louis) according to
149 manufacturer's recommendations for using a microplate reader. CB-X is an improved
150 Bradford [36] assay, compatible with all commonly used buffers and conditions in
151 protein isolation, which provides a quick estimation of protein concentration. The BSA
152 protein standard was used to get a calibration curve.
153 2.4. 2-DE protein profiles
154 2-DE was performed according to Görg et al. [37] with some modification. 2-DE
155 was carried out from 350 µg of total protein extract dissolved in lysis and rehydration (7
156 M urea, 2 M thiourea, 4% CHAPS, 0.002% bromophenol blue) buffers. Protein extracts
157 were loaded into 24-cm-long ReadyStrip IPG Strips (Bio-Rad Laboratories, Hercules)
158 with linear pH gradient of 4-7, together with 0.6% DTT and 1% IPG buffer (Bio-Rad
159 Laboratories). The IEF was performed using a PROTEAN IEF cell system (Bio-Rad
160 Laboratories). Gels were initially rehydrated for 12 h at 50 V. Rapid voltage ramping
161 was subsequently applied to reach a total of 70 kVh. After IEF, strips were equilibrated
162 for 15 min at room temperature in equilibration solution I (50 mM Tris pH 8.8, 6 M
163 urea, 2% SDS, 30% glycerol and 1% DTT) and then with equilibration solution II (50
164 mM Tris pH 8.8, 6 M urea, 2% SDS, 30% glycerol and 4% iodoacetamide) under the
165 same conditions. Second dimension electrophoresis was run on 12% (w/v) SDS-PAGE

166 gels using an Ettan DALTsix vertical slab gel system (GE, Healthcare) and Tris-
167 glycine-SDS (50 mM Tris, 384 mM glycine and 0.2% SDS) as electrode buffer. Gels
168 were run at a constant current of 32 mA at 25 ^{o}C.

169 2.5. Phosphoprotein staining with Pro-Q Diamond

170 The phosphoprotein-specific fluorescent dye Pro-Q Diamond phosphoprotein
171 stain (Pro-Q DPS, Molecular Probes, Leiden) was used as a probe for the in-gel
172 detection of phosphorylated polypeptides according to López-Pedrouso et al. [38]. 2-DE
173 gels were treated with a fixation solution (45% methanol and 10% acetic acid, for 60
174 min) and washed twice with distilled water (15 min per wash). The gels were then
175 incubated with two-fold water-diluted Pro-Q DPS (120 min), destained with destaining
176 solution (50 mM sodium acetate and 20% ACN pH 4.0) four times (30 min per wash) to
177 remove gel-bound nonspecific Pro-Q DPS, and washed again twice with distilled water
178 (5 min per wash). The Peppermint (Molecular Probes) phosphoprotein marker was
179 added to protein extracts before 2-DE to validate the specificity of the recognition of
180 phosphoproteins by Pro-Q DPS under our experimental conditions.

181 2.6. Total protein staining with SYPRO Ruby

182 The 2-DE gels were stained with SYPRO Ruby stain (Lonza, Rockland), for
183 total protein density following the manufacturer's indications. Pro-Q DPS-stained gels
184 were also post-stained with SYPRO Ruby. 2-DE gels stained for total protein (SYPRO
185 Ruby) and phosphoprotein (Pro-Q DPS) detection were obtained for four biological
186 replicates of DFD and control meats.

187 2.6. Image analysis

188 The 2-DE gel images from both Pro-Q DPS and SYPRO Ruby staining methods
189 were acquired using a Gel Doc XR+ system (Bio-Rad Laboratories). Image analysis of

190 digitalized gels was performed through PDQuest Advanced software v. 8.0.1 (Bio-Rad
191 Laboratories). 2-DE gels were matched across biological replicates and volume of each
192 spot was quantitatively determined after background subtraction and normalization
193 using total density of validated spots across all replicate gels. The p*I* and M_r of spots
194 were determined from their position on the IEF-strips and standard molecular mass
195 markers ranging from 15 to 200 kDa (Fermentas, Ontario), respectively.
196 2.7. Preparation of samples for MS
197 Spots of interest were excised from the gel taking care to maximize the protein-
198 to-gel ratio. Only the most intense stained region at center of the spot was excised to
199 avoid extracting an excess of gel matrix. Excised pieces were subjected to in-gel
200 digestion with trypsin as described [39]. Briefly, excised spots were cut into pieces and
201 washed with Milli-Q-water. Afterward, the pieces were dehydrated with ACN and
202 dried in a vacuum centrifuge. Gel pieces were further digested with 1 µg/mL of trypsin
203 (Promega, Madison) in 50 mM ammonium bicarbonate pH 8 to a final volume of 30 µL
204 (overnight at 37 ºC).
205 For MALDI TOF/TOF MS analysis gel pieces were reduced with 10 mM DTT
206 (Sigma-Aldrich, St. Louis) in 50 mM ammonium bicarbonate (Sigma-Aldrich) and
207 alkylated with 55 mM iodoacetamide (Sigma- Aldrich) in 50 mM ammonium
208 bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate in
209 50% methanol (HPLC grade, Scharlau, Barcelona), dehydrated by addition of ACN
210 (HPLC grade) and dried in a SpeedVac. Modified porcine trypsin (Promega) was added
211 to the dry gel pieces at a final concentration of 20 ng/µl in 20 mM ammonium
212 bicarbonate, incubating them at 37 °C for 16 h. Peptides were extracted three times by

213 20 min incubation in 40 µL of 60% ACN in 0.5% HCOOH. The resulting peptide
214 extracts were pooled, concentrated in a SpeedVac and stored at −20 °C.

215 2.8. LC-MS/MS analysis

216 Peptide digests were acidified with acetic acid, cleaned on a C_{18} MicroSpin
217 column (The Nest Group, South-borough) and analyzed by LC-MS/MS using a Agilent
218 1260 HPLC series system (Agilent Technologies, Santa Clara) coupled to an LIT-Velos
219 mass spectrometer (Thermo Fisher, San Jose). Peptide separation was performed on a
220 BioBasic-18 RP column (0.18 mm x 150 mm) (ThermoHypersil-Keystone, Bellefonte),
221 using 0.15% acetic acid in Milli-Q-water and 98% ACN and 0.15% acetic acid as
222 mobile phases A and B, respectively. A 90 min linear gradient from 5 to 40% B was
223 used at a flow rate of 1.5 µL/min. The spray voltage used was 3.5 kV; N_2 flow, 8
224 arbitrary units, while the capillary temperature was 230 ºC. Peptides were analyzed in
225 the positive mode from 400 to 1600 amu (two microscans), followed by four data-
226 dependent MS/MS scans (two microscans), using an isolation width of 3 amu and a
227 normalized collision energy of 35%. Fragmented masses were set in dynamic exclusion
228 for 2 min after the second fragmentation event, and singly charged ions were excluded
229 from MS/MS analysis. MS/MS spectra were searched using SEQUEST (Proteome
230 Discoverer 1.4 package, Thermo Fisher), against the *B. taurus* UniProt/SwissProt
231 database (release 2013_10; 31.983 entries), which also included their respective decoy
232 sequences. The following constraints were used for the searches: semi-tryptic cleavage
233 with up to two missed cleavage sites and tolerances 1.2 Da for precursor ions and 0.5
234 Da for MS/MS fragment ions. The variable modifications allowed were methionine
235 oxidation, carbamidomethylation of cysteine and acetylation of the N-terminus of the
236 protein. The database search results were subjected to statistical analysis by Protein

237 Discoverer Peptide Confidence (v.4.0), choosing a False Discoverer Rate (FDR)
238 threshold of 1-5%.
239 2.9. MALDI TOF/TOF MS analysis
240 MALDI TOF/TOF MS was used to verify protein identifications by LC-MS/MS.
241 Dried samples were dissolved in 4 µL of 0.5 % HCOOH. Equal volumes (0.5 µL) of
242 peptide and matrix solution, consisting of 3 mg CHCA dissolved in 1 mL of 50 % ACN
243 in 0.1 % TFA, were deposited onto a 384 Opti-TOF MALDI plate (Applied Biosystems,
244 Foster City) using the thin layer method. Mass spectrometric data were obtained in an
245 automated analysis loop using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems).
246 MS spectra were acquired in positive-ion reflector mode with a Nd:YAG, 355 nm
247 wavelength laser, averaging 1000 laser shots, and at least three trypsin autolysis peaks
248 were used as internal calibration. All MS/MS spectra were performed by selecting the
249 precursors with a relative resolution of 300 (FWHM) and metastable suppression.
250 Automated analysis of mass data was achieved using the 4000 Series Explorer Software
251 v. 3.5 (Applied Biosystems). PMF and peptide fragmentation spectra data of each
252 sample were combined through the GPS Explorer Software v. 3.6 using Mascot
253 software v. 2.1 (Matrix Science, Boston) to search against the *B. taurus*
254 UniProt/SwissProt database (release version 2015-02; February, 2015, 32.362 entries),
255 with 30 ppm precursor tolerance, 0.35 Da MS/MS fragment tolerance, carbamidomethyl
256 cysteine as fixed modification, oxidized methionine as variable modification and
257 permitting one missed cleavage. All spectra and database results were manually
258 inspected in detail using the above software. Protein scores greater than 56 were
259 accepted as statistically significant ($p < 0.05$), and the identification was considered as

260 positive when the protein score CI (confidence interval) was above 98%. In the case of
261 MSMS spectra, the total ion score CI was above 95%.
262 2.10. Statistical analysis
263 Standard statistical tests (Mann-Whitney U test, Spearman's correlation test,
264 etc.) were performed using the IBM SPSS Statistics 20 (SPSS, Chicago) statistical
265 software package. Non-parametric bootstrap confidence intervals (CIs) were obtained
266 for the means of the observed spot volumes in DFD and control samples by the bias-
267 corrected percentile method [40] using the software DIANA (Zapata C, unpublished).
268 DIANA is a software package written in Visual Basic for population genetics data
269 analysis. It uses random numbers from the standard multiplicative linear congruential
270 generator implemented by Schrage [41] for generating the bootstrapped empirical
271 distribution of the sample mean, a random number generator that gives very long
272 sequences of "pseudo-random" numbers that have the appearance of randomness. For
273 each set of *N* (=4) estimates of spot volume, 2,000 bootstrap samples of size *N* were
274 drawn with replacement following a Monte Carlo algorithm. Bootstrap CIs were
275 constructed from distribution of 2,000 bootstrap mean replications. The bias was
276 subsequently corrected from the proportion of bootstrap mean replications according to
277 Efron [40].
278 Quantitative change in the volume of each protein spot from control to DFD
279 samples was determined by the commonly used measure of "fold change" (*FC*). The *FC*
280 is given by
281 $$FC = V_{DFD} / V_C$$
282 where V_{DFD} and V_C are the volume means for each spot across replicate gels in DFD
283 and control meats, respectively. *FC*-values less than one were represented as their

284 negative reciprocal. Therefore, FC ranges from $-\infty$ to $+\infty$. The change in the volume
285 of each spot from control to DFD samples was also calculated using a new measure of
286 relative change (RC), which is defined as follows

287 $$RC = DV / \left| DV_{max} \right|$$

288 where $DV = V_{DFD} - V_C$ is a measure of the differential volume between samples, and
289 DV_{max} is the maximum observed value of DV over spots in the study. The RC measure
290 has the advantage that it ranges between -1.0 and +1.0 and takes a value of zero when
291 there is no volume change.

292 2.11. Bioinformatic analyses

293 Gene ontology (GO) terms for each gene, inferred from electronic annotation or
294 experimentally validated, were retrieved with QuickGO
295 (http://www.ebi.ac.uk/QuickGO/ ; European Bioinformatics Institute/EBI). Broader GO
296 terms used to generate the pie charts were retrieved from the pre-existing GO slim
297 generic subset (GO Consortium) by means of the Slimmer tool of AmiGO
298 (http://amigo1.geneontology.org/cgi-bin/amigo/slimmer; Species DB ALL, Evidence
299 code ALL). For single enrichment of GO terms analysis we used FatiGO [43], a specific
300 software within the set of functional analysis tools of Babelomics 4
301 (http://v4.babelomics.org/) that took the list of terms associated with our bovine (*B.*
302 *taurus*) genes of interest (idlist data type; Ensembl IDs) in different databases (GO
303 biological process, GO molecular function, GO cellular component, KEGG and InterPro
304 databases) and compared this list to the collection of terms associated with the rest of
305 the genome. A two tailed Fisher's exact test was used to check for significant over-
306 representation of annotations ($p < 0.05$).

307 An analysis of predicted and known functional interactions between the
308 identified proteins and other *B. taurus* proteins was performed using the STRING v9.1
309 software [44]. The “additional (white) nodes” and “interactors shown” parameters were
310 set to 1 in order to obtain a protein-protein interaction network close to the proteins
311 identified in our study.

312 2.12. Cluster analysis

313 Proteins that exhibited similar significant change in abundance from control to
314 DFD meat were grouped into clusters by an agglomerative hierarchic cluster analysis.
315 The unweighted pair-group method with arithmetic averaging (UPGMA) was used to
316 cluster the proteins from the matrix of pairwise RC-values in absolute value. A
317 UPGMA dendrogram was generated with NTSYSpc v. 2.1 software (Applied
318 Biostatistics, Setauket).

319 **3. Results**

320 3.1. Identification of control and DFD meats

321 The determination of meat quality parameters in control (non-DFD) and DFD
322 meat revealed that the those samples selected in this study represent a biological
323 material suitable for unraveling the proteome changes underlying the transformation of
324 LT bovine muscle into DFD meat as a response to PSS. Table 1 shows mean values
325 (±SE, standard error) of pH, color parameters (L*; a* and b*), WHC (cooking loss) and
326 texture (shear force and hardness) in control and DFD meat samples. Statistically
327 significant differences between the two types of meat were detected across quality
328 parameters assayed in this study by the one-tailed Mann-Whitney U test (p-value <
329 0.05). It can also be seen that the values of meat quality parameters fulfil all
330 requirements of control and DFD meats [8]. Firstly, all biological replicates exhibited

331 pH values lower than 6.0 in control meats while those of DFD meats were always
332 higher than 6.0. Secondly, meat color as measured in trichromatic space was higher for
333 L* and b* in the control cuts (highest luminosity) than in the DFD group (darkest
334 color). Thirdly, the WHC was lower in control than in DFD group. Fourthly, meats
335 from the control group showed the highest shear force and hardness as assessed by WB
336 and TPA tests, respectively. In addition, there was a statistically significant negative
337 relationship of L*, b*, shear force and hardness with pH (r_s = -0.98, n= 8; $p < 0.01$ for
338 L*; r_s = -0.91, n= 8; $p < 0.01$, for b*; r_s = -0.93, n= 8; $p < 0.01$, for shear force; and r_s = -
339 0.73, n= 8; $p < 0.05$, for hardness) as previously reported [9,45].

340 3.2. Identification of proteins linked to PSS by 2-DE and MS

341 Proteins extracted from DFD and control meat samples collected at 24 h post-
342 mortem were separated using 2-DE. Sample lyophilization, protein extraction and
343 electrophoretic methods used in the study resulted in good quality and highly
344 reproducible 2-DE gel images (Supplementary Fig. 1). Representative 2-DE gel protein
345 profiles for control and DFD meats are presented in Fig. 1. Detection, matching and
346 measurement of the volume of protein spots across gels were assessed using PDQuest
347 software. There was a set of 19 protein spots that showed greater intensity differences
348 between control and DFD groups. Specifically, 12 spots (designated as spots C1-12)
349 were overexpressed in control meats, whereas the remaining 7 spots (spots D1-7) were
350 overexpressed in DFD meats. Quantitative (i.e. different spot intensity between sample
351 groups) and qualitative (i.e. presence/absence of a given spot in control or DFD meats)
352 changes of expression were found between the two types of samples. Table 2 gives the
353 mean value (±SE) of the volume ($\times 10^{-2}$) of each protein spot in control and DFD
354 samples along with their 95% and 99% bootstrap CIs. Difference of mean volumes

355 between the two sample groups were found to be statistically significant ($p < 0.05$) for
356 all protein spots, given that their 95% CIs did not overlap (spots with quantitative
357 differences) or did not overlap zero (spots with qualitative differences).

358 Differentially abundant protein spots between sample groups were selected for
359 LC-MS/MS analysis. All protein spots were confidently identified by LC-MS/MS and
360 identifications are listed in Table 3 (see Supplementary Table 1 for more detailed
361 information). However, some identified proteins were subtracted from the final list of
362 proteins analyzed in response to PSS for the following reasons. Firstly, an apparent
363 fragmentation phenomenon was detected at six of the identified protein spots (AFG3L2,
364 spot C1; CCBP2, spot C2; MYL6B-1, spot C7; CPS1, spot C8; MYL2-1, spot C-12;
365 and CPS1, spot C12) from the comparison between the theoretical and the
366 experimentally observed M_r on 2-DE gels. This is probably due to proteolytic
367 degradation at 24 h post-mortem associated with cellular death and the meat aging
368 process [46,47]. Secondly, three identifications (spots C3, C4 and C10) corresponded to
369 uncharacterized proteins of *B. taurus*. Thirdly, the D4 protein spot contained a
370 hypothetical protein (LOC767890). Therefore, the final list of selected spots for further
371 analysis comprised a total of ten differentially abundant proteins in control and DFD
372 samples: MYL3, MYL6B, MYL2, TNNC2, STGAL1, ATP5B, TPI1, CFL2, MYLPF
373 and MYLPF-1. Protein identifications were validated from several other lines of
374 evidence: MALDI-TOF/TOF MS analysis (Supplementary Table 2); close agreement
375 between the theoretical and the 2-DE-based experimental M_r and pI (Table 3); and
376 consistent results with previously reported protein identifications in LT bovine muscle
377 based on 2-DE and MS [21,22].

378 Table 4 shows the strength of spot intensity changes between control and DFD
379 meat as measured by *FC* and *RC* statistics. There can be seen that both statistics
380 provided completely discrepant results about the relative magnitude of changed proteins
381 in muscle conversion to DFD meat. The use of the *FC* measure has some
382 inconveniences because of its range of variation (from $-\infty$ to $+\infty$). *FC* gives values of -
383 ∞/ + $\infty$for qualitative changes between sample groups when the absence of a given spot
384 can be due merely to the occurrence of protein amounts undetectable by 2-DE. In
385 contrast, the measure *RC* allows us the joint analysis of spots with qualitative and
386 quantitative expression changes under the same range of variation (i.e., from-1.0 to
387 +1.0). Consequently, the *RC* measure can provide more genuine information than *FC* to
388 assess differential protein abundance in PSS response. Applying the *RC* statistic,
389 MYL6B, MYL2, TNNC2 and MYL3 were found to be overrepresented ($RC < 0$) in
390 control samples, whereas ATP5B, TPI1, CFL2, STGAL1, MYLPF and MYLPF-1 were
391 overrepresented ($RC > 0$) in DFD samples (Table 4; Fig. 2). When proteins were
392 ordered by the magnitude of *RC* scores it turned out that MYL6B and two MYLPF
393 isoforms (MYLPF and MYLPF-1) underwent the sharpest decrease and increase,
394 respectively, from control to DFD meats (Fig. 2). In particular, the MYLPF-1 protein
395 exhibited the maximum difference of spot volume (DV_{mav}), highly represented in DFD
396 meats ($RC = +1.0$).

397 3.3. Phosphorylated fast skeletal light chain 2 (MYLPF) isoforms

398 Protein spots identified as MYLPF form part of a wider spot constellation with
399 the same M_r but different p*I*s (Fig. 1), which suggests that they are probably MYLPF
400 isoforms caused by post-translational modifications such as phophorylations and
401 acetylations [48]. We first confirmed that indeed this spot constellation is formed by

402 different MYLPF isoforms through post-translational modifications using MALDI
403 TOF/TOF MS (Supplementary Table 3). In addition, the phosphoprotein-specific
404 fluorescent dye Pro-Q DPS was used for in-gel detection of phosphorylated forms of
405 MYLPF. The specificity of the recognition of phosphoproteins by Pro-Q DPS under our
406 experimental conditions was validated using Peppermint phosphoprotein molecular
407 weight standards (data not shown). Reference (SYPRO Ruby total protein stain) and
408 phosphorylated (Pro-Q DPS phosphoprotein stain) MYLPF spot patterns on 2-DE gels
409 obtained from control and DFD meat samples are shown in Fig. 3. It was found that two
410 out of three MYLPF isoforms were phosphorylated in control samples, whereas four out
411 of five isoforms were phosphorylated in DFD ones. The most alkaline protein spot in
412 both types of samples (spot no. 1) was apparently the only unphosphorylated MYLPF
413 isoform because it was not detected in gels with Pro-Q DPS. Note that the two
414 additional protein spots found in DFD meat (spots no. 4 and no. 5) turned out to be
415 phosphorylated MYLPF isoforms.

416 3.4. Bioinformatic and cluster analyses of identified proteins in PSS response

417 Fine-grained (Supplementary Table 4) and slim versions (Supplementary Fig. 2)
418 of GO terms for each differentially abundant *B. taurus* protein were retrieved by means
419 of Quick GO and the AmiGO Slimmer tool, respectively, in order to categorize the
420 identified proteins. According to Supplementary Table 4, the set of nine differentially
421 abundant proteins were mainly involved in biological processes such as calcium ion
422 binding and cardiac muscle morphogenesis/contraction, function as proteins that bind
423 calcium ions and actin, and can be found at different locations, either outside cells such
424 as the extracellular space and the cell membrane, or inside cells, such as the
425 cytoskeleton, actin/tropomyosin complex, or nucleus. The same conclusions were

426 drawn when upper level GO slim terms were used (Supplementary Fig. 2). Moreover,
427 we also looked for significant overrepresentation of functional annotations in our set of
428 nine genes regarding the distributions of functional terms from the rest of the bovine
429 genome. Thus, FatiGO/Babelomics [43] single enrichment analysis displayed a
430 significant ($p \leq 0.05$) enrichment of InterPro and GO molecular function database terms
431 indicating calcium, cytoskeletal and actin binding (Supplementary Table 5). In addition,
432 there was a significant ($p \leq 0.05$) enrichment in proteins linked to the following KEGG
433 database pathways: Tight junction (bta04530), regulation of actin cytoskeleton
434 (bta04810), focal adhesion (bta04510) and leukocyte transendothelial migration
435 (bta04670).
436 STRING database revealed two groups of direct interactions between differently
437 abundant proteins in PSS response: a major group of structural-contractile muscle
438 proteins (TPI1 and MYL3, MYL6B, MYL2, MYLPF and TNNT1) and a minor group
439 of only two proteins involved in structural-contractile functions (CFL2) and metabolism
440 (TPI1) (Fig. 4). Note that TNNC2 was replaced by TNNT1 because STRING database
441 found no proteins by this name on *B. taurus*. MYL3 exhibited the strongest interaction
442 with MYL2, MYLPF and TNNT1 (threshold: 0.7; high confidence interval). Only two
443 proteins, namely ST6GAL1 and ATP5B, were not linked with the two interaction
444 networks, and ATP5B was linked in an interactome at a first level (i.e. when a
445 maximum of interaction with one protein of the proteome is searched by the STRING
446 software). Using evidence only for co-expression of identified proteins from STRING
447 database led to a single group of links between structural-contractile muscle proteins
448 showing more solid levels of co-expression (Supplementary Fig. 3).

449 Cluster analysis gives an alternative perspective to STRING on the co-
450 expression levels of the identified proteins based only on the information provided by
451 this study. The UPGMA dendrogram obtained from differences in *RC* (in absolute
452 value) between pairs of protein spots differentially abundant in control and DFD
453 samples is shown in Fig. 5. Cluster analysis suggests the presence of two major clusters
454 of related proteins and one outlier protein (protein MYLPF-1) distantly related to the
455 other proteins. Thus, the outlier protein MYLPF-1 followed by the cluster made up of
456 proteins MYLPF, MYL6B and MYL2 were the most differently abundant proteins
457 according to the UPGMA dendrogram.

458 **4. Discussion**

459 In this study, we report the first evidence on the changes of the proteome
460 landscape of LT bovine muscle in response to PSS. The combination of a diversity of
461 proteomic tools led to identify proteins showing statistically significant abundance
462 change in muscle conversion to DFD meat in the Rubia Gallega breed. The identified
463 proteins can be grouped into two major functional categories: structural-contractile
464 muscle proteins (MYL3, MYL6B, MYL2, MYLPF, TNNC2 and CFL2) and proteins
465 involved in metabolism (TPI1, ATP5B and ST6GAL1). Most identified proteins and
466 their modulation (increasing or decreasing) in response to PSS have a clear biological
467 significance.

468 Tenderness is considered to be one of the most important attributes contributing
469 to meat quality [19,49]. Current evidence suggests that longitudinal and lateral
470 sarcomere shrinkage, total collagen content, temperature, pH and proteolysis play a
471 central role in the tenderization process occurring during post-mortem meat aging [49-
472 51]. Sarcomere shortening during rigor mortis development increases shear force with

473 an increase in toughness during the first 24 h post-mortem [49,50]. However, significant
474 depletion of muscle glycogen reserves associated with PSS response has a well-
475 documented effect on muscle fiber characteristics that control the tenderization phase
476 during the conversion of muscle to meat [1,51]. In this study, textural tests showed that
477 DFD meat of cattle affected by PSS was significantly tenderer at 24 h of aging than beef
478 from normal cattle, as previously reported [45]. The higher pH in DFD meat enhances
479 electrostatic repulsion between the myofibrillar proteins, which contributes to less
480 lateral shrinkage of the muscle fibers [51]. However, in-depth knowledge of proteome
481 post-mortem changes which occur upon slaughter of the animal is crucial for a better
482 understanding of the mechanisms controlling tenderness. A number of studies have
483 shown that structural proteins specific to the sarcomere, including myosin light chains,
484 and many other functionally diverse proteins participate in meat tenderization [22,28-
485 31,52]. But the available evidence refers to biochemical changes underlying
486 tenderization that occurs during normal post-mortem aging of beef. It is noteworthy that
487 meat affected by PSS has particular characteristics and thereby tenderization processes
488 may be achieved by different biochemical mechanisms that in normal meat.

489 Our proteomic study revealed for the first time that several muscle myosin light
490 chains (MYL3 and MYL6B) and regulatory light chain 2 isoforms (MYL2 and
491 MYLPF) participate in the conversion of cattle muscle to DFD meat in Rubia Gallega
492 animals affected by PSS. All these proteins appeared to be involved in the main network
493 of functionally associated proteins according to the STRING database. This is not a
494 surprising result when the myosin is a major structural protein of the muscle sarcomere
495 in association with actin and other contractile proteins. The MYL3, MYL6B and MYL2
496 proteins play important structural and functional roles by supporting the structure of the

497 myosin neck region and fine-tuning the kinetics of the actin-myosin interaction [53].
498 We also found that levels of MYL3, MYL6B and MYL2 were lower in DFD than in
499 normal meat, probably because of a more intensive enzymatic degradation of myofibril
500 structure, which can contribute to explain its tenderness differential. It has been pointed
501 out that denaturation of the myosin heads can contribute to myofibrillar lateral
502 shrinkage and reduction in its ability to bind water resulting in decreased WHC [51].
503 These effects might be outweighed in DFD meat by the regulatory role of MYL2 that
504 triggers myofibril contraction by a Ca^{+2} and calmodulin-dependent myosin light chain
505 kinase, meaning that decrease of MYL2 in DFD meat may reduce sarcomere shortening.
506 PSS is consequence of a rapid response of the animal in reaction to acute
507 stressors that includes the activation and regulation of the autonomous nervous system
508 and hypothalamic-pituitary- adrenal axis with effects on muscle proteins [1]. In addition
509 to this, post-translational protein modification through phosphorylations has potential to
510 be an important molecular mechanism of rapid response to acute stressors. Protein
511 phosphorylation is a transient and reversible event that can change at very short
512 intervals of time with a key regulatory role in a diversity of cellular processes [54].
513 Recent studies suggest that phosphorylation of muscle proteins play an important role in
514 the post-mortem muscle conversion to meat and hence in meat quality [19,28]. In
515 particular, phosphoproteomic analysis of sarcoplasmic proteins in post-mortem porcine
516 muscle revealed high levels of phosphorylation in stress response proteins [55]. We
517 found that two phosphorylated fast skeletal myosin light chain 2 isoforms (i.e. MYLPF
518 and MYLPF-1) underwent the most intense relative change in muscle conversion to
519 DFD meat. They were the most phosphorylated fast skeletal myosin light chain 2
520 isoforms present only in DFD meat. Our results suggest, therefore, that these

521 phosphorylated isoforms of MYLPF are the most specific and sensitive biomarkers for
522 PSS in the LT muscle of the Rubia Gallega. It is well-recognized that MYL2 is highly
523 phosphorylated and that this phosphorylation is crucial for the regulation of MYL2 [56].
524 Levels of MYL2 phosphorylation around 30-40% have been reported in humans and pig
525 [53]. Interestingly, the N-terminal domain in the human orthologue MYL2 contains a
526 phosphorylation site that appears to have an important modulatory role in striated
527 muscle contraction [56]. It should also be highlighted that increased levels of pH in
528 DFD compared to normal meat may run against the activity of acid phosphatases [55].
529 Further research is required to assess whether phosphorylation of proteins is an
530 extended mechanism in PSS response through an intensive study of the muscle
531 phosphoproteome.

532 Our observations showed a decreased abundance of TNNC2 in DFD meat which
533 helps to explain its increased tenderness. TNNC forms part, together with troponin T
534 (TNNT) and troponin I (TNNI), of the regulatory protein complex of the troponin that is
535 central to muscle contraction in skeletal and cardiac muscles [57]. The TNNC binds
536 calcium ions and abolishes the inhibitory action of troponin complex on actin filaments.
537 In bovine muscle, the progress of degradation of the myofibrillar TNNT subunit seems
538 to be a good predictor of tenderization during normal post-mortem aging of beef [58].
539 We also found that CFL2 (cofilin, muscle isoform) was up-modulated in response to
540 PSS. Cofilin is an isoform of the actin depolymeration factor family involved in
541 regulating the actin cytoskeleton and in other multiple facets of cellular biology [59]. It
542 has the ability to bind G- and F-actin in a 1:1 ratio of cofilin to actin and is the major
543 component of intranuclear and cytoplasmic actin rods. CFL2 is a skeletal-muscle
544 specific protein localized to the thin filaments/cytoskeleton, cytoplasm and nucleus. It

545 controls reversibly actin polymerization and depolymerization in a pH-sensitive
546 manner, with maximal depolymerization at pH 8.0 and almost abolished at pH < 7.0
547 [60]. This means that the normal post-mortem pH decline from 7.0 to 5.5 due to lactic
548 acid accumulation will favor actin polymerization and sarcomere structure contraction,
549 while in DFD meat the higher pH (above 6.0) could sustain better cofilin-dependent F-
550 actin depolymerization, contributing to higher WHC and improved tenderness.

551 Non-structural proteins can also be associated with tenderization in response to
552 stress in cattle. Recent evidence shows that genes involved in immune response and
553 regulation of metabolism in Angus cattle were associated with variation in beef
554 tenderness induced by acute stress, using cDNA microarray and quantitative real-time
555 PCR expression analysis [61]. We found increased levels of glycolytic (TPI1),
556 mitochondrial membrane (ATP5B) and glycosyltransferase (ST6GAL1) proteins in
557 DFD as compared to normal meats. Higher levels of glycolytic enzymes such as TPI, an
558 enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and
559 glyceraldehyde 3-phosphate, seem to be related to meat tenderness in cattle and porcine
560 muscles [29]. A prolonged ATP generation via glycolysis contributes to both lactate and
561 hydrogen ion accumulation. As above mentioned, this increases in hydrogen ions
562 reduces electrostatic repulsion between the myofibrillar proteins and contributes to
563 lateral shrinkage of the muscle fibers. Nevertheless, augmented levels of both TPI and
564 CFL2 might lead to a higher WHC and tenderness in DFD meat by affecting the
565 intracellular sodium concentration and osmolality. In fact, STRING database and cluster
566 analyses disclosed that CFL2 and TPI1 were included in a minor cluster of associated
567 proteins and formed part of a sub-cluster with a similar level of relative change in the
568 conversion of normal to DFD meat, respectively. It is known that production of ATP by

569 glycolysis in muscle supports ATP consumption by ion pumps located in the plasma
570 membrane such as the Na^+/K^+-ATPase [62-64]. Interestingly, an interaction at the
571 plasma membrane between the Na^+/K^+-ATPase and the phosphorylated form of cofilin-
572 1 (the non-muscle counterpart of CFL2) complexed with TPI has been described, and
573 this interaction serves to feed glycolytic ATP for the Na^+/K^+-ATPase pump [65].

574 **Conclusions**

575 Our study showed that the proteome of the LT bovine muscle of the Rubia
576 Gallega breed underwent noticeable changes in response to PSS. Specifically,
577 significant differential levels of expression of ten structural-contractile skeletal muscle
578 proteins and metabolic proteins were found to be associated with PSS. Most proteome
579 changes were represented by a group of strongly interacting structural proteins
580 involving different myosin light chain isoforms and troponin C type 2. Our results also
581 suggest that changes in the proteome linked to PSS involve not only quantitative
582 differences at the level of protein abundance, but also pos-translational protein
583 modifications through phosphorylations. In particular, highly phosphorylated fast
584 skeletal myosin light chain 2 isoforms appeared to be the most informative biomarkers
585 linked to PSS response in Rubia Gallega. The functions of the identified proteins
586 contribute to a better understanding of the increases tenderness and WHC in meat from
587 animals affected by PSS. In addition, the results presented herein suggest that the new
588 measure of relative change is more efficient than the usual measure of fold change to
589 reliably compare differential protein abundance between sample groups. Finally, it is
590 widely recognized that a diversity of endogenous and exogenous animal factors affect
591 PSS response. Further systematic follow-up studies are clearly required, therefore, to

592 unravel the entire sub-proteome involved in the molecular mechanisms associated with
593 PSS in cattle.

594 **Acknowledgments**

595 The authors would like to thank the anonymous reviewers for their valuable
596 comments and suggestions to improve the quality of the article.

REFERENCES

[1] Ferguson DM, Warner RD. Have we underestimated the impact of pre-slaughter stress on meat quality in ruminants? Meat Sci 2008;80:12–9.

[2] Miranda-de la Lama GC, Villarroel M, María GA. Livestock transport from the perspective of the pre-slaughter logistic chain: a review. Meat Sci 2014;98:9-20.

[3] Grandin T. Auditing animal welfare at slaughter plants. Review Meat Sci 2010;86:56–65.

[4] Grandin T. Asseessment of stress during handling and transport. J Animal Sci 1997;75:249-75.

[5] Lawrie RA, Ledward DA. Lawrie's Meat Science. Cambridge: Woodhead Publishing Limited; 2006.

[6] Warriss PD. Meat Science: An Introductory Text. Wallingford & New York: CABI Publishing; 2006.

[7] Terlouw EMC, Arnould C, Auperin B, Berri C, Le Bihan-Duva E, Deiss V, Lefèvre F, Lensink BJ, Mounier L. Pre-slaughter conditions, animal stress and welfare: Current status and possible future research. Animal 2008;2:1501–17.

[8] Adzitey F, Nurul H. Pale soft exudative (PSE) and dark firm dry (DFD) meats: causes and measures to reduce these incidences – a mini review. Int Food Res J 2011;18:11-20.

[9] Abril M, Campo MM, Önenç A, Sañudo C, Albertí P, Negueruela AI. Beef colour evolution as a function of ultimate pH. Meat Sci 2001;58:69-78.

[10] Purchas RW, Aungsupakorn R. Further Investigations into the relationship between ultimate pH and tenderness for beef samples from bulls and steers. Meat Sci 1993;34:163-78.

[11] Newton KG, Gill CO. The microbiology of DFD fresh meats: A review. Meat Sci 1981;5:223-32.

[12] Viljoen HF, De Kock HL, Webb EC. Consumer acceptability of dark, firm and dry (DFD) and normal pH beef steaks. Meat Sci 2002;61:181-85.

[13] Moore MC, Gary GD, Hale DS, Kerth CR, Griffin DB, Savell JW, Raines CR, Belk KE, Woerner DR, Tatum JD, Igo JL, VanOverbeke DL, Mafi GG, Lawrence TE, Delmore Jr RJ, Christensen LM, Shackelfors SD, King DA, Wheeler TL, Meadows LR, O'Connor ME. National Beef Quality Audit–2011: In-plant survey of targeted carcass characteristics related to quality, quantity, value, and marketing of fed steers and heifers. J Anim Sci 2012;90:5143-51.

[14] Smith GC, Savell JW, Dolezal HG, Field TG, Gill DG, Griffin DB, Hale DS, Morgan JB, Nortcutt SL, Tatum JD, Ames R, Boleman S, Gardner B, Morgan W, Smith M. The National Beef Quality Audit. Colorado State University. Fort Collins; Oklahoma State University, Stillwater; Texas: A&M University. College Station; 1995.

[15] Bourguet C, Deiss V, Gobert M, Durand D, Boissy A, Terlouw EMC. Characterising the emotional reactivity of cows to understand and predict their stress reactions to the slaughter procedure. Appl Anim Behav Sci 2010;125:9–21.

[16] Averós X, Martín S, Riu M, Serratosa J, Gosálvez LF. Stress response of extensively reared young bulls being transported to growing-finishing farms under Spanish summer commercial conditions. Livest Sci 2008;119:174–82.

[17] Frylinck L, Strydom PE, Webb EC, du Toit E. Effect of South African beef production systems on post-mortem muscle energy status and meat quality. Meat Sci 2013;93:827–37.

[18] D’Alessandro A, Zolla L. Food safety and quality control: hints from proteomics. Food Technol Biotech 2012;50:275-85.

[19] Paredi G, Raboni S, Bendixen E, de Almeida AM, Mozzarelli A. “Muscle to meat” molecular events and technological transformations: the proteomics insight. J Proteomics 2012;75:4275-89.

[20] Bendixen E. The use of proteomics in meat science. Meat Sci 2005;71:138-49.

[21] Bouley J, Chambon C, Picard B. Mapping of bovine skeletal muscle proteins using two-dimensional gel electrophoresis and mass spectrometry. Proteomics 2004;4:1811-24.

[22] Laville E, Sayd T, Morzel M, Blinet S, Chambon C, Lepetit J, Renand G, Hocquette JF. Proteome changes during meat aging in tough and tender beef suggest the importance of apoptosis and protein solubility for beef aging and tenderization. J Agric Food Chem 2009;57:10755-64.

[23] Shibata M, Matsumoto K, Oe M, Ohnishi-Kameyama M, Ojima K, Nakajima I, Muroya S, Chikuni K. Differential expression of the skeletal muscle proteome in grazed cattle. J Anim Sci 2009;87:2700-08.

[24] Marcos B, Mullen AM. High pressure induced changes in beef muscle proteome: Correlation with quality parameters. Meat Sci 2014;97:11-20.

[25] Bjarnadóttir SG, Hollung K, Faergestad EM, Veiseth-Kent E. Proteome changes in bovine *longissimus thoracis* muscle during the first 48 h postmortem: shifts in energy status and myofibrillar stability. J Agric Food Chem 2010;58:7408–14.

[26] Kim NK, Cho S, Lee SH, Park HR, Lee CS, Cho YM, Choy YH Yoon D, Im SK, Park EW. Proteins in *longissimus* muscle of Korean native cattle and their relationship to meat quality. Meat Sci 2008;80:1068-73.

[27] Suman SO, Rentfrow G, Nair MN, Joseph P. 2013 EARLY CAREER ACHIEVEMENT AWARD— Proteomics of muscle- and species-specificity in meat color stability. J Anim Sci 2014;92:875-82.

[28] D'Alessandro A, Marrocco C, Rinalducci S, Mirasole C, Failla S, Zolla L. Chianina beef tenderness investigated through integrated Omics. J Proteomics 2012;75:4381-98.

[29] Ouali A, Gagaoua M, Boudida Y, Becila S, Boudjellal A, Herrera-Mendez CH, Sentandreu MA. Biomarkers of meat tenderness: present knowledge and perspectives in regards to our current understanding of the mechanisms involved. Meat Sci 2013;95:854-70.

[30] Guillermin N., Bonnet M, Jurie C, Picard B. Functional analysis of beef tenderness. J Proteomics 2011;75:352-65.

[31] Zapata I, Zerby HN, Wick M. Functional proteomic analysis predicts beef tenderness and the tenderness differential. J. Agric Food Chem 2009;57:4956-63.

[32] Carpentier SC, Dens K, Van den Houwe I, Swennen R, Panis B. Lyophilization, a practical way to store and transport tissues prior to protein extraction for 2-DE analysis? Pract Proteomics 2007;1:64-9.

[33] Møller A. Analysis of Warner Bratzler shear force pattern with regard to myofibrillar and connective tissue components of tenderness. Meat Sci 1980;5:247-60.

[34] Bourne MC. Texture profile analysis. Food Technol 1978;32:62-6.

[35] GE Healthcare. Handbook: 2-D Electrophoresis Using Inmobilized pH Gradients, Principles & Methods. Piscataway: GE Healthcare. 2004.

[36] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Chem 1976;72:248-54.

[37] Görg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. The current state of two-dimensional electrophoresis with immobilized pH gradients Electrophoresis 2000;21:1037-53.

[38] López-Pedrouso M, Alonso J, Zapata C. Evidence for phosphorylation of the major seed storage protein of the common bean and its phosphorylation-dependent degradation during germination. Plant Mol Biol 2014;84:415-28.

[39] Jensen ON, Wilm M, Shevchenko A, Mann M. Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. Methods Mol Biol 1999;112:513–30.

[40] Efron B. The Jackknife, the Bootstrap and Other Resampling Plans. CBMS-NSF Regional Conference Series in Applied Mathematics No. 38. Philadelphia: Society for Industrial and Applied Mathematics; 1982.

[41] Schrage L. A more portable Fortran random number generator. ACM Transactions Mathematical Software 1979;5:132-38.

[42] Sokal RR, Rohlf FJ. Biometry. New York: W.H. Freeman and Company, 2012.

[43] Al-Shahrour F, Díaz-Uriarte R, Dopazo J. FatiGO: A web tool for finding significant associations of Gene Ontology terms with groups of genes. Bioinformatics 2004; 20:578-80.

[44] Franceschini A, Szklarczyk D, Frankild S, Kuhn M., Simonovis M, Roth A, Lin J, Minguez P, Bork P, von Merin C, Jensen LJ. STRING v9.1: protein-protein

interaction networks with increased coverage and integration. Nucleic Acids Res 2013;41:D808-15.

[45] Silva JA, Patarata L, Martins C. Influence of ultimate pH on bovine meat tenderness during ageing. Meat Sci 1999;52:453-59.

[46] Taylor RG, Geesink GH, Thompson VF, Koohmaraie M, Goll DE. Is Z-disk degeneration responsible for postmortem tenderization? J Anim Sci 1995;73:1351-67.

[47] Geesink GH, Koohmaraie M. 1999. Effect of calpastatin on degradation of myofibrillar proteins by μ-calpain under postmortem conditions. J. Anim. Sci 1999;77:2685-92.

[48] Zhu K, Zhao J, Lubman DM, Miller FR, Barder TJ. Protein p*I* shifts due to posttranslational modifications in the separation and characterization of proteins. Anal Chem 2005;77:2745-55.

[49] Maltin C, Balcerzak R, Tilley R, Delday M. Determinants of meat quality: tenderness. Proc Nutr Soc 2003:62:337-47.

[50] Koohmarie M. Biochemical factors regulating the toughening and tenderization processes of meat. Meat Sci;1996;S193-201.

[51] Pearce KL, Rosenvold K, Andersen HJ, Hopkins DL. Water distribution and mobility in meat during the conversion of muscle to meat and ageing and the impacts on fresh meat quality attributes−a review. Meat Sci 2011;89:111-124.

[52] Anderson MJ, Lonergan SM, Huff-Lonergan E. Myosin light chain 1 release from myofibrillar fraction during post-mortem aging is a potential indicator of proteolysis and tenderness of beef. Meat Sci 2012;90:345-51.

[53] Doroszko A, Polewicz D, Cadete VJJ, Sawicka J, Jones M, Szczesna-Cordary D, Cheung P-Y, Sawicki G. Neonatal asphyxia induces the nitration of cardiac myosin light chain 2 (MLC2) which is associated with cardiac systolic dysfunction. Shock 2010;34:592-600.

[54] Schubert P, Hoffman MD, Sniatynski MJ, Kast J. Advances in the analysis of dynamic protein complexes by proteomics and data processing. Anal Bioanal Chem 2006;386:482-93.

[55] Huang H, Larsen MR, Karlsson AH, Pomponio L, Costa LN, Lametsch R. Gel-based phosphoproteomics analysis of sarcoplasmic proteins in post-mortem porcine muscle with pH decline rate and time differences. Proteomics 2011;11:4063-76.

[56] Szczesna-Cordary D. Regulatory light chains of striated muscle myosin. Structure, function and malfunction. Curr Drug Targets Cardiovasc Haematol Disord 2003;3:187-97.

[57] Pearson AM, Young RB. Muscle and Meat Biochemistry. San Diego: Academic Press. 1989.

[58] Penny IF, Dransfield E. Relationship between toughness and troponin T in conditioned beef. Meat Sci 1979;3:135-41.

[59] Munsie LN, Desmond CR, Truant R. Cofilin nuclear-cytoplasmic shuttling affects cofilin-actin rod formation during stress. J Cell Sci 2012;125:3977-88.

[60] Papalouka V, Arvanitis DA, Vafiadaki E, Mavroidis M, Papadodima SA, Spiliopoulou CA, Kremastinos DT, Kranias EG, Sanoudou D. Muscle Lim Protein interacts with cofilin 2 and regulates F-actin dynamics in cardiac and skeletal muscle. Mol Cell Biol 2009;29:6046-58.

[61] Zhao C, Tian F, Yu Y, Luo J, Mitra A, Zhan F, Hou Y, Liu G, Zan L, Updike MS, Song J. Functional genomic analysis of variation on beef tenderness induced by acute stress in Angus cattle. Comp Funct Genom 2012;2012:756284.

[62] Clarke FM, Masters CJ. On the association of glycolytic enzymes with structural proteins of skeletal muscle. Biochim Biophys Acta 1975;381:37-46.

[63] Campbell JD, Paul RJ. The nature of fuel provision for the Na+, K(+)-ATPase in porcine vascular smooth muscle. J Physiol 1992;447:67-82.

[64] Cantiello HF. Actin filaments stimulate the Na(+)/K(+)-ATPase. Am J Physiol 1995;269:F637-43.

[65] Jung J, Yoon T, Choi EC, Lee K. Interaction of cofilin with triose-phosphate isomerase contributes glycolytic fuel for Na,K-ATPase via Rho-mediated signaling pathway. J Biol Chem 2002;277:48931-7.

Figure captions

Fig. 1 - Representative 2-DE gel protein profiles of control (top) and DFD (bottom) meat samples from the *longissimus thoracis* bovine muscle. Protein spots with significantly different abundance between the two groups of samples are marked and numbered. The numbers indicated in the gels correspond to the Tables 2-4.

Fig. 2 - Relative change (*RC*) in the volume of protein spots with significantly different abundance in the *longissimus thoracis* bovine muscle conversion to DFD meat.

Fig. 3 - Representative 2-DE gel sections for protein spots of fast skeletal myosin regulatory light chain 2 (MYLPF) isoforms from control (left) and DFD (right) meat samples of the *longissimus thoracis* bovine muscle. MYLPF isoforms stained for total protein (SYPRO Ruby; top) and subsequently stained for phosphorylated protein (Pro-Q DPS, bottom) are shown. Isoforms with the same number are located on the same position (p*I* and M_r) across gels stained for total and phosphorylated protein.

Fig. 4 - Protein-protein interaction networks of differentially abundant stress-related protein spots in the *longissimus thoracis* bovine muscle, according to STRING confidence view. The network nodes (circles) are proteins, the edges represent known or predicted functional associations and line thickness is a rough indicator for the strength of the association (threshold: 0.4, medium confidence interval).

Fig. 5 - Dendrogram derived from unweighted pair-group method with arithmetic averaging (UPGMA) cluster analysis based on the matrix of pairwise differences in the relative change (*RC*) of protein spots (in absolute value) with significantly different abundance in the *longissimus thoracis* bovine muscle conversion to DFD meat.