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1 **Inverse relationships between biomarkers and beef tenderness according to contractile**  
2 **and metabolic properties of the muscle**

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15 **Abstract**

16 In previous proteomic analyses, we established a list of proteins biomarkers of beef  
17 tenderness. In the present study we quantified the relative abundance of 21 of these proteins  
18 by Dot-blot technique in the *Longissimus thoracis* and *Semitendinosus* muscles of 71 young  
19 bulls from three breeds: Aberdeen Angus (AA), Limousin (LI) and Blond d'Aquitaine (BA).  
20 For both muscles overall tenderness was estimated by sensory analysis, shear force was  
21 measured with a Warner-Bratzler instrument, and an index combining sensory and  
22 mechanical measurements was calculated. Multiple regressions based on relative abundances  
23 of these proteins were used to propose equations of prediction of the three evaluations of  
24 tenderness. Hsp70-1B appeared a good biomarker of low tenderness in the three breeds and in  
25 the two muscles. Proteins such as Lactate dehydrogenase-B, Myosin heavy chain IIX, small  
26 Heat Shock Proteins (Hsp27, Hsp20 and  $\alpha$ B-crystallin) were related to tenderness but  
27 inversely according to the muscle and breed. The results demonstrate that prediction of  
28 tenderness must take into account muscle characteristics and animal type.

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32 **Key words**

33 Biomarkers, beef tenderness, skeletal muscle, heat shock proteins, prediction, Dot-blot

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## INTRODUCTION

35 Beef quality includes sensory quality traits (tenderness, flavour, juiciness, colour, etc),  
36 nutritional value, healthiness and technological quality (which all refer to intrinsic quality  
37 traits) as well as issues like animal welfare, environmental concerns, traceability, etc (which  
38 refer to extrinsic quality traits). A major cause of consumer dissatisfaction is the high and  
39 uncontrolled variability in sensory beef quality, especially tenderness<sup>1</sup>. Muscle characteristics  
40 (fiber type, collagen, intramuscular lipids) can only explain up to 30% of the variability in  
41 tenderness<sup>2-4</sup>. Another problem is that meat intrinsic quality can only be determined at the  
42 time of eating, i.e. after slaughter and cooking, which hampers the production of beef of  
43 consistent good quality. In order to better control sensory quality, it is necessary to have tools  
44 to predict eating quality, especially tenderness, in live animals. Therefore, the beef sector is  
45 looking for biological or molecular indicators to identify live animals with desired quality  
46 attributes, to help beef producers to choose the most appropriate production system, animal  
47 types and markets. To meet this objective, several genomics programs combining genomics,  
48 transcriptomics, proteomics, computational biology and biochemistry have been carried out  
49 over the past years (for review: <sup>5-9</sup>). Using 2-D electrophoresis techniques, comparisons of  
50 two groups of high *versus* low tenderness allowed the identification of proteins of which  
51 abundance was associated with tenderness<sup>6, 8, 10, 11</sup>. These proteins are representative of  
52 several biological functions: muscle structure, contraction, energetic metabolism, cellular  
53 stress and proteolysis<sup>7, 12</sup>. The objective of the present study was to test the predictive power  
54 of the 21 proteins most strongly associated with tenderness in another group of experimental  
55 young bulls. To do so, we analysed the relationships between protein abundances and  
56 tenderness in young bulls from three beef breeds differing in their precocity and physiological  
57 characteristics. The French Blond d'Aquitaine breed is highly muscled with low  
58 intramuscular fat content<sup>13</sup>, the Angus breed is known to be fat, producing marbled meat,  
59 while Limousin French breed has intermediate properties<sup>14</sup>. Two muscles with differences in  
60 metabolic characteristics and tenderness: *Longissimus thoracis* (LT) and *Semitendinosus*  
61 (ST) were studied. The abundances of the 21 proteins were quantified by the immunological  
62 technique Dot-blot developed by Guillemin *et al.*<sup>15</sup> allowing the simultaneous analysis of  
63 large number of samples for one protein. Relationships between tenderness traits and protein  
64 relative abundance were evaluated using multiple regression analyses.

65

66

## MATERIAL AND METHODS

67 The study was carried out in compliance to the current French welfare  
68 recommendations for the use of experimental animals.

### 69 *Animals and samples*

70 The study was conducted using 71 young entire males of three pure breeds: Aberdeen  
71 Angus (AA) (n =21), Limousin (LI) (n =25) and Blond d'Aquitaine (BA) (n =25). Animals  
72 (12 month-old at start) were assigned to a 100 day finishing period before slaughter. They  
73 were housed in groups of 4 animals of the same breed in 6 x 6 m pens with straw bedding,  
74 individually fed and weighed every 2 weeks. Diets consisted of concentrate (75 %) and straw  
75 (25 %). Animals were slaughtered at the same age (around 17 months) and final live weight  
76 (around 665 kg) in order to avoid weight and age effects on muscle characteristics and beef  
77 meat quality.

78 All bulls were directly transported in a lorry (3 x 2 m) from the experimental farm to  
79 the experimental abattoir situated at 1 km from the rearing building, with 2 bulls of the same  
80 home pen per transport to avoid social isolation stress. After unloading, they were slaughtered  
81 within 3 min in the slaughterhouse of INRA institute (Saint-Genès-Champagnelle, France) in  
82 compliance with the current ethical guidelines for animal welfare. Bulls were stunned by  
83 captive bolt prior to exsanguination.

84 Muscle samples from *Longissimus thoracis* (LT, mixed fast-oxido-glycolytic muscle  
85 23% of type I fibers, 36 % IIA and 39 % of IIX) and *Semitendinosus* (ST, mixed fast  
86 glycolytic 8 % of type I fibers, 24 % of IIA and 64 % of IIX), were excised from the carcass  
87 of each animal within 15 minutes after slaughter. Muscle samples were immediately frozen in  
88 liquid nitrogen and stored at -80°C until protein extraction for protein markers quantification.  
89 Samples of the two muscles for sensory evaluation and mechanical measurement were cut  
90 into steaks 24 hours after slaughter and placed in sealed plastic bags under vacuum and kept  
91 between 2–4°C for 14 days for ageing, then frozen and stored at -20°C until analysis.

### 92 *Meat quality evaluation*

93 LT and ST samples were grilled on a preheated grill at 310°C, resulting in an internal  
94 cooked temperature of 55°C. For sensory analysis, a trained sensory panel (12 experienced  
95 panellists) evaluated the steak samples of the same muscle. The panel evaluated overall  
96 tenderness attribute on a continuous and unstructured scale with scores from 0 to 10 (0 = hard  
97 – 10 = tender)<sup>16, 17</sup>.

98 Toughness of cooked meat was further evaluated instrumentally by Warner-Bratzler  
99 shear force (WBSF) using INSTRON 5944 as described by Lepetit and Culioli <sup>18</sup>. Force at  
100 rupture during shear compression testing was expressed in N/cm<sup>2</sup>.

101 Sensory and mechanical values of tenderness were used to compute a synthetic  
102 tenderness index by combining standardized normal sensory and mechanical  
103 tenderness/toughness values<sup>19</sup>. This was calculated for each muscle as the difference within  
104 each breed between the standardized values of tenderness score minus the standardized value  
105 of the WBSF measure. This index was suggested to take into account the very close genetic  
106 correlation underlying the moderate phenotypic correlation observed between both traits <sup>20</sup>,  
107 suggesting both traits are under the control of common genes.

### 108 *Dot-Blot analysis*

109 The 21 proteins analysed are described in **Table 1**. The conditions for use and  
110 specificity of primary antibodies against these 21 proteins in bovine muscle have been  
111 determined previously by Guillemain *et al.* <sup>21</sup> using western blot techniques (**Table 2**).

112 Total protein extractions were performed according to Bouley *et al.* <sup>22</sup> in a  
113 denaturation extraction buffer (8.3M urea, 2M thiourea, 1% DTT, 2% CHAPS). The protein  
114 concentration was determined using the Bradford protein assay <sup>23</sup>. Protein extractions were  
115 stored at -20°C.

116 Relative abundances of proteins were evaluated following the Dot-blot technique as  
117 described by Guillemain *et al.* <sup>21</sup>. Briefly, protein samples were spotted in quadruplicate on a  
118 nitrocellulose membrane with the Minifold I Dot blot from Schleicher & Schuell Biosciences  
119 (Germany) and hybridised with the specific antibody of each protein, with conditions  
120 described in **Table 2**. Secondary fluorescent-conjugated IRDye 800CW antibodies (anti-  
121 mouse, anti-sheep and anti-rabbit) were supplied by LI-COR Biosciences (Lincoln, Nebraska,  
122 USA) and used at 1/20000. Subsequently, membranes were scanned by an Odyssey (LI-COR  
123 Biosciences, Lincoln, NA, USA) scanner at 800 nm. Protein relative abundance for each  
124 sample, given in arbitrary units, was normalised by comparison to a reference sample  
125 constituted by mixing all samples from young bulls from this experiment in equal proportions.  
126 The technical coefficient of variation of this technique is in average of 9% <sup>21</sup>.

### 127 *Statistical analysis*

128 Analysis of variance was performed using the GLM procedure of SAS for repeated  
129 measured (Version 9.1, 2002; SAS Institute Inc.). The effects of breed, muscle-type (LT vs

130 ST) and breed x muscle-type interaction are reported. When significant effects were detected,  
131 differences were evaluated by the PDIFF option of SAS.

132 Principal component analyses were performed using the factor procedure of SAS to  
133 study the overall relationships between the studied variables (tenderness traits and protein  
134 relative abundances).

135 Multiple regression analyses were carried out using XLStat 2009 software to explain  
136 overall tenderness, WBSF and tenderness index traits for the two muscles separately,  
137 presenting the 21 protein biomarkers as potential explanatory variables, as well as breed. The  
138 'optimal model' explaining maximal variability option was used with 'maximal 4 variables'.  
139 The percentage of variability in meat quality parameter explained by proteins is based on the  
140 adjusted  $r^2$  value of the regression analysis x 100%.

141 Absence of colinearity was verified for each model. Condition indices and variance  
142 proportions were produced using the COLLIN option of SAS, with components identified as  
143 collinear if they possessed both a high condition index greater than 10 and a proportion of  
144 variation greater than 0.5 for two or more variables.

## 145 RESULTS

### 146 *Tenderness traits*

147 The values of tenderness evaluated by three ways are presented in **Table 3**. For the LT  
148 muscle, significant breed effects were found for overall tenderness only, AA bulls being more  
149 tender than BA and LI bulls (**Table 3**). In the ST muscle, significant differences between  
150 breeds were observed for the WBSF and the tenderness index. A significant muscle x breed  
151 interaction was observed for WBSF and tenderness index. Limousin ST muscle being tougher  
152 than BA, while AA had intermediate values (**Table 3**). Overall tenderness (**Table 3**) was  
153 much higher in the LT. For WBSF, this was only a tendency.

### 154 *Protein relative abundances*

155 Significant breed effects were found for at least one of the two muscles for most of the  
156 proteins except for Hsp70-1B, MyBP-H, and SOD1 (**Table 4**). The two muscles of AA bulls  
157 presented higher levels of  $\alpha$ B-crystallin, Hsp27, Eno3 and PRDX6 and lower levels of  
158 MyHC-IIx. Most proteins showed an effect of muscle or a muscle x breed interaction, apart  
159 from MyLC-1F, MyBP-H, SOD1 and  $\mu$ -calpain (**Table 4**).

### 160 *Relationships between biomarkers and tenderness traits*

161 *Descriptive analysis*

162 The principal component analysis (PCA) illustrating the relationships between relative  
163 abundances of protein biomarkers and tenderness traits evaluated by i) sensory analysis  
164 (overall tenderness), ii) Warner-Bratzler shear force (WBSF) and iii) tenderness index are  
165 presented in Figure 1 (a-d). In the two muscles, overall tenderness and tenderness index were  
166 positively correlated, and both were negatively correlated with the WBSF

167 - *LT muscle*

168 As illustrated in **Figure. 1a**, high overall tenderness scores were positively associated  
169 with small Hsp proteins (Hsp27, Hsp20,  $\alpha$ B-crystallin), Eno3, structural protein CapZ- $\beta$  and  
170 antioxidants PRDX6 and DJ-1 when considered across breeds. Low tenderness scores were  
171 associated with proteins of the Hsp70 family (Hsp70-8, Hsp70-1A/B and Hsp70/Grp75), or  
172 related to fast glycolytic muscle fibres (e.g. LDH-B, MyHC-IIx). The average loadings of the  
173 different breeds (**Figure. 1b**) differed on the first axis (BA<LI<AA;  $p=0.0001$ ) and on the  
174 second axis, the AA bulls differed from the other breeds (AA>LI, BA;  $p=0.001$ ). Compared to  
175 LI and BA, LT muscles of AA bulls were characterised by higher abundances of small Hsp  
176 (Hsp27, Hsp20,  $\alpha$ B-crystallin), Hsp40, Eno3, CapZ- $\beta$ , PRDX6, DJ-1 and lower abundances of  
177 LDH-B and MyHC-IIx (cf **Table 4**).

178 - *ST muscle*

179 In the ST muscles, overall tenderness scores were positively associated with LDH-B  
180 and MyHC-IIx (**Figure. 1c**) and negatively with small Hsp's (Hsp27, Hsp20,  $\alpha$ B-crystallin),  
181 Hsp40, Eno3, MyHC-I, CapZ- $\beta$ , PRDX6, DJ-1. On the first axis (**Figure. 1d**), AA differed  
182 significantly ( $p < 0.0001$ ) from the other breeds (LI, BA < AA) and on the second axis all  
183 breeds differed significantly (AA < LI < BA;  $p < 0.01$ ). As in the LT AA were characterised  
184 by higher abundances of small Hsp, Hsp40, Eno3, MyHC-I, CapZ- $\beta$ , PRDX6, DJ-1 and lower  
185 abundances of LDH-B and MyHC-IIx.

186 - *Comparison of the PCA's of the LT and ST muscles*

187 The PCA demonstrates inverse relationships between some proteins and tenderness in  
188 the two muscles. MyHC-IIx and LDH-B were positively associated with tenderness in ST  
189 muscle and negatively in LT. In contrast, proteins from the small Hsp family, Eno3, Hsp40,  
190 CapZ- $\beta$ , PRDX6 and DJ-1 were negatively correlated with tenderness in ST and positively in

191 LT muscle. In addition, the graphs show that the Angus breed was associated with high  
192 tenderness values in LT muscle and with low tenderness in the ST muscle.

### 193 *Regression analyses*

194 Multiple regression analyses carried out on the pooled data of the three breeds showed  
195 that, for the LT and ST muscles, the proteins explained only 10 - 17% of the variability of the  
196 three tenderness measurements (**Tables 5-7**). The models for overall tenderness retained  
197 (**Table 5**) LDH-B, PRDX6 and Hsp20, Hsp70-1A/B for the ST muscle; Hsp20 and MyHC-  
198 IIX, Hsp70-1B for LT muscle. The models for WBSF retained Hsp70-1B for the two muscles  
199 and MyHC-IIX for the ST muscle (**Table 6**). The models of the tenderness index retained  
200 MyHC-IIX, for the ST and for the LT muscle. The LT tenderness index was further correlated  
201 with  $\alpha$ -actin and with Hsp70-1A/B (**Table 7**).

202 When breeds were considered separately, the prediction power (adjusted  $r^2$ ) improved,  
203 although it varied according to breed and tenderness measurement (**Tables 5 to 7**). The  
204 models for LI bulls had most predictive power. The model of overall tenderness of the ST  
205 muscle of LI bulls retained (**Table 5**) fast MyHC, Hsp70/Grp75 and PRDX6 explaining 53%  
206 of the variability between animals. MyLC-1F and Hsp70-1A/B explained 35% of variability  
207 in overall tenderness of the LT muscle between LI bulls. The model for WBSF explained  
208 even 60% of the variability of the LI breed in both muscles (**Table 6**). Proteins retained in the  
209 models were MyHC-IIX and -II for the ST muscle and LDH-B, Hsp70-1A/B and MDH1 for  
210 the LT muscle (**Table 6**). The model for the tenderness index for both muscles had high  
211 predictive power, explaining 73 and 66% of the variability for the ST and LT, respectively  
212 (**Table 7**).

213 Correlations between predicted and measured values for each tenderness trait among  
214 the two studied muscles are shown (**Figure 2, a-f**).

215 For the AA bulls, none of the 21 quantified protein biomarkers could predict WBSF of  
216 LT muscle (**Table 6**). In the ST muscle, WBSF was predicted by Eno3 and Hsp70-1A/B  
217 (positively) and by  $\alpha$ B-crystallin (negatively). Overall tenderness and tenderness index were  
218 better predicted in the ST than in the LT muscle.

219 For the BA bulls, prediction powers were similar in the two muscles with better  
220 prediction for overall tenderness by Hsp70-1A/B (negatively) in ST and by DJ-1 (positively)  
221 and MyBP-H (negatively) in LT muscle (**Table 5**). The predictions of WBSF and tenderness  
222 index were lower than in the two other breeds (**Table 6 and 7**).



223 Overall, results demonstrate that among the 21 quantified proteins, Hsp70-1A/B was  
224 often retained in the prediction models of the different breeds, and negatively correlated with  
225 the different tenderness measurements in both muscles. Proteins representing fast glycolytic  
226 fibre types such as MyHC-IIx or LDH-B were correlated with tenderness in the two muscles  
227 for the three tenderness traits but the direction of the correlation depended on the muscle, as  
228 also illustrated in the PCA (**Figure 1**): they were positively correlated with tenderness  
229 measurements in the ST and negatively in the LT muscle. The glycolytic Eno3 enzyme was  
230 correlated with the three tenderness indicators in the ST muscle of the AA bulls. PRDX6 was  
231 correlated with ST overall tenderness and tenderness index but never with WBSF.

## 232 DISCUSSION

233 The first objective of this study was to determine in two different muscles of young  
234 Aberdeen Angus, Limousin and Blond d'Aquitaine bulls the relationships between tenderness  
235 indicators and several protein biomarkers previously identified as good predictors of meat  
236 tenderness<sup>6</sup>. The second objective was to propose prediction equations of tenderness based on  
237 the abundances of the biomarkers. Among the quantified proteins, Hsp70-1A/B was the only  
238 protein associated with the different tenderness indicators in the two muscles and the three  
239 breeds. Other proteins including LDH-B, MyHC-IIx, and various small Hsp's were associated  
240 with tenderness, but oppositely in the ST compared to the LT muscle. These data suggest that  
241 different mechanisms underlie tenderness some which are common between both muscles and  
242 while others differ. The underlying mechanisms appear to be related to the contractile and  
243 metabolic properties of the muscle and they differ in the sense that the direction of the  
244 correlation with tenderness depends on the muscle.

### 245 *Muscle and breed specific biomarkers of tenderness*

246 Our findings show that MyHC-IIx and LDH-B are positively and negatively correlated  
247 with tenderness of the ST muscle (fast glycolytic) and LT muscle (fast oxido-glycolytic,  
248 respectively. These opposite correlations are in agreement with earlier studies. For example,  
249 Chaze *et al.*<sup>19</sup> showed in young bulls from three main French beef breeds that in the LT  
250 muscle several proteins representing fast glycolytic properties were negatively correlated with  
251 tenderness and several proteins corresponding to slow oxidative properties were positively  
252 correlated with tenderness. Studying the same muscle in young Blond d'Aquitaine, Morzel *et*  
253 *al.*<sup>24</sup> found Succinate dehydrogenase, an oxidative enzyme, to be a good marker of  
254 tenderness. In Charolais young bulls, fast proteins such as Troponin T fast isoforms,

255 phosphoglucomutase, fast MyHC, glycogen phosphorylase were found to be potential  
256 biomarkers of toughness and slow MyHC, ATP synthase  $\beta$ , ApoBEC were associated with  
257 increased tenderness<sup>12</sup>. Other studies based on fibre types and enzyme activities showed  
258 positive relationships between slow oxidative fibre types and tenderness in the LT and a  
259 negative relationships in the ST muscle<sup>2</sup>. This was further confirmed in a meta-analysis  
260 combining more than 332,000 data on fibre types and enzyme activities of these two muscles  
261 in several types of cattle<sup>4</sup>. The present results indicate also that in the case of a fast glycolytic  
262 muscle such as ST, muscles containing a greater proportion of fast glycolytic fibres will  
263 produce more tender meat and in the case of a more oxidative muscle such as LT, muscles  
264 containing a greater proportion of slow oxidative fibres will produce more tender meat. In the  
265 present study, LT meat of AA bulls was more tender, and ST meat was less tender compared  
266 to the LI and BA breeds. This is coherent with the opposite correlations described above.  
267 Irrespectively of breed, for the LT muscle, the more glycolytic it is, the less tender it is, and  
268 for the ST muscle, the more glycolytic it is, the more it is tender. The LT of AA was less  
269 glycolytic compared to the other breeds, and consequently more tender. The ST of AA was  
270 less glycolytic compared to the other breeds, and consequently, less tender. Other factors,  
271 such as lipid content, may of course also play a significant role.

272 The positive correlation between MyHC-IIx and tenderness observed in the LT muscle  
273 of the AA bulls may seem to contrast with the above idea. Other authors found similarly,  
274 positive relationships between fast glycolytic type and tenderness in LT muscle from animals  
275 with muscles with oxidative characteristics. For example, D'Allessandro *et al.*<sup>25</sup> in  
276 *Longissimus dorsi* from Chianina beef cattle observed that the tender meat group on the basis  
277 of WBSF was characterized by higher levels of glycolytic enzymes.

278 Overall, existing results demonstrate that the contractile and metabolic properties of  
279 muscle play a major role in the elaboration of tenderness.

280 The present study found other opposite associations between proteins and tenderness  
281 according to the muscle or breed. Thus, proteins from the small Hsp family (Hsp20, 27 and  
282  $\alpha$ B-crystallin) were inversely associated with tenderness depending on two muscles. These  
283 proteins were negatively associated with tenderness in the ST muscle and positively in the LT  
284 muscle. These data are in agreement to the results of Guillemin *et al.*<sup>26</sup> which demonstrated  
285 that in ST muscle, Hsp from both Hsp70 family and small sHsp family were inversely  
286 correlated with tenderness as observed in the present study. Nevertheless, these Hsp's were  
287 not correlated with tenderness of the LT muscle. In a previous experiment with Blond

288 d'Aquitaine young bulls we found inverse relationships between sHsp abundances and  
289 tenderness in ST and LT muscles<sup>10</sup>. This could demonstrate that the functions of sHsp depend  
290 on the contractile and metabolic properties of the muscle. Guillemain *et al.*<sup>27</sup> showed that slow  
291 oxidative fibres have the highest abundance of  $\alpha$ B-crystallin. This is in accordance with our  
292 data showing higher abundances in LT than in ST muscle and earlier data reported by  
293 Guillemain *et al.*<sup>26</sup> showing higher abundances in Charolais young bulls and AA than in LI  
294 and BA.

295 Overall, these results allow understanding why the correlations between one  
296 biomarker and tenderness could be inversed as described in the literature. Our results give  
297 explanations as described in **Figure 3**. These relationships according to muscle and breed  
298 types need to be confirmed in other muscles and animal types.

299 In the present study, PRDX6, an antioxidant enzyme was associated with tenderness  
300 mainly of the ST muscle, but in opposite directions, depending on the breed. This is in  
301 accordance with earlier results. Guillemain *et al.*<sup>27</sup> found that PRDX6 is a biomarker of low  
302 tenderness of the ST muscle in young Charolais bulls while Jia *et al.*<sup>28</sup> reported an over  
303 expression of PRDX6 in tender meat in young Norwegian Red bulls. The antioxidant action  
304 of PRDX6 is based on the hydrolysis of hydrogen peroxides and by facilitating repair of  
305 damaged cell membranes *via* reduction of peroxidised phospholipids<sup>7,29</sup>. Antioxidant  
306 enzymes such as PRDX6 and also SOD1 are involved in the protection of the cell against  
307 oxidative stress which is causal of free radicals of oxygen, resulting in formation of protein  
308 aggregates<sup>30</sup>. These aggregates may hamper the tenderization process of the meat. In  
309 agreement with this finding, D'Alessandro *et al.*<sup>25</sup> proposed that oxidative stress promotes  
310 meat tenderness and elicits heat shock protein responses. Ouali and co-workers<sup>7,31</sup> proposed  
311 that the first step of the conversion of muscle into meat is the onset of apoptosis. This death  
312 process is energy dependent and involve a large number of proteins included Hsp families.  
313 D'Alessandro *et al.*<sup>25</sup> discusses the possible causes of opposite relationships between  
314 abundances of oxidative enzymes and tenderness. The authors speculate that *postmortem*  
315 metabolism in tender and tough meat is subtly modulated *via* higher levels of specific  
316 enzymes and amino acidic residue phosphorylation in a breed-specific fashion. Results of the  
317 present study showing that PRDX6 is positively associated with overall tenderness in AA and  
318 negatively in LI young bulls are in accordance with this suggestion. Guillemain *et al.*<sup>27</sup>  
319 showed that the relationship between antioxidant enzymes such as PRDX6 and also SOD1  
320 was more significant in Charolais steers than in young bulls, suggesting that gender may also

321 play a role. They showed that SOD1 was correlated with tenderness only in steers, which may  
322 explain the absence of an association between tenderness and SOD1 in the present study.

323 Overall, these data demonstrate that the contractile and metabolic properties of muscle  
324 play a major role in the elaboration of tenderness. It is likely that mechanisms underlying the  
325 determinism of tenderness involve several biological processes such as apoptosis, oxidative  
326 stress, and proteolysis which depend probably on these muscle properties.

### 327 ***Hsp70-1A/B a good candidate biomarker of meat tenderness in the three breeds***

328 Proteins from the Hsp70 family (Hsp70-1A/B, Hsp70-8, GRP 75) were recently found  
329 to be related to tenderness<sup>8</sup>. In the present study, among Hsp70's, only Hsp70-1A/B (gene  
330 HSPA1B) also called Hsp70-2 depending on species, was negatively associated to overall  
331 tenderness and tenderness index and positively with WBSF in the two muscles across the  
332 three breeds.

333 The members of the Hsp70 family serve a variety of roles: i) they act as molecular  
334 chaperones facilitating the assembly of multi-protein complexes, ii) they participate in the  
335 translocation of polypeptides across cell membranes and to the nucleus, and iii) they help in  
336 the proper folding of nascent polypeptide chains<sup>32</sup>. In the *Diaphragma* muscle from Holstein-  
337 Friesian cattle, Sugimoto *et al.*<sup>33</sup> showed that misfolding of energy-related proteins due to  
338 Hsp70 deficiency might lead to protein aggregation and muscle fiber degeneration. This is in  
339 coherence with the negative association with tenderness observed in the present study.  
340 Moreover, the study of Crawford and Horowitz<sup>34</sup> showed that in particular scaffolding  
341 proteins and chaperone proteins such as Hsp90 and 70 are required for individual steps in the  
342 assembly of myofibril. The study of Miyabara *et al.*<sup>35</sup> showed that Hsp70 improves structural  
343 and functional recovery of skeletal muscle after disuse atrophy. This is in agreement with  
344 several studies showing that Hsp70 is one of the most important heat shock protein for  
345 maintenance of cell integrity during normal cellular growth as well as under  
346 pathophysiological conditions<sup>36,37</sup>. These data suggest that Hsp70 is important for  
347 maintaining structural, ultrastructural and functional properties of skeletal muscle. Possibly,  
348 Hsp70-1A/B plays also an important role in structural modifications during *post-mortem*  
349 ageing. Hsp70-1A/B has further an anti-apoptotic role in skeletal muscle. Gao *et al.*<sup>36</sup>  
350 reported a function of Hsp70-1A/B in regulating TNF- $\alpha$ -induced cell apoptosis. By forming a  
351 complex Hsp70/CHIP/ASK1, Hsp70 promotes ASK1 proteasomal degradation and prevents  
352 TNF- $\alpha$ -induced cell apoptosis. Hsp70 proteins are also known to sequester pro-apoptotic

353 factors such as BCL-2<sup>38</sup>. These data are in agreement with the important role of apoptosis in  
354 meat ageing<sup>7,39</sup>.

355 The present study found that in contrast to Hsp70-1A/B, the association between the  
356 relative abundance of the other Hsp70's and tenderness traits differed according to breed and  
357 muscle. This may be explained by different regulation of the expression of these Hsp70's in  
358 the muscle. In the present study, Hsp70/Grp75 and Hsp70-8 were less abundant in AA than in  
359 BA, particularly in the LT muscle. However, Hsp70-1A/B abundance did not differ between  
360 breeds, in the two muscles. These data are in agreement with the results of Guillemin *et al.*<sup>27</sup>  
361 who showed no effect of gender on Hsp70-1A/B abundance between steers and young bulls.  
362 This would explain the association between Hsp70-1A/B and tenderness across muscles and  
363 breeds observed in the present study. The lack of consistent associations between tenderness  
364 and Hsp70/Grp75, may be explained by the fact that it is exclusively expressed in the  
365 mitochondrial matrix, and that it is involved in the translocation and folding of nascent  
366 polypeptide chains of both nuclear and mitochondrial origin<sup>40</sup>.

367 These findings suggest that Hsp70-1A/B could be a relatively general biomarker of  
368 tenderness in different muscles and breeds compared to proteins discussed earlier which  
369 appear to be quite strongly muscle or breed specific.

370 In conclusion, this study shows that some biomarkers of tenderness such as MyHC IIX,  
371 LDH-B and small Hsp are dependent of the contractile and metabolic properties of the muscle  
372 (cf **Figure 3**), explaining their opposite relationships with tenderness in the ST compared to  
373 the LT muscle and in AA compared to LI and BA bulls. It further shows that Hsp70-1A/B is a  
374 biomarker of low beef tenderness across the breeds and muscles studied. All these biomarkers  
375 need further testing in various muscles from cattle of different genders and breeds before they  
376 can be used as biomarkers in routine immunological testing.

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## Figure captions

**Figure 1.** Principal component analysis. **a)** and **c)** Distribution of protein abundance and meat tenderness traits (overall tenderness, WBSF and tenderness index) for the LT and ST muscles respectively; **b)** and **d)** Distribution of the three breeds on the first two principal axes (mean  $\pm$  standard errors) for LT and ST muscles respectively

**Figure 2.** Correlations between predicted and measured beef tenderness traits (for tenderness, WBSF and tenderness index) using best models for the three breeds among LT (a, b and c) and ST muscles (d, e, f). Adjusted R-squares corresponding to the prediction equations shown in Tables (5 – 7) for each breed are given. Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin.

**Figure 3.** Schematic illustration of the relationships between muscle metabolic and contractile properties and tenderness depending on the muscle.

Left: For the *Longissimus thoracis* (fast oxido-glycolytic muscle), higher degrees of fast glycolytic properties are associated with lower tenderness. Right: For the *Semitendinosus* (fast glycolytic muscle) higher degrees of fast glycolytic properties are associated with higher tenderness.

Consequently biomarkers associated with contractile and metabolic properties of the muscles such as MyHC IIX and LDH-B, are positively or negatively associated with tenderness of the ST and LT muscles, respectively. The opposite is found for other biomarkers such as small Hsp's (Hsp20, Hsp27,  $\alpha$ B-crystallin).

ST and LT muscles from Angus breed (AA) have lower levels of fast glycolytic properties than those of Limousin (LI) and Blond d'Aquitaine (BA) breeds. Therefore, their LT and ST muscles are more and less tender, respectively, than those of the LI and BA breeds.

**Table 1.** List of the 21 protein biomarkers of beef tenderness investigated in this study<sup>1</sup>.

<b>Protein name</b>	<b>Gene</b>	<b>UniProt ID</b>
<b>Heat Shock Proteins</b>		
$\alpha$ B-Crystallin	CRYAB	<b>P02511</b>
Hsp20	HSPB6	<b>O14558</b>
Hsp27	HSPB1	<b>P04792</b>
Hsp40	DNAJA1	<b>P31689</b>
Hsp70-1A/B	HSPA1B	<b>P08107</b>
Hsp70-8	HSPA8	<b>P11142</b>
Hsp70-Grp75	HSPA9	<b>P38646</b>
<b>Metabolism</b>		
Eno3 (Enolase 3)	ENO3	<b>P13929</b>
LDH-B (Lactate Dehydrogenase Chain B)	LDHB	<b>P07195</b>
MDH1 (Malate Dehydrogenase 1)	MDH1	<b>P40925</b>
<b>Structure</b>		
CapZ- $\beta$ (F-actin-capping protein subunit $\beta$ )	CAPZB	<b>P47756</b>
$\alpha$ -actin	ACTA1	<b>P68133</b>
MyLC-1F (Myosin Light Chain 1F)	MYL1	<b>P05976</b>
MyBP-H (Myosin Binding Protein H)	MYBPH	<b>Q13203</b>
MyHC-I (Myosin Heavy Chain-I)	MYH7	<b>P12883</b>
MyHC-II (MyHC IIa+IIx+IIb)	MYH2	<b>Q9UKX2</b>
MyHC-IIx (Myosin Heavy Chain-IIx)	MYH1	<b>P12882</b>
<b>Oxidative resistance</b>		
DJ-1 (Parkinson disease protein 7)	PARK7	<b>Q99497</b>
PRDX6 (Cis-Peroxiredoxin)	PRDX6	<b>P30041</b>
SOD1 (Superoxide Dismutase Cu/Zn)	SOD1	<b>P00441</b>
<b>Proteolysis</b>		
$\mu$ -calpain	CAPN1	<b>P07384</b>

<sup>1</sup> List of protein biomarkers of beef tenderness established by our group in previous proteomic studies (For review: Guillemin *et al.*, 2011; Picard *et al.*, 2010; Picard *et al.*, 2012a,b; 2013)

**Table 2.** Suppliers and conditions for each primary antibody used in this study.

Target protein	Antibody references	Dilution <sup>1</sup>
<b>Heat Shock Proteins</b>		
$\alpha$ B-crystallin	Monoclonal anti-bovine Assay Designs SPA-222	1/500
Hsp20	Monoclonal anti-human Santa Cruz HSP20-11:SC51955	1/200
Hsp27	Monoclonal anti-human Santa Cruz HSP27 (F-4):SC13132	1/3000
Hsp40	Monoclonal anti-human Santa Cruz HSP40-4 (SPM251):SC-56400	1/250
Hsp70-1A/B	Monoclonal anti-human Abnova HSPA1B (M02), clone 3B7	1/2000
Hsp70-8	Monoclonal anti-bovine Santa Cruz HSC70 (BRM22):SC-59572	1/250

Hsp70-Grp75	Monoclonal anti-human RD Systems Clone 419612	1/250
<b>Metabolism</b>		
Eno3	Monoclonal anti-human Abnova Eno3 (M01), clone 5D1	1/45000
LDH-B	Monoclonal anti-human Novus LDHB NB110-57160	1/50000
MDH1	Monoclonal anti-pig Rockland 100-601-145	1/1000
<b>Structure</b>		
CapZ- $\beta$	Monoclonal anti-human Abnova CAPZB (M03), clone 4H8	1/250
$\alpha$ -actin	Monoclonal anti-Rabbit Santa Cruz $\alpha$ -actin (5C5):SC-58670	1/1000
MyLC-1F	Polyclonal anti-human Abnova MYL1 (A01)	1/1000
MyBP-H	Monoclonal anti-human Abnova MYBPH (M01), clone 1F11	1/4000
MyHC-I	Monoclonal anti-bovine Biocytex 5B9	1/2000
MyHC-II <sup>2</sup>	Monoclonal anti-bovine Biocytex 15F4	1/4000
MyHC-IIx	Monoclonal anti-bovine Biocytex 8F4	1/500
<b>Oxidative resistance</b>		
DJ-1	Polyclonal anti-human Santa Cruz DJ-1 (FL-189):SC-32874	1/250
PRDX6	Monoclonal anti-human Abnova PRDX6 (M01), clone 3A10-2A11	1/500
SOD1	Polyclonal anti-rat Acris SOD1 APO3021PU-N	1/1000
<b>Proteolysis</b>		
$\mu$ - calpain	Monoclonal anti-bovine Alexis $\mu$ -calpain 9A4H8D3	1/1000

<sup>1</sup> Dilution of each antibody was defined according to Guillemin *et al.* (2011).

<sup>2</sup> MyHC II corresponds to fast MyHC: IIa+IIx+IIb isoforms

**Table 3.** Effect of breed and muscle on beef tenderness trait evaluated using three analyses methods.

Variables	Muscle	Breed (B) <sup>1</sup>			SEM <sup>2</sup>	Muscle (M) <sup>3</sup>		Significance <sup>4</sup>		
		AA 21	BA 25	LI 25		LT 70	ST 68	B	M	B x M
Overall tenderness	LT <sup>5</sup>	5.27 <sup>a</sup>	4.85 <sup>b</sup>	4.75 <sup>b</sup>	0.08	4.94 <sup>a</sup>	4.58 <sup>b</sup>	*	**	ns
	ST	4.58	4.66	4.49	0.05			ns		
WBSF <sup>6</sup>	LT	40.62	44.24	41.69	1.27	42.27	44.90	ns	t	*
	ST	45.91 <sup>a,b</sup>	41.35 <sup>b</sup>	47.80 <sup>a</sup>	1.01			*		
Tenderness index	LT	0.67	-0.14	-0.26	0.20	0.04	-0.03	ns	ns	*
	ST	-0.14 <sup>a,b</sup>	0.58 <sup>a</sup>	-0.56 <sup>b</sup>	0.18			*		

<sup>1</sup> Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin.

<sup>2</sup> standard error of mean

<sup>3</sup> Muscle designation: LT: *Longissimus thoracis*, ST: *Semitendinosus*

<sup>4</sup> t<0.1, \* <0.05, \*\* < 0.001

<sup>5</sup> Least square means in the same row for breed and muscle effects not followed by a common letter (a-c) differ significantly:  $P < 0.05$ .

<sup>6</sup> Warner-Bratzler Shear Force test.

**Table 4.** Breed, muscle and breed x muscle interaction effects on the 21 protein biomarkers of beef tenderness.

Variables	Muscle	Breed (B) <sup>1</sup>			SEM <sup>2</sup>	Muscle (M) <sup>3</sup>		Significance <sup>4</sup>		
		AA 21	BA 25	LI 25		LT 71	ST 69	B	M	B x M
$\alpha$ B-crystallin <sup>5</sup>	LT	26.5 a	16.1 b	18.4 b	0.84	20.0 a	8.9 b	***	***	*
	ST	12.5a	7.0b	7.9b	0.45			***		
Hsp20	LT	20.2	17.5	17.9	0.51	18.5 a	13.2 b	t	***	ns
	ST	13.6	12.9	13.3	0.35			ns		
Hsp27	LT	28.7 a	19.0 b	21.4 b	1.08	22.7 a	16.0 b	**	***	ns
	ST	21.0a	13.7b	14.3b	0.60			***		
Hsp40	LT	18.1 a	17.1 <sup>a,b</sup>	16.1 b	0.26	17.0 a	13.0 b	**	***	ns
	ST	13.8	12.8	12.6	0.22			*		
Hsp70-1A/B	LT	17.6	17.8	19.3	0.46	18.3 a	12.5 b	ns	***	ns
	ST	12.0	12.6	12.8	0.29			ns		
Hsp70-8	LT	15.8 b	17.5 a	16.6 <sup>a,b</sup>	0.22	16.7 a	15.6 b	*	**	ns
	ST	15.0	16.0	15.8	0.19			t		
Hsp70/Grp75	LT	9.1 c	16.6 a	12.6 b	0.44	13.0	12.6	***	ns	***
	ST	13.3	12.6	12.0	0.24			ns		
Eno3	LT	17.5 a	14.9 <sup>a,b</sup>	13.4 b	0.56	15.1 a	13.4 b	*	*	ns
	ST	15.5a	13.3b	12.9b	0.35			**		
LDH-B	LT	10.2 c	19.1 a	14.2 b	0.59	14.7	13.4	***	t	**
	ST	11.2c	15.0a	13.5b	0.33			***		
MDH1	LT	12.3 b	15.4 a	14.1 <sup>a,b</sup>	0.45	14.1	14.9	*	ns	*
	ST	15.4	15.1	14.2	0.37			ns		
CapZ- $\beta$	LT	19.3 a	15.9 b	15.4 b	0.42	16.7 a	14.0 b	**	***	*
	ST	14.9	13.4	13.8	0.28			ns		
$\alpha$ -actin	LT	16.9 b	19.5 a	16.3 b	0.40	17.6	17.6	**	ns	**
	ST	17.5	17.4	17.9	0.31			ns		
MyLC-1F	LT	14.9 <sup>a,b</sup>	15.5 <sup>a</sup>	14.3 <sup>b</sup>	0.20	14.9	15.2	*	ns	ns
	ST	15.0	15.7	15.0	0.18			ns		
MyBP-H	LT	14.9	13.3	13.7	0.84	14.0	12.7	ns	ns	ns
	ST	13.8	12.4	12.0	0.36			ns		
MyHC-I	LT	17.7	16.9	18.4	0.33	17.7 a	12.4 b	ns	***	**
	ST	14.3 <sup>a</sup>	10.2 <sup>c</sup>	12.4 <sup>b</sup>	0.35			***		
MyHC-II	LT	13.7 <sup>c</sup>	16.5 <sup>a</sup>	15.0 <sup>b</sup>	0.30	15.1 b	16.8 a	**	***	*
	ST	16.3	16.9	17.2	0.22			ns		
MyHC-IIx	LT	4.2 <sup>c</sup>	23.3 <sup>a</sup>	14.3 <sup>b</sup>	1.08	14.5 <sup>b</sup>	24.34 <sup>a</sup>	***	***	**
	ST	18.4 <sup>c</sup>	28.6 <sup>a</sup>	24.8 <sup>b</sup>	0.74			***		
DJ-1	LT	17.1	16.0	16.6	0.33	16.5 a	13.5 b	ns	***	ns
	ST	14.6a	13.1b	13.0b	0.26			*		
PRDX6	LT	15.5 a	13.4 b	13.3 b	0.22	14.0 b	16.1 a	***	***	ns
	ST	17.7a	15.6b	15.2b	0.23			***		
SOD1	LT	17.6	15.8	15.6	0.42	16.3	16.7	ns	ns	ns
	ST	16.1	10.2	12.4	1.22			ns		
$\mu$ -calpain	LT	14.2 b	15.9 a	14.0 b	0.28	14.7	14.4	**	ns	ns
	ST	13.6b	15.2a	14.1a,b	0.26			*		

<sup>1</sup> Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin.

<sup>2</sup> standard error of mean

<sup>3</sup> Muscle designation: LT: *Longissimus thoracis*, ST: *Semitendinosus*

<sup>4</sup>  $t < 0.1$ , \*  $< 0.05$ , \*\*  $< 0.001$ , \*\*\*  $< 0.0001$

<sup>5</sup> Least square means in the same row for breed and muscle effects not followed by a common letter (a-c) differ significantly:  $P < 0.05$ .

**Table 5.** Equations of best models (parameters including the direction and level of significance) to predict overall tenderness across breeds and for each breed

Breeds <sup>1</sup>	Parameter 1	P- value <sup>2</sup>	Parameter 2	P- value	Parameter 3	P- value	Parameter 4	P- value	Predictive power <sup>3</sup>	P- value model
<i>Semitendinosus</i> muscle										
AA	+ PRDX6	**	– Eno3	*	– $\alpha$ -actin	‡	–	–	43	*
BA	– Hsp70-1A/B	*	+ Replicate	*	–	–	–	–	39	*
LI	+MyHC-II	***	– PRDX6	**	+ Hsp70/Grp75	‡	–	–	53	**
All breeds	– Hsp20	*	+ LDH-B	‡	+ PRDX6	‡	– Hsp70-1A/B	*	14	*
<i>Longissimus thoracis</i> muscle										
AA	– MyLC-1F	**	+ MyHC-IIx	‡	+ Replicate	‡	–	–	35	‡
BA	+ DJ-1	***	– MyBP-H	‡	+ Replicate	*	–	–	40	*
LI	– Hsp70-1A/B	*	+ MyLC-F1	*	–	–	–	–	35	*
All breeds	– MyHC-IIx	‡	+ Hsp20	‡	– Hsp70-1A/B	‡	–	–	17	*

<sup>1</sup> Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin

<sup>2</sup> Significance of differences: ‡:  $P=0.06$ ; †:  $P<0.05$ ; \*:  $P<0.01$ ; \*\*:  $P<0.001$ ; \*\*\*:  $P<0.0001$

<sup>3</sup> (%) of variability between animals explained by the model.



**Table 6.** Equations of best models (parameters including the direction and level of significance) to predict WBSF across breeds and for each breed

Breeds <sup>1</sup>	Parameter 1	P- value <sup>2</sup>	Parameter 2	P- value	Parameter 3	P- value	Predictive power <sup>3</sup>	P- value model
<i>Semitendinosus</i> muscle								
AA	- $\alpha$ B-Crystallin	‡	+ Eno3	*	+ Hsp70-1A/B	‡	36	‡
BA	- MyHC-IIx	±	+ Eno3	‡	- $\alpha$ -actin	‡	22	‡
LI	- MyHC-IIx	*	- MyHC-II	‡	- Replicate	***	60	***
All breeds	- MyHC-IIx	*	+ Hsp70-1A/B	‡	-	-	15	*
<i>Longissimus thoracis</i> muscle								
AA	-	-	-	-	-	-	-	-
BA	- $\alpha$ -actin	‡	-	-	-	-	21	‡
LI	+ LDH-B	**	- MDH1	**	+ Hsp70-1A/B	**	60	***
All breeds	+ Hsp70-1A/B	*	+ Replicate	‡	-	-	10	*

<sup>1</sup> Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin

<sup>2</sup> Significance of differences: ‡:  $P=0.1$ ; †:  $P<0.05$ ; \*:  $P<0.01$ ; \*\*:  $P<0.001$ ; \*\*\*:  $P<0.0001$

<sup>3</sup> (%) of variability between animals explained by the model.

**Table 7.** Equations of best models (parameters including the direction and level of significance) to predict tenderness index across breeds and for each breed

Breeds <sup>1</sup>	Parameter 1	P- value <sup>2</sup>	Parameter 2	P- value	Parameter 3	P- value	Parameter 4	P- value	Predictive power <sup>3</sup>	P- value model
<i>Semitendinosus</i> muscle										
AA	+ $\alpha$ B-Crystallin	‡	+ PRDX6	‡	– Eno3	*	–	–	47	*
BA	– Hsp70-1A/B	‡	+ Replicate	‡	–	–	–	–	24	*
LI	– $\alpha$ B-Crystallin	*	+ Hsp40	*	+ MyHC-II	**	– PRDX6	**	73	***
All breeds	+ MyHC-IIx	‡	+ Replicate	*	–	–	–	–	12	*
<i>Longissimus thoracis</i> muscle										
AA	– Eno3	‡	+ Hsp27	*	– CapZ- $\beta$	+	–	–	29	*
BA	– CapZ- $\beta$	‡	+ Hsp40	‡	+ $\alpha$ -actin	*	– Hsp70-8	*	28	‡
LI	– LDH-B	***	+ MDH1	**	+ CapZ- $\beta$	*	– Hsp70-1A/B	***	66	***
All breeds	+ $\alpha$ -actin	‡	– Hsp70-1A/B	*	– MyHC-IIx	*	–	–	17	**

<sup>1</sup> Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, LI: Limousin

<sup>2</sup> Significance of differences: ‡:  $P=0.1$ ; †:  $P<0.05$ ; \*:  $P<0.01$ ; \*\*:  $P<0.001$ ; \*\*\*:  $P<0.0001$

<sup>3</sup> (%) of variability between animals explained by the model.

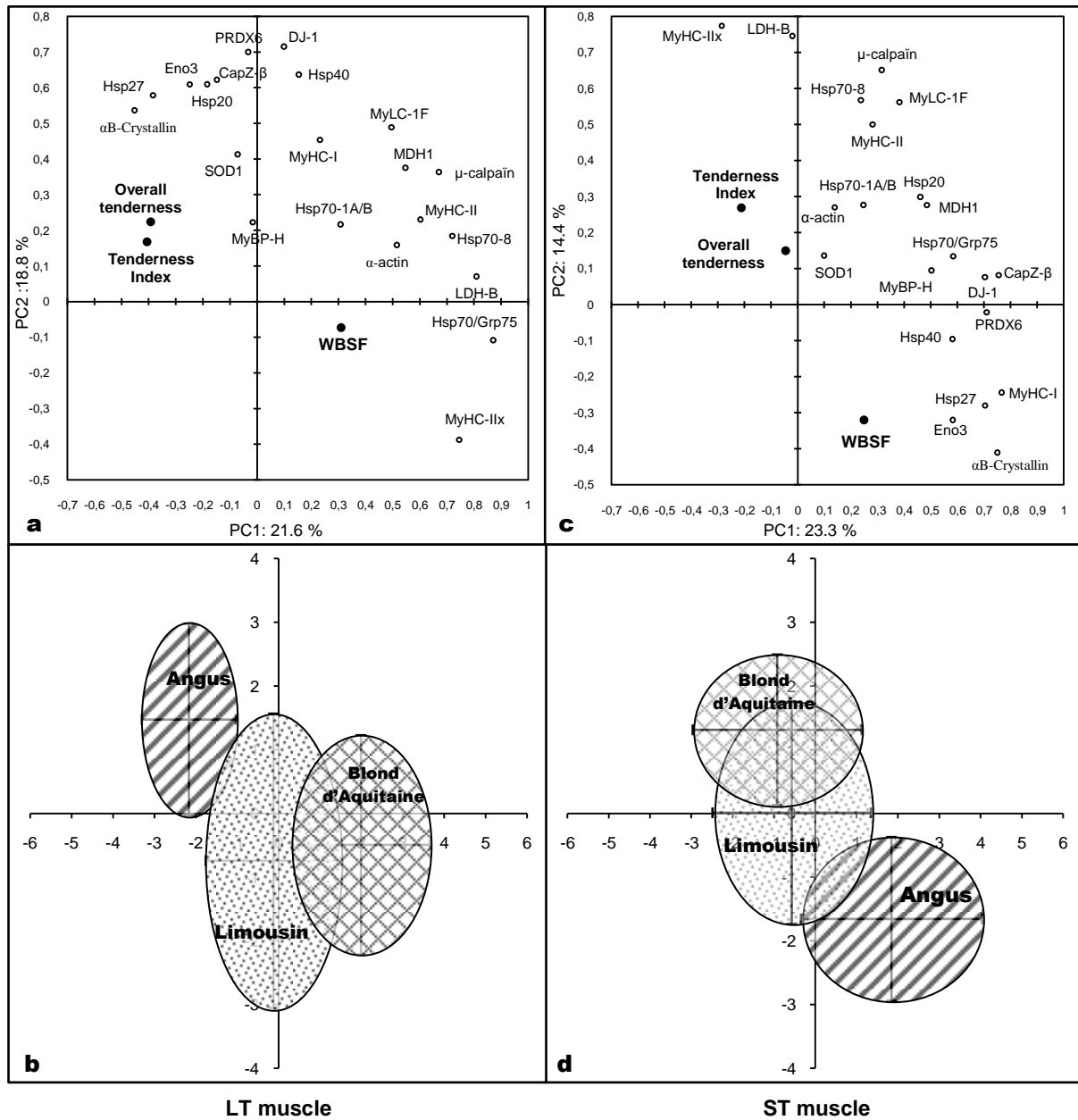


Figure 1.

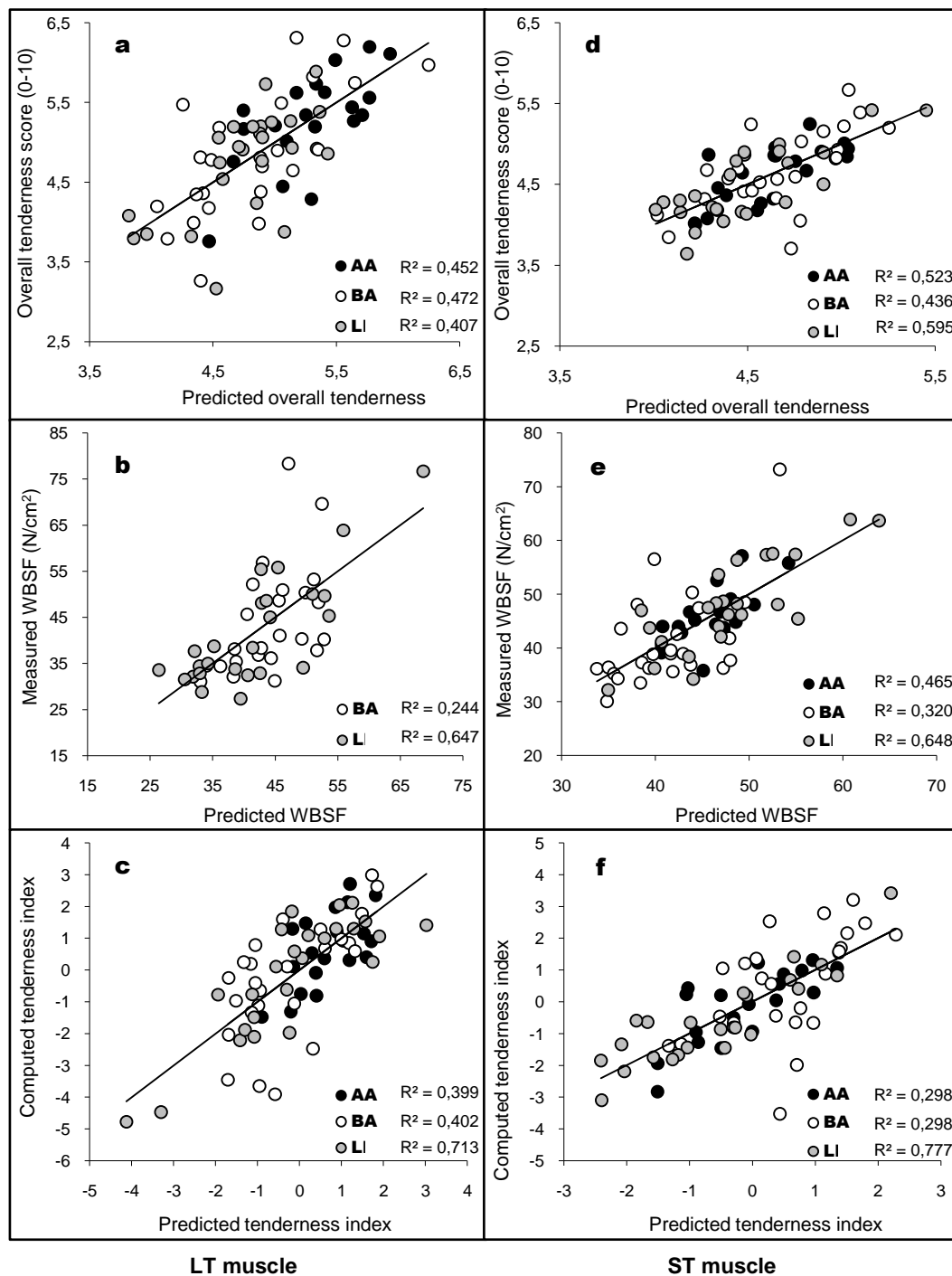
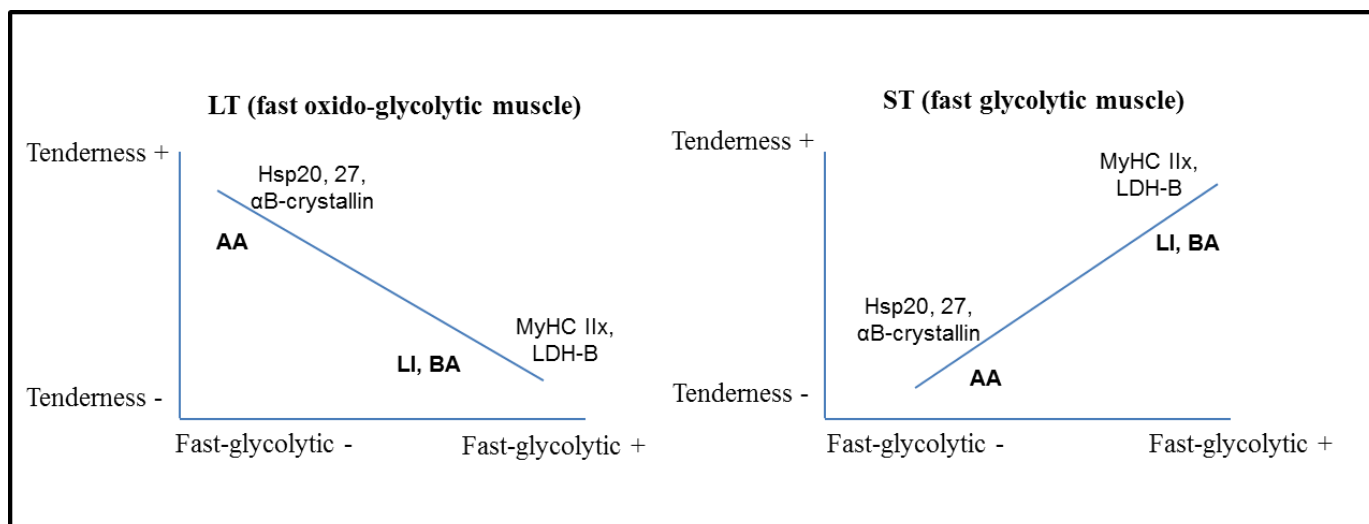


Figure 2.



**Figure 3.**

