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1 **Associations between protein biomarkers and pH and color traits in *Longissimus***
2 ***thoracis* and *Rectus abdominis* muscles in PDO Maine-Anjou cull cows**

3
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17
18 **ABSTRACT**

19 This study investigated the relationships between a list of 23 protein biomarkers with CIE-
20 *L*a*b** meat color traits and ultimate pH on *Longissimus thoracis* and *Rectus abdominis*
21 muscles of 48 PDO Maine-Anjou cows. The technological parameters were correlated with
22 several biomarkers and were in some cases muscle-dependent. More biomarkers were related
23 to pH_u in LT than in RA muscle. Some consistencies were found, by the common correlation
24 of pH_u with MyHC-IIa and MyHC-IIx. The pH_u of LT muscle was also correlated with other
25 cytoskeletal entities and proteins belonging to metabolism and cellular stress. In contrast to
26 the relationships found between biomarkers and LT pH_u, more proteins were related to the
27 instrumental color coordinates in RA than in LT muscle. The regression equations were
28 parameter and muscle-dependent. Certain of the retained proteins explained more than one
29 color coordinate. Hsp70-Grp75, was positive in the models of *L**, *a**, *b** and *C** of LT and of
30 *b** in the RA muscle. Further Heat shock proteins were strongly related with the meat color
31 coordinates in both muscles. The involvement of metabolic enzymes and myofibrillar proteins
32 in meat color development was also verified in this experiment. This study confirmed once
33 again the importance of numerous biological pathways in beef color.

34 **Keywords:** beef; cows; meat color; pH; protein biomarkers; prediction.

35

36 INTRODUCTION

37 Color, tenderness, juiciness and flavor are the most important attributes of beef eating
38 quality¹. Among these, beef color is the first attribute evaluated by consumers and is the major
39 factor which determines and impacts on the decisions of meat purchase at point of
40 sale². Myoglobin (Mb), a protein present in the sarcoplasm of muscular fibres, is responsible
41 of the state of color in post-mortem (p-m) meat. Mb is an unstable chemical compound and
42 when the oxygen availability is high, it changes to oxymyoglobin (OxyMb) giving meat a
43 bright red color². However, if the oxygen level is low, an oxidation reaction may occur and
44 metmyoglobin (MetMb) of brown color is formed. The above mentioned reactions may be
45 reversible in relation to the amount of oxygen on the meat surface and to other factors. The
46 amount of Mb in muscle fibres may also affect the variations in meat color³ and was reported
47 to be related to mitochondria and its functional status⁴.

48 The implementation of high-throughput analytical tools over the last two decades was an
49 important step towards a better understanding of the complex biological systems that define
50 muscle to meat conversion^{5,6}. Thus, numerous studies have focused on the factors influencing
51 meat color and a recent comprehensive review by Joseph *et al.*⁷ highlighted the importance of
52 the use of proteomic approaches to unravel the mechanisms behind the variations in meat
53 color. For example, Sayd *et al.*⁸ investigated the biochemical mechanisms on sarcoplasmic
54 proteome components responsible of the change in pork color stability. The authors found 22
55 proteins or fragments differentially abundant in *Semimembranosus* muscle between light and
56 dark meat. In beef, Joseph *et al.*⁹ were the first to characterize the sarcoplasmic proteins of
57 two muscles with different color stability. They identified 16 differentially abundant proteins,
58 which included antioxidant and chaperone proteins and enzymes involved in energy
59 metabolism. More recently, we have analyzed the relationships between a list of protein
60 biomarkers of tenderness belonging to myriad biological pathways¹⁰ and color development
61 and pH decline in *Longissimus* muscles of young bulls¹¹. The main correlated proteins were
62 involved in apoptosis, oxidative stress or had chaperone activities.

63 The rate of p-m pH decline influences further the biochemical reactions and structural
64 characteristics of the muscles¹². In order to understand the molecular aspects of meat quality
65 development more fully, we have recently performed an extensive study using a proteomic
66 approach and found a number of protein biomarkers, including elements of muscle fibre type
67 to be related with pH parameters¹¹. Hence, the objectives of this study using the PDO

68 (Protected Designation of Origin) Maine-Anjou cows and two different muscles (*Longissimus*
69 *thoracis* (LT, ribeye steak) and *Rectus abdominis* (RA, flank steak)) sampled early p-m were
70 for the first time **i**) to compare the muscle characteristics and meat quality of the two sampled
71 muscles of the breed; **ii**) to relate the abundances of a list of 23 protein biomarkers with meat
72 color coordinates and ultimate pH; **iii**) to predict these technological meat quality traits with
73 this list of biomarkers using multivariate analyses and **iv**) to elucidate the underlying
74 mechanisms of meat color on PDO Maine-Anjou cull cows. The PDO Maine-Anjou breed
75 was the second (since 2004) among the 4 breeds allowed to be used in France for PDO meat
76 production. This PDO is composed of around 80% of cows, younger than 10 years of age,
77 having calved at least once and a minimal carcass weight of 380 kg. Steers over 30 months of
78 age with carcass weight of 400 kg minimum can also be found (20%). PDOs are of special
79 importance for the valorization of local breeds and the specifications of animal products under
80 PDO pay an increasing attention. Thus, there is a great interest as an initiative of the breeders
81 association, for the implementation of high-throughput techniques, likely omics techniques,
82 for meat quality management of PDO meat to guarantee great quality for the consumers.

83 MATERIALS AND METHODS

84 *Animals and sampling*

85 A total of forty-eight French Rouge des Prés cull cows (hereafter PDO Maine-Anjou breed,
86 originated in the northwestern part of France) of an average of 67 months old at slaughter
87 were collected from a cooperative of livestock farmers located in the department of Maine-et-
88 Loire (France). Before slaughter, all animals were food deprived for 24 h and had free access
89 to water. The animals were slaughtered in a commercial abattoir (Elivia, Lion d'Angers,
90 49220, France), stunned using captive-bolt pistol prior to exsanguination and dressed
91 according to standard commercial practice. Slaughtering was performed in compliance with
92 French welfare regulations and respecting EU regulations (Council Regulation (EC) No.
93 1099/2009). After slaughter, the hot carcasses were weighed and their classification was
94 graded according to the European beef grading system (CE 1249/2008). In this study, mean
95 hot carcass weight and carcass conformation were 434 ± 30 kg and 4.8 ± 0.8 , respectively. The
96 carcasses were not electrically stimulated and they were chilled and stored at 4°C until 24 h p-
97 m. Muscle samples from the 6th and 7th ribs of *Longissimus thoracis* (LT, mixed fast oxido-
98 glycolytic muscle) and from the dorsal part of *Rectus abdominis* (RA, slow oxidative) were
99 excised from the right-hand side of the carcass of each animal 24 h after slaughter. These two

100 muscles were chosen because **i)** sampling of *M. longissimus* leads to a significant depreciation
101 of the carcass, other easier-to-sample muscles and low depreciative such as *M. rectus*
102 *abdominis* need to be studied, **ii)** the two muscles are known to differ in their contractile and
103 metabolic properties^{21,22} (representing different anatomical regions and divergent growth and
104 functionalities) and **iii)** no overall characterization of their proteome in PDO Maine-Anjou
105 breed was conducted. So, RA muscle might appear as an alternative to LT for fast and routine
106 physico-chemical analysis for studies on beef quality. After muscle sampling, the epimysium
107 was carefully dissected and about 110 g of each muscle sample was taken. Part of the samples
108 was frozen in liquid nitrogen and kept at -80°C until analyzed for determination of enzyme
109 activities, protein extraction for Dot-Blot analysis and myosin heavy chain (MyHC)
110 quantification. The other part was used for meat color coordinates determination.

111 ***pH measurement***

112 The ultimate pH of all cull cows LT and RA muscles were measured 24h p-m using a pH
113 meter (HI9025, Hanna Instruments Inc., Woonsocket, RI, USA) equipped with a glass
114 electrode suitable for meat penetration. For LT muscle the measurements were done between
115 the 6th and 7th rib and for RA muscle at the center of the dorsal part of the muscle. For each
116 animal, five measurements were made (positioned on a horizontal line with about 1.5 cm
117 between two measurements) and the average value was used for analysis.

118 ***Color measurement of meat***

119 The color of the surface meat was monitored at 24h p-m on the two muscles using a
120 portable colorimeter (Minolta Cameras CR 400, Japan) (CIELAB coordinates, light source
121 D65, 8-mm diameter measurement area, 0° standard observer). D65 was chosen as the
122 illuminant because it closely approximates daylight¹³. Calibration was performed by using a
123 white plate supplied by the manufacturer prior to the color determination. Fresh cut slices of
124 muscles of not less than 2.5 cm thick and overwrapped were left on a polystyrene tray to
125 refrigerate at 4°C for 1h to allow blooming. Instrumental meat color measurements were
126 recorded for lightness (L^* = the lightness component, which ranges from 0 to 100 (from black
127 to white)), and the parameters, redness (a^* = from green if negative to red if positive) and
128 yellowness (b^* = from blue if negative to yellow if positive). The parameters Chroma (C^*),
129 related to the intensity of color (higher when a^* or b^* are high), and hue angle (h^*), related to
130 the change of color from red to yellow, were calculated using the CIE L^* , a^* , and b^* values
131 with the following equations: $C^* = [(a^{*2} + b^{*2})^{1/2}]$ and $h^* = [\text{ATAN}^{-1} (b^*/a^*)]$. The

132 measurements were done in three replicate series, at nine different locations on each steak to
133 obtain three measures for each muscle and an average value was used for analysis.

134 ***Enzyme activities measurement***

135 Glycolytic and oxidative metabolism was determined by the measurement of enzyme
136 activities of respectively lactate dehydrogenase (LDH; EC 1.1.1.27) and isocitrate
137 dehydrogenase (ICDH; EC 1.1.1.42), using the protocol of Jurie *et al.*¹⁴. These enzymes
138 representative of different steps of the glycolytic and oxidative pathways are currently used
139 for the characterization of the metabolic types of beef muscles¹⁵. Briefly, two hundred mg of
140 frozen muscle was thawed, ground and homogenized with a Polytron during 15 s in a 5%
141 (w/v) solution buffer of 10 mM Trizma-Base (pH 8.0), 250 mM sucrose and 2 mM EDTA.
142 One aliquot of homogenate was centrifuged at 6000g for 15min at 4°C for determination of
143 LDH and ICDH activities. Enzyme activities (means of triplicate) were measured at 25°C
144 using an automatic spectrophotometric analyser (UVIKON 923, Biotek-Instruments,
145 Winooski, VT, USA). The results were expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ of wet muscle.

146 ***Electrophoresis separation of Myosin Heavy Chain (MyHC) isoforms***

147 In addition to the two biomarkers of muscle metabolism described above, three other
148 biomarkers corresponding to Myosin Heavy Chain (MyHC) isoforms were quantified
149 according to Picard *et al.*¹⁶. For that, we have used an adequate SDS-PAGE. Briefly, the
150 myofibrillar proteins were extracted using a buffer containing 0.5 M NaCl, 20 mM Na
151 Pyrophosphate, 50 mM Tris, 1 mM EDTA and 1 mM Dithiothreitol. After homogenization of
152 approximately 100 mg of frozen muscle in 5 mL of the extraction buffer using a Polytron
153 (x22 000) and an incubation time of 5 min at 4°C on ice, the sample was subjected to a
154 centrifugation at 5000 x g for 10 min at 4°C. Following centrifugation, the supernatant was
155 diluted 1:1 (v/w) with glycerol at 87% and stored at -20°C until use. The samples were then
156 mixed with an equal volume of loading buffer (basic 2 x Laemmli) containing 4% SDS (w/v),
157 125 mM Tris (pH 6.8), 20% glycerol (v/v), 10% β -mercaptoethanol (v/v) and 0.02% pyronin
158 Y (w/v), incubated at room temperature for 10 min and then heated (70°C) in a water bath for
159 10 min. The protein extracts (10 μg) were loaded per well onto 0.75-mm-thick gels and were
160 separated using 9.2% polyacrylamide gels. The lower running buffer consisted of 50mM Tris
161 (base), 75mM glycine and 0.05% w/v SDS. The upper running buffer was at 2x
162 the concentration of the lower running buffer and β -mercaptoethanol (0.07% v/v) was added.
163 Ten micrograms of protein extracts were loaded per well onto 0.75-mm-thick gels mounted

164 on a Mini-Protean II Dual Slab Cell electrophoretic system (Bio-Rad). The migration was
165 carried out at a constant voltage of 70 V for 30 hours at 4°C. Controls of bovine muscle
166 containing three (MyHC-I, IIA and IIX) or four (MyHC-I, IIA, IIX and IIB) muscle fibers were
167 added at the extremities of each gel. After migration, the gels were fixed in 30% (v/v) ethanol
168 and 5% acetic acid (v/v) and then stained with colloidal Coomassie Blue R250 for 24 h. Gels
169 were destained in a 30% ethanol (v/v) and 5 % acetic acid (v/v) solution until the background
170 was sufficiently cleared. After staining in a Coomassie Blue dye solution, the gels were
171 scanned and the proportions of the different MyHCs bands were quantified by densitometry
172 with ImageQuant Software 5500 (Amersham Biosciences/GE Healthcare) and expressed in
173 percentage. The quantification of the bands revealed no existence of MyHC-IIb¹⁶ isoform in
174 PDO Maine-Anjou breed, thus only MyHC-I, IIA and IIX isoforms are reported.

175 ***Extraction of proteins for Dot-Blot analysis***

176 Total protein extractions were performed to use subsequently the soluble fractions for Dot-
177 Blot analysis according to Bouley *et al.*¹⁷ and as recently reported by Gagaoua *et al.*¹⁸. Briefly,
178 80 mg of muscle was homogenized using a polytron (x22 000) in a denaturation/extraction
179 buffer containing 8.3M urea, 2M thiourea, 1% DTT and 2% CHAPS. After 30 min of
180 centrifugation at 10 000g at 8 °C, the supernatant was stored at -20°C until use for protein
181 assay and biomarkers quantification by Dot-Blot.

182 ***Protein content determination***

183 The protein concentrations of the protein extracts used in this study were determined
184 according to the dye binding method of Bradford¹⁹ using the Bio-Rad Protein assay (Bio-Rad
185 Laboratories Inc.). Bovine serum albumin (BSA) at a concentration of 1 mg/mL was used as
186 standard and the protein content was calculated from the standard curve.

187 ***Immunological protein quantification***

188 The abundance of 18 protein biomarkers (including intact molecules and their fragments)
189 corresponding to six different biological functions (**Table 1**): ***heat shock proteins*** (α B-
190 crystallin, Hsp20, Hsp27, Hsp40, Hsp70-1A, Hsp70-1B, and Hsp70-Grp75); ***muscle fibre***
191 ***structure*** (α -actin, MyLC-1F and MyBP-H); ***energy metabolism*** (ENO3 and PGM1);
192 ***proteolysis*** (μ -calpain and m-calpain); ***oxidative resistance*** (DJ-1, Prdx6 and SOD1) and
193 ***apoptosis*** (TP53) were determined as recently reported by Gagaoua *et al.*¹⁸ using specific
194 antibodies. The specificity of the antibodies was checked by western blots. An antibody was

195 considered specific against the studied protein when only one band at the expected molecular
196 weight was detected by western blot. Optimal dilution ratios for each of the 18 antibodies
197 were determined at the same time, using the conditions indicated by the supplier of the
198 reactant and adapted to bovine muscle samples¹¹. Conditions retained and suppliers for all
199 primary antibodies dilutions are reported in **Table 1**.

200 Protein extracts (15 µg) of each of the LT (n = 48) and RA (n = 48) muscle samples were
201 spotted (four replications per muscle sample) on a nitrocellulose membrane with the Minifold
202 I Dot-Blot apparatus from Schleicher & Schuell Biosciences (Germany) in a random order on
203 the 96-spot membrane. In addition, a mixed standard sample (15 µg) was deposited for data
204 normalization. The Dot-Blot membranes were air-dried for 5 min, blocked in 10% PBS milk
205 buffer at 37°C for 20 min, and then incubated to be hybridized with the specific primary
206 antibody of each protein. Subsequently, the membranes were incubated at 37°C for 30 min
207 with the anti-mouse fluorochrome-conjugated LICOR-antibody IRDye 800CW (1 mg/mL).
208 Infrared fluorescence detection was used for quantification of the relative protein abundances.
209 Subsequently, the membranes were scanned using the Odyssey NIR imager (LI-COR
210 Biosciences), with an 800 nm laser, a 169 µm spatial resolution and a fixed gain of 5. Finally,
211 the Dot-Blot images were quantified with GenePix PRO v6.0 (Axon). Each dot volume was
212 calculated as the total dot intensity from which the median local background value multiplied
213 by the dot area was subtracted. Because Dot-Blot offers the possibility of replicates, a data-
214 prefiltering approach was implemented to eliminate outlier values mainly due to dust. The
215 exclusion technique of outliers was based on the Medium Absolute Difference (MAD) and
216 applied before repeated values were averaged. Finally, to make the data comparable between
217 assays, the data were normalized using a regression-approach based on the used mix standard
218 specific for each muscle. Thus, relative protein abundances were based on the normalized
219 volume and expressed in arbitrary units.

220 *Statistical analysis*

221 Data were analyzed using Statistical Analysis System (SAS) software version 9.2 (SAS
222 Institute, Cary, NJ, USA). Before analysis, raw data means were scrutinized for data entry
223 errors and outliers. To determine the significance of differences between the least squares
224 means of color coordinates, ultimate pH and proteins between the two muscles, PROC GLM
225 procedure of SAS was performed. Significant differences between LT and RA muscles were
226 performed using Tukey's test at a significance level of $P < 0.05$.

227 The PROC CORR of SAS was used to compute the Pearson's correlations of coefficients
228 between the 23 biomarkers with ultimate pH and color coordinates parameters. Correlation
229 values were considered significant at $P < 0.05$.

230 Principal components analyses (PCA) were carried out using the PROC PRINCOMP
231 procedure of SAS to visually illustrate the biomarkers, the color coordinates and pH
232 according to the studied muscles (LT vs. RA). The overall Kaiser's Measure of Sampling
233 Adequacy of the performed PCA was 0.76. To confirm that the two muscles are correctly
234 separated (discriminated) by the PCA, a *K*-means cluster analysis ($k = 2$ or 3) was performed.
235 This was done using the variability explained by the axes of the PCA with eigenvalues > 1.0
236 after *Z*-scores calculation. *K*-means clustering is a widely used classification method that
237 generates a specific number of classes (non-hierarchical). In our case, the Euclidean matrix
238 distance was applied to classify the samples into 2 or 3 classes to confirm the separation of the
239 two muscles using the variables projected on the PCA.

240 In addition, for each color coordinate parameter and pH_u, a PCA was carried out using
241 only the correlated biomarkers. They aimed to illustrate visually the correlated biomarkers
242 with each technological quality trait.

243 Multiple regression analyses were performed using PROC REG of SAS to create best
244 models for pH and color coordinates (as dependent variables, x) using the 23 protein
245 biomarkers (as independent variables, y) for each muscle. For that, the option “optimal
246 model” was chosen to produce the model with the highest r^2 value. The r^2 value expressed as
247 a percentage will be referred to as the % of variability explained. The maximal number of
248 explanatory variables (among the 23 proteins) to be retained in the models was set at 4 to
249 meet to the principle of parsimony (one variable each ten observations (animals)). Variables
250 that were significant but contributed less than 2% in terms of explanatory power (r^2) were
251 excluded from the model. Partial R-squares, regression coefficients, t-values and significance
252 of each retained variable are given for the models. Regression analyses were further
253 conducted on the above dependant variables using both muscles (LT and RA) as one data set.
254 For that, the data of LT and RA muscles were first standardized for muscle effect using the
255 Proc Standard of SAS 9.2 to obtain *Z*-scores. A *Z*-score represents the number of standard
256 deviations for each observation relative to the mean of the corresponding animal: $z = \frac{x-\mu}{\sigma}$
257 where x is the raw value, μ is the mean of the population for each muscle and σ is the standard
258 deviation of the same population. Finally, the absence of collinearity was systematically

259 verified for each regression model, by producing condition indices and variance proportions
260 using the COLLIN procedure of SAS. Variables were identified as collinear if they possessed
261 both a high condition index > 10 and a proportion of variation > 0.5 for two or more traits¹¹,
262 ¹⁵.

263 RESULTS

264 *Instrumental meat color coordinates, ultimate pH and protein biomarkers*

265 The results of instrumental color and ultimate pH values of LT and RA muscles are
266 presented in **Table S2**. The results revealed that there are no significant differences for
267 Lightness (L^*) and hue (h^*) between the two muscles. The Redness (a^*), Yellowness (b^*) and
268 Chroma (C^*) indices were significantly different ($P < 0.001$) between LT and RA muscles.
269 The three parameters were the highest in LT muscle. The ultimate pH (pHu) was significantly
270 different ($P < 0.001$) between the two muscles and was the highest in RA muscle. The
271 findings showed that the lower the pHu is, the highest are the redness and yellowness values.

272 Mean and standard deviations of the biomarkers abundances are summarized in **Table S2**
273 except those of myosin heavy chains isoforms presented in **Figure 1**. Among the quantified
274 biomarkers, nearly most proteins (18 from 23) were significantly different ($P < 0.05$) between
275 the two muscles. Globally, Hsp proteins are higher abundant in RA than LT muscle.
276 Significant differences ($P < 0.05$) were found for α B-crystallin, Hsp27 and Hsp70-1A. The
277 abundances of Hsp20, Hsp40, Hsp70-1B and Hsp70-Grp75 were not different between the
278 two muscles. Among the four metabolism proteins, they were all significantly different ($P <$
279 0.05). ENO3 and ICDH were the highest in RA, and LDH and PGM1 were the highest in LT
280 muscle. All the structural proteins quantified were significantly different between the two
281 muscles. The abundances of α -actin, MyBP-H and MyHC-IIa were the highest in LT muscle.
282 Those of MyLC-1F, MyHC-I and MyHC-IIx isoforms were the highest in RA muscle. The
283 abundances of the proteins of oxidative stress, namely DJ-1, Prdx6 and SOD1 were
284 exclusively the highest in RA muscle. Significant differences were also obtained for the
285 proteolytic enzymes. Thus, μ -calpain was the highest in LT and m-calpain was the highest in
286 RA muscle. Finally, TP53, a protein representative of apoptosis, was found to differ
287 significantly ($P < 0.001$) between the two muscles and was the highest in RA muscle.

288 The PCA computed using color parameters, ultimate pH and abundances of the 23
289 biomarkers is given on **Figure 2**. The score plot on the first and second principal components

290 showed a clear separation between the two muscles, and this differentiation was mainly
291 caused by the first principal component (PC). Together, the two first PCs accounted for 40.6%
292 of the total variance, with the first and second components explaining 26.8% and 13.8% of the
293 total variability, respectively. It allowed discriminating efficiently the two studied muscles on
294 the two first axes (LT on the right (red circles) and RA on the left (green circles)). The
295 separation was confirmed using a *K*-means clustering by choosing the option to construct two
296 or three classes. As can be seen on **Figure 2a**, the option of two clusters, separated perfectly
297 the 48 animals, regrouping in classes 1 and 2 only observations of LT and RA muscle,
298 respectively. Moreover, the option of three clusters (**Figure 2b**) allowed obtaining three
299 groups and once again the observations of RA muscle were all grouped together from those of
300 LT muscle subdivided by this option into LT class1 and 2, respectively. This confirms that
301 PCA allowed, by means of the studied variables, the separation of LT and RA muscles.
302 Finally, further PCAs were performed for each muscle using all the variables and are
303 presented as supplementary data (**Figure S2**).

304 Correlations calculated per muscle showed that L^* was correlated with b^* ($r = 0.52$, P
305 <0.001) in LT muscle only. For both muscles, a^* was strongly correlated with b^* . Chroma
306 (C^*) was found to be slightly correlated ($r = 0.36$, $P <0.05$) with the hue angle (h^*) in LT
307 muscle only. Finally, pHu was not correlated ($P > 0.1$) with color coordinates in both muscles.

308 *Relationships between meat color coordinates, pHu and biomarker abundances*

309 The significant correlations found between the relative abundances of biomarkers and the
310 technological quality traits for LT and RA muscles are given in **Table 2**, respectively. For LT
311 muscle, only L^* , h^* and pHu were correlated with the abundances of protein biomarkers and
312 most of them, mainly structural and metabolic proteins, were with pHu. Lightness (L^*) was
313 correlated with four proteins, negatively with Hsp20 ($r = -0.26$, $P <0.1$) and Hsp40 ($r = -0.29$,
314 $P <0.05$) and positively with PGM1 ($r = 0.32$, $P <0.05$) and TP53 ($r = 0.34$, $P <0.05$). Hue
315 angle was correlated positively with three proteins, namely Hsp70-1A ($r = 0.26$, $P <0.1$),
316 ENO3 ($r = 0.32$, $P <0.05$) and TP53 ($r = 0.31$, $P <0.05$). Finally, pHu was correlated with
317 eight proteins, negatively with Hsp70-1A ($r = -0.24$, $P <0.1$), PGM1 ($r = -0.46$, $P <0.01$),
318 LDH ($r = -0.46$, $P <0.01$), α -actin ($r = -0.34$, $P <0.05$), MyLC-1F ($r = -0.38$, $P <0.01$) and
319 MyHC-IIx ($r = -0.38$, $P <0.01$) and positively with ICDH ($r = 0.44$, $P <0.01$) and MyHC-IIa
320 ($r = 0.33$, $P <0.05$).

321 For RA muscle, the technological quality traits were all found to correlate with several
322 biomarkers. L^* was correlated negatively with ICDH ($r = -0.26$, $P < 0.1$) and MyHC-I ($r = -$
323 0.33 , $P < 0.05$) and positively with LDH ($r = 0.28$, $P < 0.05$) and MyHC-IIx ($r = 0.34$, P
324 < 0.05). a^* was correlated with two proteins only, negatively with Hsp70-1A ($r = -0.29$, P
325 < 0.05) and positively with μ -calpain ($r = 0.27$, $P < 0.1$). b^* was correlated with five proteins,
326 three of them are large Hsp70s (negatively with Hsp70-1A ($r = -0.30$, $P < 0.05$) and Hsp70-
327 1B ($r = -0.24$, $P < 0.1$) and positively with Hsp70-Grp75 ($r = 0.24$, $P < 0.1$)). The two other
328 proteins are PGM1 ($r = 0.29$, $P < 0.05$) and Prdx6 ($r = 0.27$, $P < 0.1$). C^* was correlated with
329 four proteins, negatively with two Hsp70 proteins (Hsp70-1A ($r = -0.31$, $P < 0.05$) and
330 Hsp70-1B ($r = -0.25$, $P < 0.1$)) and positively with ENO3 ($r = 0.29$, $P < 0.05$) and μ -calpain (r
331 $= 0.25$, $P < 0.1$). Hue angle was slightly correlated with three proteins, positively with α -actin
332 ($r = 0.26$, $P < 0.1$) and Prdx6 ($r = 0.24$, $P < 0.1$) and negatively with MyHC-IIa ($r = -0.26$, P
333 < 0.1). Finally, independently of muscle type, pHu was correlated with two structural proteins,
334 positively with MyHC-IIa ($r = 0.33$, $P < 0.05$) and negatively with MyHC-IIx ($r = -0.32$, P
335 < 0.05). A graph showing these common correlations is given in **Figure S1a,b**.

336 The biomarkers that were significantly correlated with color coordinates and pHu of LT
337 and RA muscle and given above, were introduced into PCAs (**Figure 3** and **4**). For LT muscle,
338 the first two PCAs axes explained 65.4, 71.4, and 64% of the variability of L^* (**Figure 3a**), h^*
339 (**Figure 3b**), and pHu (**Figure 3c**), respectively between animals. For RA muscle, the first
340 two axes of the PCAs explained 71.3, 63.1, 65.1 and 74.1% of the variability of L^* (**Figure**
341 **4a**), b^* (**Figure 4b**), h^* (**Figure 4c**) and C^* (**Figure 4c**), respectively between animals. The
342 PCAs of a^* and pHu which were correlated with two biomarkers only are not illustrated.

343 **Regression analyses of meat color coordinates and pHu for LT muscle**

344 The regression models of LT muscle using color coordinates and pHu as dependent and
345 biomarker proteins abundances as independent variables are presented in **Table 3**. The models
346 were significant but less powerful and they explained between 14 and 30% of variability. The
347 model of L^* explained 30% of the variability between animals ($P < 0.01$) by three Hsp
348 proteins: Hsp70-Grp75 (positive) and Hsp40 and Hsp20 (both negative). The model of a^*
349 explained 21% of the variability ($P < 0.05$) by Hsp27 (negative) and ICDH and Hsp70-Grp75
350 (both positive). The model of b^* explained 14% of the variability ($P < 0.05$) by Hsp70-Grp75
351 and MyHC-I (both positive) and Hsp20 (negative). The C^* model explained 25% of the
352 variability ($P < 0.05$) by Hsp70-Grp75 and ICDH (both positive) and MyHC-IIa and Hsp20

353 (both negative). For the four above models (L^* , a^* , b^* and C^*), Hsp70-Grp75 was already
354 retained as an explanatory variable. The h^* model explained 23% of the variability ($P < 0.01$)
355 by three new variables not retained for the other color coordinates, namely the glycolytic
356 enzymes ENO3 and PGM1 (both positive) and MyBP-H (negative). Finally, the model of
357 ultimate pH included two variables, ICDH (positive) and Hsp70-1A (negative), and explained
358 27% of the variability ($P < 0.001$). In this model, ICDH alone explained 20% of the
359 variability.

360 ***Regression analyses of meat color coordinates and pHu for RA muscle***

361 The regression models of RA muscle using color coordinates and pHu as dependent and
362 biomarker proteins abundances as independent variables are presented in **Table 4**. The models
363 were significant but less powerful and they explained between 14 and 33% of variability. The
364 model of L^* explained 33% of the variability between animals ($P < 0.01$) and retained three
365 structural proteins. MyHC-IIx and Hsp27 were positive and MyBP-H and α -actin were
366 negative in the model equation. The model of a^* explained 29% of the variability ($P < 0.01$)
367 by Hsp70-1A (negative) and μ -calpain, Prdx6 and ICDH (all positive). The model of b^*
368 explained 33% of the variability ($P < 0.001$) and highly predictive compared to the LT b^*
369 model. It retained Hsp70-1A (negative) and Prdx6 and Hsp70-Grp75 (both positive) as
370 explaining variables. The C^* , h^* and pHu models explained between 14 and 19% of the
371 variability and all retained MyHC-IIa as an explanatory variable. The h^* model was explained
372 by structural proteins only (both α -actin and MyHC-IIa). Overall, the retained variables are
373 trait and muscle dependant.

374 ***Regression analyses of meat color coordinates and pHu for both LT and RA muscles***

375 The explanatory powers of regression analyses using Z-scores for both muscles to explain
376 common color coordinates and pHu as one dataset (**Table S3**) were very weak and ranges
377 from 11 to 17%. For certain models, the retained variables are shared with the LT and RA
378 muscles, likely for L^* , a^* , h^* and pHu models.

379 **DISCUSSION**

380 This study investigated the relationship between a list of proteins so far validated as
381 potential biomarkers of meat tenderness with meat color coordinates and pHu in two muscles
382 (*Longissimus thoracis* and *Rectus abdominis*) of PDO Maine-Anjou cows. It allowed us to
383 explore consistencies and differences among the two muscles and revealed thanks to the

384 studied biomarkers some biological mechanisms that may explain the meat color and pHu for
385 this breed. Further, it allowed us to use these prime candidate biomarkers to show the strength
386 of their relationships with the meat traits studied using multivariate analysis.

387 *Differences and similarities between muscle characteristics of LT and RA*

388 The ultimate pH values of all individuals in both muscles were below the threshold of 6.0
389 and were within the normal range for cattle²⁰ but significantly different between the two
390 muscles. The pHu of RA was higher than that of LT muscle. Higher pHu values were already
391 reported in RA muscle²¹. This may be in part related to the type of metabolism of the studied
392 muscles, as RA is known to be less glycolytic than LT²². Also, the composition of fibre type
393 is well-accepted as a vital factor responsible for these variations. When comparing RA and LT
394 muscles, ICDH activity was higher and LDH and PGM1 activities were lower for RA muscle
395 (**Table S2**). These findings may be linked with the higher proportion of oxidative MyHC-I
396 and the lower proportion of MyHC-IIa fibres in RA muscle (**Figure 1**). These properties
397 confirmed the less glycolytic and more oxidative metabolism in RA muscle as previously
398 reported²². Also, muscles with predominantly type I fibres have lower glycogen
399 concentrations than those with type II or fast twitch muscles²³. These earlier findings and the
400 results of this study could explain why higher oxidative and lower glycolytic metabolisms are
401 noted in RA than for LT muscle, hence explaining part of the differences in pH values.

402 The data showed that the lower the ultimate pH is, the highest are redness and yellowness
403 for LT muscle. The fact that differences were detected could be attributed to the variation in
404 pH values²⁴. Also, beef muscle profiling studies showed that physico-chemical and
405 biochemical characteristics of individual muscles in a carcass vary significantly and may
406 impact color attributes differently²⁵. Nevertheless, in contrast to several earlier studies²⁰, no
407 relationship was observed between pHu values and meat color coordinates. Also, no
408 significant difference was found for lightness between the two muscles. This finding is
409 unexpected as it has been shown that differences in muscle structure due to changes caused by
410 protein denaturation may lead to differences in light dispersion and thus L^* values. Changes
411 in L^* values have been reported to be subtle²⁵. We can suppose that the findings may be due to
412 the same variation of bound and free water within the muscle fibres causing a scattering and
413 reflectance of light altering the lightness of the meat. Although a^* and b^* values could
414 reflect important color changes, basing the comparisons on just one parameter may not tell
415 the complete color history. Color is a three-dimensional attribute better described by its hue,

416 lightness and saturation properties. The sample's C^* values for the two muscles had a similar
417 behavior to that of a^* and b^* values, increasing in LT samples and decreasing in RA samples,
418 but h^* -values did not differ between the two muscles. The low h^* angle obtained in both
419 muscles indicates less meat browning²⁶ and, therefore, a benefit to PDO Maine-Anjou meat
420 quality. Furthermore, the higher C^* of LT muscle compared to RA muscle indicates a more
421 vibrant color and the LT muscle can be considered more beneficial from a meat quality
422 perspective.

423 *Relationship between biomarkers in the early post-mortem period and ultimate pH*

424 To date, no single factor can explain more than 50% of the variation in pH_u which is often
425 associated with p-m muscle glycogen content and metabolism. Although the molecular and
426 cellular mechanisms that regulate p-m pH decline are not yet fully understood, proteomic
427 strategies implemented over the last decade have revealed numerous biological pathways to
428 be involved¹¹. Thus, the present study is the first to link between a list of protein biomarkers
429 so far validated for meat tenderness with ultimate pH of the two muscles of PDO Maine-
430 Anjou cows.

431 The results showed clearly and in agreement to the well documented literature data^{10, 18, 27,}
432 ²⁸, that the relationships are muscle-dependent. More biomarkers are related to pH_u in LT
433 than in RA muscle. Nevertheless, some consistencies exist. MyHC-IIa (positive) and MyHC-
434 IIx (negative) were correlated to pH_u in both LT and RA. The correlation between pH_u and
435 MyHC-IIx was recently reported for LT muscle in young Blond d'Aquitaine bulls¹¹ but the
436 direction is inversed for PDO Maine-Anjou breed (negative). MyHC-IIx content is usually
437 associated with glycogen amount²⁹ and a potential for a faster metabolism, which may explain
438 the relationship with pH decline, thus suggesting this protein as a prime candidate biomarker
439 of pH_u independently of muscle and breed. Overall, muscles composed of type II fibres are
440 more susceptible to p-m glycolysis than muscles composed of type I fibres³⁰, and these may
441 explain the relationships with pH decline.

442 pH_u in LT muscle was correlated with further structural proteins and others belonging to
443 metabolism and Heat shock proteins pathways. Earlier studies reported that pH_u was related
444 with levels of various muscle proteins present in beef and pork muscles early p-m^{11, 31}. The
445 relationships with α -actin and MyLC-1F have been for example already described by
446 Gagaoua *et al.*¹¹. In coherence with our findings, previous proteomic studies found that during
447 the early p-m period, actin fragments are released and that pH decline and actin degradation

448 were closely related⁵. Actin, a component of microfilaments, is the second most abundant
449 protein in skeletal muscle⁶ and was reported to undergo intense degradation during the p-m
450 period⁵. Higher actin content was found in LT compared to RA muscle, and this may be
451 related to the differences in the final pHu values. MyLC-1F is a member of the myosin light
452 chain family; which is the principal component of myofibrils associated both with myosin
453 heavy chain heads and actin³². It is highly expressed in fast-twitch skeletal muscle and prone
454 to phosphorylation³³. In p-m muscle, protein phosphorylation is known to play important roles
455 in meat quality development by regulating the activities of glycolytic enzymes, hence
456 explaining the relationship with pHu. These data are in agreement with the results presented
457 by Choi *et al.*³⁴ who reported that meat with faster glycolysis rates contain less MyLC.

458 pHu of LT muscle was further related with proteins of energy metabolism, negatively with
459 glycolytic (PGM1 and LDH) and positively with oxidative (ICDH) enzymes. In the early p-m
460 period, as long as glycolytic enzymes are not inhibited by low pH, glycolysis will continue,
461 thus contributing to the acidification process. Changes in energy metabolism in beef LT
462 muscle early p-m were investigated using proteomic studies. A clear shift in p-m energy
463 metabolism was indicated. For example, an increase of PGM1 during ageing has been
464 reported³⁵. PGM1 catalyzes the production of 3-phosphoglycerate and ATP from 1,3-
465 bisphosphoglycerate.

466 As expected, LDH was also related to pHu. It is a key glycolytic enzyme that catalyzes the
467 inter-conversion of pyruvate and lactate, with concomitant inter-conversion of NADH and
468 NAD⁺. It is among the metabolic enzymes which are already identified by proteomic studies
469 to be related with meat qualities⁶. Recently and in agreement to our findings Liang *et al.*³⁶
470 reported that LDH was negatively correlated with changes in intramuscular pH levels.

471 ICDH involved in the tricarboxylic acid (TCA) cycle was already identified to play a great
472 role in p-m beef metabolism³⁷. It was retained in the explanatory model of LT muscle and
473 overall model for both muscles. In LT muscle, this enzyme explained 20% of the variability
474 of pHu. Although the explanatory powers of the models are weak (<27%), the biological
475 significances of the retained variables are of great interest for a better understanding of the
476 mechanisms.

477 Hsp70-1A was both slightly correlated with pHu and retained in the prediction model of
478 LT muscle. In a recent study by our group, we showed that large Hsp proteins are related to
479 pH decline¹¹. Heat shock proteins (HSPs), also called stress proteins, are considered to be

480 molecular chaperones which play a universal physiological role in maintaining cellular
481 homeostasis³⁸. Pulford *et al.*³⁹ showed that variation in bull beef quality due to ultimate
482 muscle pH is in part related to Hsp proteins levels. The correlation with pH may be explained
483 as a response to cellular stress since they have been reported to be induced in skeletal muscle
484 and to bind to the structural proteins to maintain homeostasis.

485 ***Relationships between biomarkers and meat color coordinates***

486 Inversely to the relationships found between biomarkers and LT pHu, more proteins were
487 related to instrumental color coordinates in RA than in LT muscle. For LT muscle, only L^*
488 and h^* values were correlated with 4 and 3 proteins, respectively. In RA muscle, all the color
489 coordinates were correlated with several biomarkers, 4 proteins with L^* , 2 with a^* , 5 with b^* ,
490 4 with C^* and 3 with h^* .

491 The findings of this study confirm the muscle-dependent relationship between the
492 biomarkers and meat quality traits. The regression equations are also trait and muscle-
493 dependent and there is no universal model. Nonetheless, some consistencies exist and some
494 proteins entered in the regression equations of more than one coordinate. For example,
495 Hsp70-Grp75 (a glucose regulated protein) was retained (positive) in the models of L^* , a^* , b^*
496 and C^* of LT muscle and of b^* in the RA. The central role of this biomarker for meat quality
497 was already reported⁴⁰. It was found to be related to meat color coordinates in LT muscle of
498 different young bulls, namely Blond d'Aquitaine¹¹. Grp75 is a cytoprotective chaperone
499 triggered by glucose deprivation, Ca^{2+} influx, agents perturbing glycolysis or oxidative
500 stress⁴¹. The lack of oxygen and nutrients in p-m muscle might lead to accumulation of ROS
501 in the mitochondria, the end of ATP production and a deregulation of Ca^{2+} which may set off
502 apoptosis⁵. Apoptosis was reported to play significant role in the muscle to meat conversion
503 process⁵. In the present study, Grp75 content soon after slaughter was the highest in LT
504 muscle characterized also by high abundances of μ -calpain and low ultimate pH value. Earlier
505 studies reported that μ -calpain activity is influenced by Grp75 activity⁴². Grp75 was also
506 reported to enhance Ca^{2+} trafficking from endoplasmic reticulum towards mitochondria and
507 cytosol⁴³. Possibly, increased Ca^{2+} levels may exert regulatory effects on many enzymes,
508 namely μ -calpain and proteins involved in metabolic and apoptotic processes. A biological
509 mechanism involving Grp75 in muscle to meat conversion was recently described by our
510 group^{11, 18}. By these mechanisms, structural proteins may be altered and once their reflectance
511 is affected, a direct relationship with meat color may occur. Grp75 could also interact with

512 antioxidant enzymes regulating its activity during the depletion of redox potential NADH¹⁸,
513 thus affecting meat color traits.

514 Further Hsp proteins were in this study closely related with the meat color coordinates in
515 both LT and RA muscles. These findings are in agreement to several proteomic studies
516 aiming to understand meat color^{9, 44-46}. Small and large Hsp proteins were involved. For
517 example, Hsp70-1A was negatively correlated with a^* , b^* and C^* in RA muscle. The
518 potential role of this inducible chaperone in meat color development was recently reported in
519 several studies^{9, 11, 31}. Hsp70 (HSPA1A/HSPA1B), a crucial member of Hsps, is believed to
520 play various protective roles in skeletal muscle and regulating apoptosis. Furthermore, small-
521 Hsp proteins (Hsp20 and 27) were negatively related with L^* and already entered in the
522 regression equations of L^* , a^* , b^* and C^* of LT muscle. In agreement to our findings, the
523 expression levels of Hsp27 were negatively correlated with L^* and a^* of Korean native
524 cattle⁴⁷. Similarly, in earlier studies Hsp27 was overabundant in dark porcine
525 *Semimembranosus* muscles⁸. Hsp27 play a central role in organizing and protecting
526 myofibrillar structures, as suggested by its localization to the Z-line and the I-band in muscle⁶.
527 In addition to these activities, Hsp20 and 27 have the ability to collaborate to control the
528 redox status⁴⁸. We suppose that the involvement of Hsps in meat color may be in part related
529 to their protective role against protein denaturation known to affect the reflectance of meat
530 aspects.

531 Metabolic enzymes were also correlated with color coordinates and entered in the
532 regression equations of both muscles. For example, glycolytic enzymes were earlier found to
533 be related with instrumental beef color parameters^{9, 40, 44, 45}. PGM1 was positively correlated
534 with L^* in LT muscle and with b^* in RA muscle. Recently, a positive correlation was reported
535 between PGM1 and a^* values of beef LT muscle⁴⁴. Previous investigations reported
536 significant correlations of PGM1 with instrumental color parameters of pork meat.
537 Kwasiborski *et al.*³¹ reported a negative correlation with a^* values. In broiler breast, a
538 negative correlation was also reported with L^*_{24h} ⁴⁵. Phosphorylation increases the enzymatic
539 activity of this protein, thus accelerating the conversion of glucose-1-phosphate to glucose-6-
540 phosphate, which in turn favors the generation of substrates necessary for regeneration of
541 NADH⁴⁴. NADH is an important co-factor in enzymatic and non-enzymatic metmyoglobin
542 reduction². ENO3 was in this study positively correlated with h^* in LT muscle and with C^* in
543 RA muscle. The enzyme was also retained as an explanatory variable (first) in the h^* model
544 of LT muscle. ENO3 is involved in the conversion of 2-phosphoglycerate to

545 phosphoenolpyruvate during glycolysis. Joseph *et al.* reported that ENO3 was over-abundant
546 in color-stable *Longissimus lumborum* compared with color-labile *Psoas major*⁹ and a
547 positive correlation was found with a^* values. ENO3 expression was reported to be
548 influenced by differences in glucose levels and deprivation of oxygen supply⁴⁹. These
549 research findings were interpreted to indicate that ENO3 was a critical enzyme related to meat
550 color. Finally, LDH was found to be slightly related with L^* in RA muscle. LDH may help
551 replenish the pool of p-m NADH. Production of reducing equivalents via this enzyme seemed
552 to be used for metmyoglobin reduction⁷ and thus affect the color coordinates of beef meat.

553 Antioxidant proteins are slightly related in this study with instrumental color parameters.
554 Only Prdx6 was correlated with b^* and h^* coordinates and entered in the regression equations
555 of a^* and b^* of RA muscle. It is also retained in the models of a^* , b^* and C^* for both muscles
556 when grouped as one data set. Prdx6, a bifunctional protein with both glutathione peroxidase
557 (GPx, antioxidative) and phospholipase A2 (PLA2) activities was reported by our group to
558 play a significant role in pH decline of LT muscle of young bull breeds¹¹. To date little is
559 known about the pathways by which Prdx6 may impact meat color. It was suggested that the
560 production of Prdx6 during storage could be regarded as counteraction against the meat
561 discoloration, as the antioxidant protein contributes to protection against meat
562 discoloration^{9,11}. Further, Joseph *et al.* found that Prdx2, a member of peroxiredoxins
563 superfamily, overabundant in bovine *Longissimus lumborum*, was a good model for color-
564 stable muscle. The expression of peroxiredoxins may be considered an indicator for meat
565 discoloration.

566 The involvement of myofibrillar proteins in meat color development was also confirmed in
567 this study. Several structural proteins were correlated with L^* and h^* in RA muscle but never
568 in LT muscle. Also, several among them entered in the regression equations of color
569 coordinates. Little studies have reported relationships between myofibrillar proteins and meat
570 color traits, since most of them investigated the sarcoplasmic proteins only. For example, the
571 association between slow twitch oxidative fibres (type I) and L^* has recently been
572 reported¹¹ and may be related to their relatively high content of myoglobin, which explains
573 part of the variation in meat color. These fibres contain high levels of inducible Hsp70
574 (closely related to a^* , b^* and C^*), of which the potential role has been described above.
575 Otherwise, as an important regulatory protein of the thick filament, MyBP-H has significant
576 effects on length, thickness, and lateral alignment of myosin filaments, thus its involvement in
577 meat color may be of great significance.

578 The findings of the present study indicated that the protein biomarkers related to pH and
579 meat color traits would be of primary importance for the beef industry, which has to satisfy
580 the increasing consumer expectations. The present study used for the first time Dot-Blot as a
581 fast and routine throughput technique, to quantify pre-selected proteins of interest in two
582 muscles of PDO Maine-Anjou. This study confirmed once again the importance of several
583 biological pathways in beef color and some of them are muscle-dependent. Looked at in more
584 detail, ultimate pH is influenced by many biological pathways. The knowledge of these
585 processes allows a faster understanding of similarities but also differences that may exist
586 according to each muscle. The proteins related to both technological parameters were also
587 reported to play great roles in the determinism of meat tenderness trait^{5, 6, 10, 27}, thus common
588 biological pathways may be shared. In addition, the variables were able to separate between
589 the two muscles and some of them would be used for the authenticity of muscle cuts. Thus,
590 the meat industry may benefit from the use of these biomarkers, once validated, as a tool for
591 investigation, monitoring or decision/management of meat depending on the desired quality
592 trait.

593 **Notes**

594 The authors declare no competing financial interest.

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Figure captions

Figure 1. Electrophoretic separation (A) of MyHC isoforms in m. *Longissimus thoracis* and m. *Rectus abdominis*. The different wells correspond to animals ($n = 4$) selected randomly. Samples of bovine *Longissimus thoracis* with three (C1) and four (C2) types of MyHC were used as controls. Proportion (B) and variance analysis of the different MyHC isoforms (I, IIA, IIX) detected in the two muscles after densitometry analysis. *** $P < 0.001$. Fibre types: I, slow oxidative fibre; IIA fast oxido-glycolytic fibre; IIX, fast glycolytic fibre. Fibre types IIB were not detected in PDO Maine-Anjou cows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure 2. Principal component analysis: A) distribution of protein biomarker abundances and technological meat traits (instrumental meat color and ultimate pH) for both LT and RA muscles of the PDO Maine-Anjou cull cows; B and C) bi-plots showing the distribution of the two muscles (LT in red and RA in green; or RA in green and LT in blue and yellow, respectively) on the first two principal axes according to the K -means classification. B) A bi-plot obtained after $k = 2$, showing the perfect separation of LT (in red) and RA (in green) muscles using the variables projected by the PCA. C). A bi-plot obtained after $k = 3$, showing the perfect separation of RA (in red) from LT muscle and the clustering of this later into two sub-classes (LT_class1 (in blue) and LT_class2 (in yellow)) with a very weak separation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure 3. Principal component analyses showing relationships (from Table 2) between (A) Lightness (L^*), (B) hue (h^*) and Chroma (C^*) pHu and the correlated biomarkers on *Longissimus thoracis* muscle of PDO-Maine Anjou.

Figure 4. Principal component analyses showing relationships (from Table 2) between (A) Lightness (L^*), (B) Yellowness (b^*), (C) Hue (h^*) and (D) Chroma (C^*) and the correlated biomarkers on *Rectus abdominis* muscle of PDO-Maine Anjou. The PCA of Redness (a^*) and pHu which were correlated with two biomarkers only are not performed.

Table 1. List of the eighteen protein biomarkers quantified using the Dot-Blot technique (Gagaoua *et al.* 2015). The suppliers and conditions for each primary antibody used in this study are given.

Protein biomarker name (gene)	UniProtKB ID	Monoclonal (Mo) or Polyclonal (Po) antibodies references	Antibody dilutions
<i>Heat Shock Proteins</i>			
α B-Crystallin (<i>CRYAB</i>)	P02511	Mo. anti-bovine Assay Designs SPA-222	1/500
Hsp20 (<i>HSPB6</i>)	O14558	Mo. anti-human Santa Cruz HSP20-11:SC51955	1/200
Hsp27 (<i>HSPB1</i>)	P04792	Mo. anti-human Santa Cruz HSP27 (F-4):SC13132	1/3000
Hsp40 (<i>DNAJ1</i>)	P31689	Mo. anti-human Santa Cruz HSP40-4 (SPM251):SC-56400	1/250
Hsp70-1A (<i>HSPA1A</i>)	Q27975	Mo. anti-human RD Systems MAB1663	1/2000
Hsp70-1B (<i>HSPA1B</i>)	Q27965	Mo. anti-human Abnova HSPA1B (M02), clone3B7	1/3000
Hsp70-Grp75 (<i>HSPA9</i>)	P38646	Mo. anti-human RD Systems Clone 419612	1/250
<i>Metabolism</i>			
Enolase 3 (<i>ENO3</i>)	P13929	Mo. anti-human Abnova Eno3 (M01), clone 5D1	1/45000
PGM 1 (<i>PGM1</i>)	Q08DP0	Mo. anti-human Abnova PGM1 (M01), clone 3B8-H4	1/8000
<i>Structure</i>			
α -actin (<i>ACTA1</i>)	P68133	Mo. anti-Rabbit Santa Cruz α -actin (5C5):SC-58670	1/1000
MyLC-1F (<i>MYL1</i>)	P05976	Po. anti-human Abnova MYL1 (A01)	1/1000
MyBP-H (<i>MYBPH</i>)	Q13203	Mo. anti-human Abnova MYBPH (M01), clone 1F11	1/4000
<i>Oxidative resistance</i>			
DJ-1 (<i>PARK7</i>)	Q99497	Po. anti-human Santa Cruz DJ-1 (FL-189):SC-32874	1/250
Prdx6 (<i>PRDX6</i>)	P30041	Mo. anti-human Abnova PRDX6 (M01), clone 3A10-2A11	1/500
SOD1 (<i>SOD1</i>)	P00441	Po. anti-rat Acris SOD1 APO3021PU-N	1/1000
<i>Proteolysis</i>			
μ -calpain (<i>CAPN1</i>)	P07384	Mo. anti-bovine Alexis μ -calpain 9A4H8D3	1/1000
m-calpain (<i>CAPN2</i>)	Q27971	Mo. anti-bovine ABR M-calpain MA3-942	1/1000
<i>Apoptosis</i>			
Tumor protein p53 (<i>TP53</i>)	P67939	Mo. anti-human Abnova TP53, clone HR231, MAB1969	1/1000

Abbreviations: PGM1: Phosphoglucomutase 1; MyLC: Myosin Light chain; MyBP-H: Myosin Binding protein H; DJ-1: Protein deglycase 1; Prdx6: Peroxiredoxin-6; SOD: Superoxide dismutase [Cu-Zn].

Table 2. Pearson correlations between technological traits and biomarkers in *Longissimus thoracis* and *Rectus abdominis* muscle samples. Only the significant correlations are shown.

Biomarkers	<i>Longissimus thoracis</i>			<i>Rectus abdominis</i>					
	<i>L</i> *	<i>h</i> *	pHu	<i>L</i> *	<i>a</i> *	<i>b</i> *	<i>C</i> *	<i>h</i> *	pHu
Heat shock proteins									
Hsp20	-0.26t								
Hsp40	-0.29*								
Hsp70-1A		+0.26t	-0.24t		-0.29*	-0.30*	-0.31*		
Hsp70-1B						-0.24t	-0.25t		
Hsp70-Grp75						+0.24t			
Metabolism									
ENO3		+0.32*					+0.25t		
PGM1	+0.32*		-0.46**			+0.29*			
ICDH			+0.44**	-0.26t					
LDH			-0.46**	+0.28t					
Structure									
α -actin			-0.34*					+0.26t	
MyLC-1F									
MyHC-I			-0.38**	-0.33*					
MyHC-IIa			+0.33*				-0.26t		+0.33*
MyHC-IIx			-0.38**	+0.34*					-0.32*
Oxidative stress									
Prdx6						+0.27t		+0.24t	
Proteolysis									
μ -calpain					+0.27t		+0.25t		
Apoptosis									
TP53	+0.34*	+0.31*							

Significance: *t* < 0.1; * < 0.05; ** < 0.01

The coefficients in bold character show the robust and common correlations found for both LT and RA muscles and the two muscles grouped in one data set using *Z*-scores.

Table 3. Regression equations of meat color coordinates and pHu of *Longissimus thoracis* samples from PDO Maine-Anjou cull cows using protein biomarkers.

Dependent variable	R-square	S.E	Entered independent variable ¹	Partial R ²	Regression coefficient	t-value	P-value ²
Lightness (L*)	0.30**	0.14	Hsp70-Grp75	0.08	+0.50	+3.50	0.001
		0.15	Hsp40	0.16	-0.40	-2.67	0.010
		0.14	Hsp20	0.06	-0.27	-1.91	0.046
Redness (a*)	0.21*	0.17	Hsp27	0.04	-0.47	-2.87	0.006
		0.16	ICDH	0.06	+0.41	+2.59	0.013
		0.15	Hsp70-Grp75	0.11	+0.36	+2.50	0.016
Yellowness (b*)	0.14*	0.16	Hsp70-Grp75	0.05	+0.39	+2.43	0.019
		0.16	Hsp20	0.04	-0.47	-1.95	0.045
		0.15	MyHC-I	0.05	+0.24	+1.53	0.070
Chroma (C*)	0.25*	0.16	ICDH	0.05	+0.44	+2.81	0.007
		0.16	MyHC-IIa	0.05	-0.44	-2.83	0.007
		0.15	Hsp70-Grp75	0.07	+0.41	+2.78	0.008
		0.15	Hsp20	0.08	-0.34	-2.19	0.034
Hue (h*)	0.23**	0.14	ENO3	0.10	+0.43	+3.03	0.004
		0.16	MyBP-H	0.08	-0.41	-2.62	0.012
		0.15	PGM1	0.05	+0.26	+1.77	0.053
Ultimate pH	0.27***	0.13	ICDH	0.20	+0.46	+3.56	0.000
		0.13	Hsp70-1A	0.07	-0.27	-2.08	0.040

¹ Variables are shown in order of their entrance in the prediction models.

² Significance of the models: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4. Regression equations of meat color coordinates and pHu of *Rectus abdominis* muscle samples from PDO Maine-Anjou cull cows using protein biomarkers.

Dependent variable	R-square	S.E	Entered independent variable ¹	Partial R ²	Regression coefficient	t-value	P-value ²
Lightness (L*)	0.33**	0.14	MyHC-IIx	0.12	+0.52	+3.79	0.000
			Hsp27	0.07	+0.43	+3.00	0.003
			MyBP-H	0.07	-0.36	-2.62	0.012
			α -actin	0.07	-0.27	-2.08	0.043
Redness (a*)	0.29**	0.14	Hsp70-1A	0.09	-0.45	-3.28	0.002
			μ -calpain	0.09	+0.37	+2.73	0.009
			Prdx6	0.05	+0.26	+1.93	0.049
			ICDH	0.06	+0.25	+1.83	0.054
Yellowness (b*)	0.33***	0.13	Hsp70-1A	0.09	-0.48	-3.62	0.000
			Prdx6	0.12	+0.37	+2.90	0.005
			Hsp70-Grp75	0.12	+0.35	+2.72	0.009
Chroma (C*)	0.19**	0.14	ICDH	0.10	-0.39	-2.81	0.007
			MyHC-IIa	0.09	+0.32	+2.29	0.026
Hue (h*)	0.17*	0.14	α -actin	0.08	+0.30	+2.17	0.035
			MyHC-IIa	0.07	-0.29	-2.13	0.038
Ultimate pH	0.14*	0.14	MyHC-IIa	0.11	+0.32	+2.33	0.025
			TP53	0.04	+0.20	+1.44	0.070

¹ Variables are shown in order of their entrance in the prediction models.

² Significance of the models: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

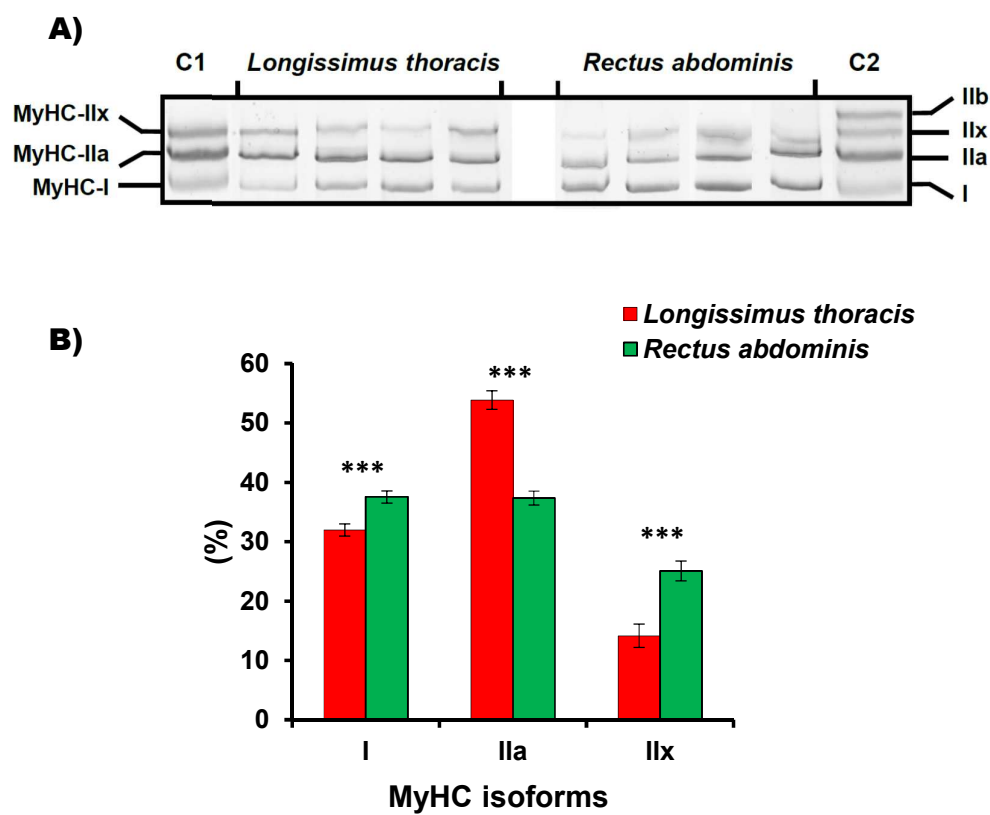


Figure 1.

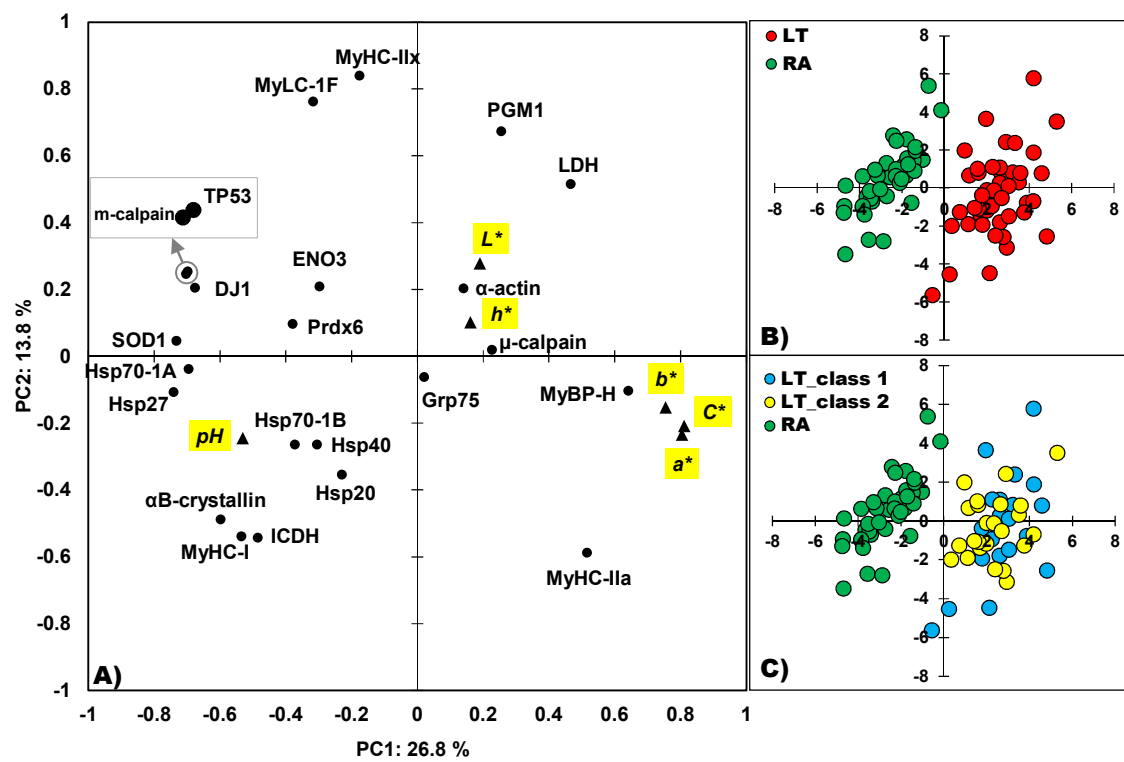


Figure 2.

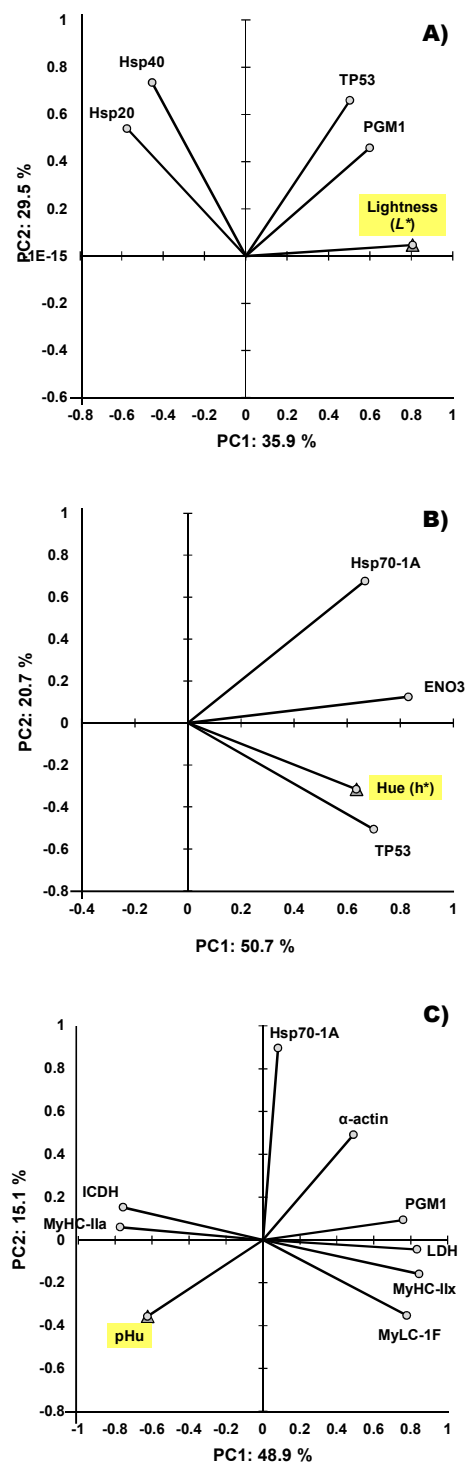


Figure 3.

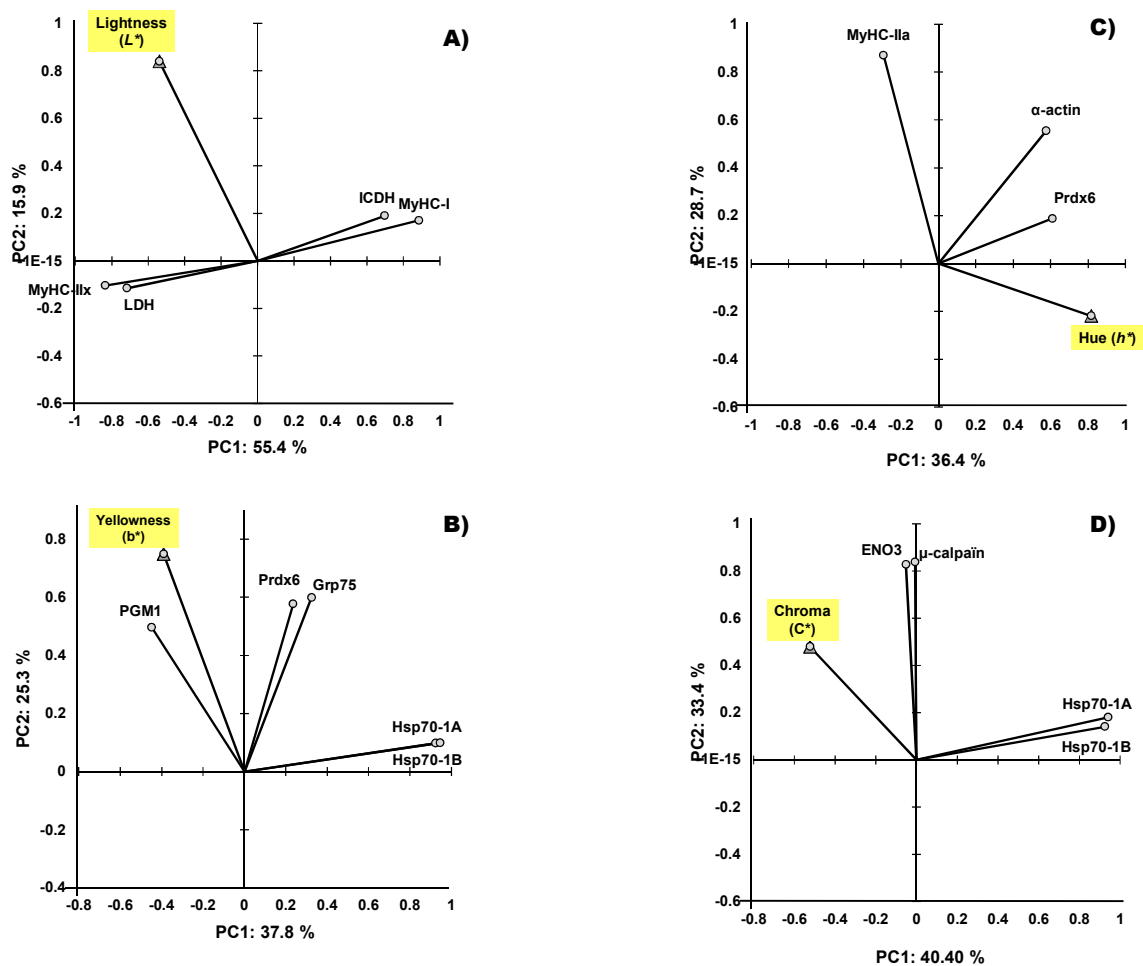
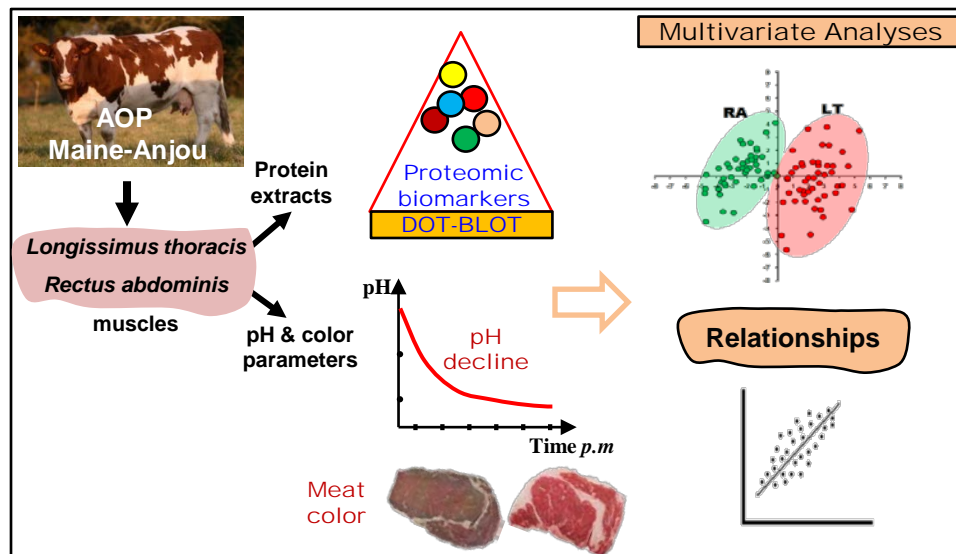


Figure 4.

TOC



Associations between protein biomarkers and pH and color traits in *Longissimus thoracis* and *Rectus abdominis* muscles in PDO Maine-Anjou cull cows

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Associated Content

Supporting Information

Supplementary Table S1. Variance analysis of the instrumental meat color and ultimate pH (LSmeans \pm SD) of the *Longissimus thoracis* and *Rectus abdominis* muscles of the PDO Maine-Anjou dairy cull cows.

Supplementary Table S2. LSmeans \pm SD and effect of muscle type (*Longissimus thoracis* vs. *Rectus abdominis*) on the protein expression of the studied biomarkers on PDO Maine-Anjou cows. Protein abundances were quantified in arbitrary units unless stated.

Supplementary Table S3. Regression equations of meat color coordinates and pHu of both *Longissimus thoracis* and *Rectus abdominis* muscle samples from PDO Maine-Anjou cull cows using protein biomarkers.

Supplementary Figure S1. Pearson correlation analyses between myosin heavy chains fibers (MyHC-IIx, MyHC-IIa and MyHC-I) with ultimate pH of both muscles (LT, red circles and RA, green circles) of the PDO Maine-Anjou cows. The correlations of ultimate pH with **A**) MyHC-IIx ($r = -0.35$; $P = 0.0005$); **B**) MyHC-IIa ($r = +0.32$; $P = 0.001$) and **C**) MyHC-I ($r = +0.19$; $P = 0.067$). The first graphs (**A** and **B**) illustrate the common and robust correlations found for both muscles.

Supplementary Figure S2. Principal components analysis performed by muscle. Projection of the variables (left) and bi-plot (right) for **A)** *Longissimus thoracis* and **B)** *Rectus abdominis* muscles of PDO Maine-Anjou, respectively.

Table S1. Variance analysis of the instrumental meat color and ultimate pH (LSmeans \pm SD) of the *Longissimus thoracis* and *Rectus abdominis* muscles of the PDO Maine-Anjou dairy cull cows.

Traits	<i>Longissimus thoracis</i>	<i>Rectus abdominis</i>	SEM	<i>p</i> -value
Lightness (<i>L</i> *)	39.85 \pm 2.17	39.56 \pm 1.91	0.21	ns
Redness (<i>a</i> *)	8.96 \pm 1.25	6.07 \pm 1.02	0.19	***
Yellowness (<i>b</i> *)	6.88 \pm 1.37	4.49 \pm 0.90	0.17	***
Chroma (<i>C</i> *)	11.32 \pm 1.67	4.56 \pm 1.28	0.25	***
Hue (<i>h</i> *)	37.28 \pm 4.19	36.36 \pm 3.62	0.40	ns
Ultimate pH	5.60 \pm 0.11	5.73 \pm 0.14	0.01	***

Significance: ns: not significant; *** < 0.001

Table S2. LSmeans \pm SD and effect of muscle type (*Longissimus thoracis* vs. *Rectus abdominis*) on the protein expression of the studied biomarkers on PDO Maine-Anjou cows. Protein abundances were quantified in arbitrary units unless stated.

Biomarkers	<i>Longissimus thoracis</i>	<i>Rectus abdominis</i>	SEM	<i>p</i> -value
Heat shock proteins				
α B-crystallin	248 \pm 95	327 \pm 101	11.1	***
Hsp20	176 \pm 50	178 \pm 44	4.9	ns
Hsp27	81 \pm 19	120 \pm 24	3.0	***
Hsp40	133 \pm 27	136 \pm 19	2.5	ns
Hsp70-1A	119 \pm 26	163 \pm 37	4.1	***
Hsp70-1B	194 \pm 57	210 \pm 42	5.4	ns
Hsp70-Grp75	145 \pm 32	138 \pm 14	2.6	ns
Metabolism				
ENO3	144 \pm 36	161 \pm 21	3.3	**
PGM1	96 \pm 31	79 \pm 17	2.7	*
ICDH ¹	1.07 \pm 0.4	1.38 \pm 0.4	0.04	***
LDH ¹	705 \pm 110	620 \pm 75	10.6	***
Structure				
α -actin	121 \pm 33	110 \pm 17	2.9	*
MyLC-1F	63.6 \pm 15	79.7 \pm 14	1.7	***
MyBP-H	126 \pm 18	63 \pm 37	4.5	***
Oxidative stress				
DJ-1	92 \pm 16	114 \pm 10	1.8	***
Prdx6	108 \pm 20	118 \pm 16	2.0	**
SOD1	87 \pm 42	153 \pm 21	5.0	***
Proteolysis				
μ -calpain	146 \pm 37	130 \pm 14	3.1	*
m-calpain	96 \pm 14	118 \pm 13	1.8	***
Apoptosis				
TP53	97 \pm 20	129 \pm 16	2.5	***

¹: Metabolic enzyme activities expressed in $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$.

Significance: ns: not significant; P * < 0.05; P ** < 0.01; P *** < 0.001

Table S3. Regression equations of meat color coordinates and pHu of both *Longissimus thoracis* and *Rectus abdominis* muscle samples from PDO Maine-Anjou cull cows using protein biomarkers.

Dependent variable	R-square	S.E	Entered independent variable ¹	Partial R ²	Regression coefficient	t-value	P-value ²
Lightness (L*)	0.14**	0.11	MyHC-IIx	0.05	+0.32	+3.00	0.003
		0.12	Hsp27	0.05	+0.34	+2.94	0.004
		0.11	Hsp20	0.04	-0.22	-2.10	0.038
Redness (a*)	0.17**	0.11	Hsp70-1A	0.05	-0.32	-2.99	0.004
		0.10	μ-calpain	0.04	+0.27	+2.71	0.008
		0.11	Prdx6	0.05	+0.24	+2.24	0.028
		0.10	ICDH	0.03	+0.19	+1.94	0.050
Yellowness (b*)	0.16**	0.11	PGM1	0.05	+0.33	+3.02	0.003
		0.10	Prdx6	0.04	+0.25	+2.54	0.013
		0.10	MyBP-H	0.03	-0.23	-2.24	0.028
		0.11	MyHC-I	0.04	+0.22	+2.08	0.041
Chroma (C*)	0.13**	0.11	Prdx6	0.06	+0.26	+2.40	0.018
		0.11	Hsp70-1A	0.02	-0.25	-2.34	0.021
		0.10	μ-calpain	0.05	+0.23	+2.25	0.027
Hue (h*)	0.11*	0.10	TP53	0.05	+0.23	+2.29	0.020
		0.10	MyBP-H	0.03	-0.20	-2.01	0.040
		0.10	PGM1	0.03	+0.18	+1.79	0.060
Ultimate pH	0.16***	0.10	MyHC-IIa	0.11	+0.25	+2.51	0.014
		0.10	ICDH	0.05	+0.24	+2.41	0.018

¹ Variables are shown in order of their entrance in the prediction models.

² Significance of the models: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

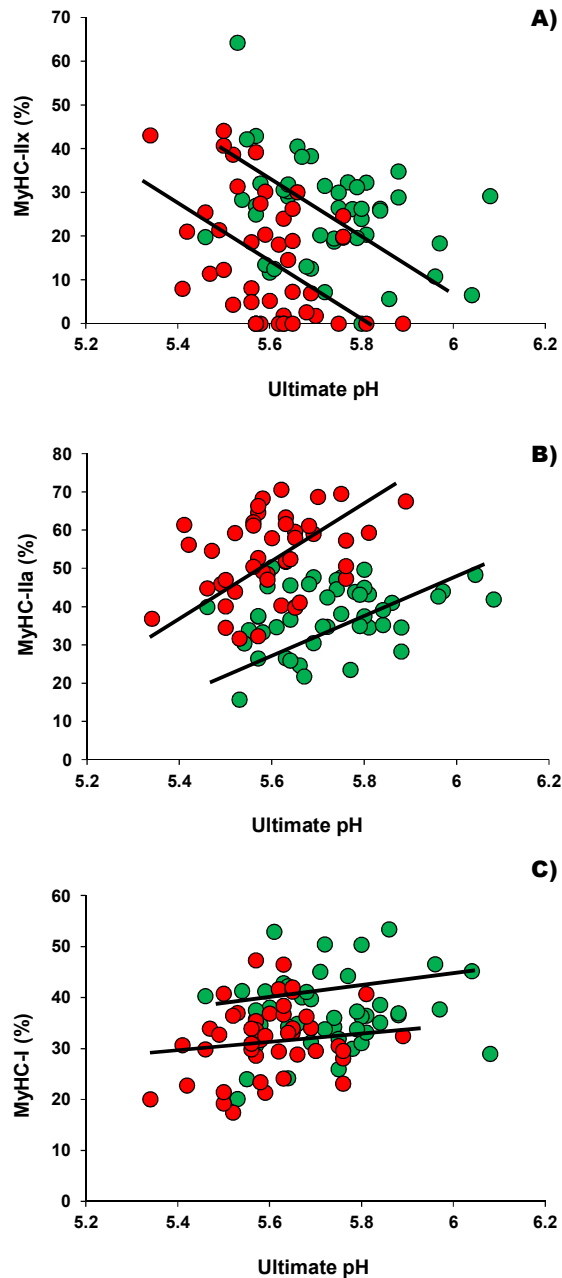


Figure S1. Pearson correlation analyses between myosin heavy chains fibers (MyHC-IIx, MyHC-IIa and MyHC-I) with ultimate pH of both muscles (LT, red circles and RA, green circles) of the PDO Maine-Anjou cows. The correlations of ultimate pH with **A**) MyHC-IIx ($r = -0.35$; $P = 0.0005$); **B**) MyHC-IIa ($r = +0.32$; $P = 0.001$) and **C**) MyHC-I ($r = +0.19$; $P = 0.067$). The first graphs (**A** and **B**) illustrate the common and robust correlations found for both muscles.

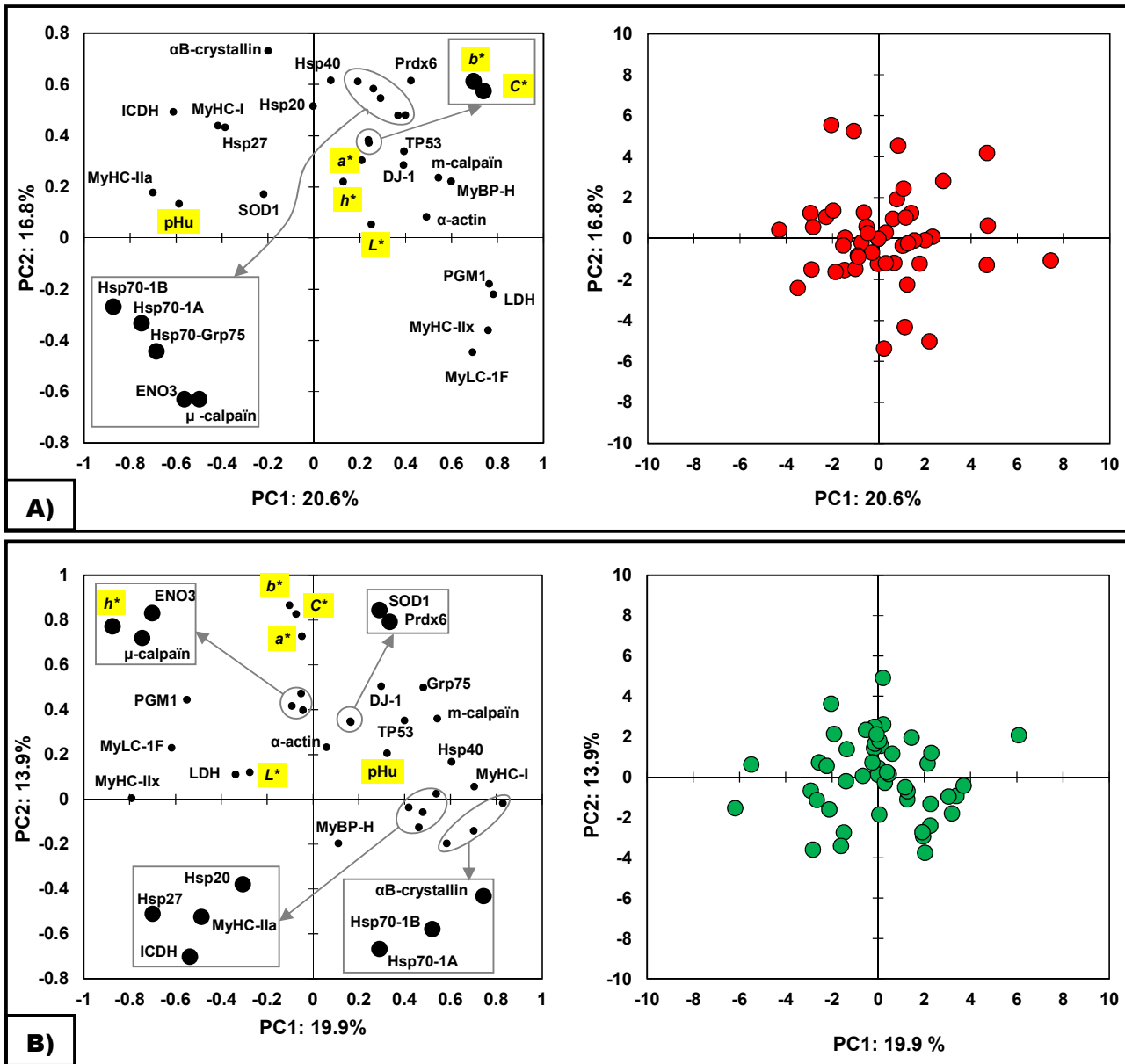


Figure S2. Principal components analysis performed by muscle.

Projection of the variables (left) and bi-plot (right) for **A)** *Longissimus thoracis* and **B)** *Rectus abdominis* muscles of PDO Maine-Anjou, respectively.