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**The study of protein biomarkers to understand the biochemical processes underlying
beef color development in young bulls**

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Abstract

This study investigates relationships between 21 biomarkers and meat color traits of *Longissimus thoracis* muscles of young Aberdeen Angus and Limousin bulls. The relationships found allowed to propose metabolic processes underlying meat color. The color coordinates were related with several biomarkers. The relationships were in some cases breed-dependent and the variability explained in the regression models varied between 31 and 56%. The correlations between biomarkers and color were sometimes opposite between breeds. The PCA using the 21 biomarkers and the instrumental color coordinates showed that these variables discriminated efficiently between the two studied breeds. Results are coherent with earlier studies on other beef breeds showing that several proteins belonging to different but partly related biological pathways involved in muscle contraction, metabolism, heat stress and apoptosis are related to beef color. The results suggest that in future, biomarkers may be used to classify meat cuts sampled early *post-mortem* according to their forthcoming color.

Keywords: Beef; Young bulls; Meat color; Biomarkers; Biological Mechanisms; Regression

1. Introduction

In recent years, the search for biomarkers to predict and understand the biological mechanisms underlying the conversion of muscle into meat and the different characteristics of meat is receiving considerable attention (Ouali et al., 2013; Picard & Gagaoua, 2017). Sophisticated foodomic techniques are used to identify genes (genomics), gene-transcripts (transcriptomics), proteins (proteomics), and metabolites (metabolomics), which correlate with meat quality traits, including meat color (Gagaoua, Terlouw, Micol, et al., 2015b; Joseph, Nair, & Suman, 2015; Picard, Gagaoua, & Hollung, 2017). Color is an important quality trait as it influences the attractiveness of beef at purchase (Carpenter, Cornforth, & Whittier, 2001). Consumers often have to decide on purchase of meat using visual cues as other sensory traits such as tenderness, juiciness and flavor cannot be verified (Carpenter et al., 2001; Chamhuri & Batt, 2013). Hence, predicting meat color quality from a single on-line measurement has great commercial potential, particularly if this measurement can predict the status of the whole muscle or even of the entire carcass.

In cattle, studies using protein biomarkers to predict or explain meat color development after animal bleeding are scarce and relatively recent (Canto et al., 2015; Gagaoua, Couvreur, Le Bec, Aminot, & Picard, 2017; Gagaoua, Terlouw, Micol, et al., 2015a; Gagaoua, Terlouw, & Picard, 2016; Gagaoua, Terlouw, Micol, et al., 2015b; Joseph, Suman, Rentfrow, Li, & Beach, 2012; Wu et al., 2015). The objective of the present study is to help produce a list of molecular markers which may predict meat color or be used in the selection of beef cattle to produce better and stable color meat. Several candidate biomarkers have been identified with significant effects on meat color trait. For example, the study of (Joseph et al., 2012) investigated the sarcoplasmic proteins of two muscles of undisclosed beef breeds with different color stability, *Longissimus thoracis* (stable color) and *Psoas major* (unstable color) and identified 16 differentially abundant proteins, some of which belong to antioxidant proteins and chaperones. More recently, our laboratory investigated the potential use of a list of protein biomarkers known to be related to meat tenderness (Picard & Gagaoua, 2017; Picard et al., 2014), to relate them to CIE- $L^*a^*b^*$ color traits in young Blond d'Aquitaine bulls (Gagaoua, Terlouw, Micol, et al., 2015b). The main correlated proteins were revealed to be involved in apoptosis, oxidative stress, or had chaperone activities. The same study further reports regression models of CIE- $L^*a^*b^*$ color traits.

The objectives of the present study were **i**) to investigate the associations between the same list (Gagaoua, Terlouw, Micol, et al., 2015b) of protein biomarkers and meat color coordinates in the *Longissimus thoracis* muscle of young bulls of two different breeds, Aberdeen Angus (known as an early-maturing breed and fat, thus producing marbled meat) and Limousin (known as a late-maturing breed with less fat); **ii**) to show the strength of the relationship between the relevant protein biomarkers and meat color traits using regression analysis, and **iii**) to use this information to identify the biological mechanisms potentially underlying meat color traits.

2. Materials and Methods

2.1. Animals, handling and slaughtering

This study is part of the European FP6 Integrated Project ProSafeBeef (FOODCT-2006-36241) under the INRA reference AQ284 (Gagaoua, Terlouw, Boudjellal, & Picard, 2015). A total of 42 young bulls of two pure breeds were used: Aberdeen Angus (AA, n= 21) and Limousin (LIM, n= 21). None of the animals was double-muscled. At 12 months of age, the animals were subjected to a 105 day finishing period until slaughter. The feeding regime given *ad libitum* was based on straw (25%) and concentrate (75%) and the animals were housed in groups of four animals of the same breed in 6 × 6 m pens with straw bedding. Animals were slaughtered at the same age (around 17 months) and at similar live weights (around 665 kg) to avoid weight and age effects on muscle characteristics and beef meat quality. All bulls were directly transported in a lorry (3 × 2 m) from the experimental farm to the experimental abattoir situated at 1 km from the rearing building, with two bulls of the same home pen per transport to avoid social isolation stress. After unloading, they were slaughtered within 3 min in the slaughterhouse of INRA Auvergne-Rhône-Alpes Research center in compliance with the current French welfare recommendations for the use of experimental animals. Bulls were stunned by penetrative captive bolt prior to exsanguination. Before slaughter, all animals were food deprived for 24 hours, but had free access to water. The carcasses were not electrically stimulated and they were chilled and stored at 4°C up to 24 h post-mortem.

2.2. Muscle sampling

Muscle samples from *Longissimus thoracis* (LT, mixed fast oxido-glycolytic muscle) were excised 45 min *post-mortem* from the carcass (6th rib) of each animal. The epimysium was carefully dissected. Part of the samples was frozen in liquid nitrogen and kept at -80°C until

protein extractions for Dot-Blot analysis or Myosin Heavy Chain (MyHC) isoforms quantification. At 24h *post-mortem* another part was excised and used for meat color coordinates determination.

2.3. Color measurement

The color of the surface meat at 24h *post-mortem* was monitored according to (Gagaoua, Terlouw, Micol, et al., 2015b) on the *Longissimus thoracis* muscle, at the sixth rib level, using a portable colorimeter (Minolta Cameras CR 400, Japan) (CIE-LAB coordinates, light source D 65, 8-mm diameter measurement area, 0° standard observer). Calibration was performed by using a white plate supplied by the manufacturer prior to the color determination. Fresh cut slices of muscles of not less than 2.5 cm thick and overwrapped, were left on a polystyrene tray to refrigerate at 4°C for 1h to allow blooming. Color coordinates were calculated in the CIE-LAB system. L^* (lightness) is measured from 0 (black) to 100 (white), a^* (redness) has a negative value for green and a positive value for red and b^* (yellowness) values have a negative value for blue and a positive value for yellow. Measurements were taken at three locations on each steak and averaged. Mathematical operations were used to calculate color intensity or Chroma (C^*), related to the intensity of color by using the formula $C^* = [(a^{*2} + b^{*2})^{1/2}]$ (Gil et al., 2008).

2.4. Protein content determination

Protein concentration was determined according to the dye binding method of Bradford (Bradford, 1976) using the Bio-Rad Protein assay (Bio-Rad Laboratories Inc.). Bovine serum albumin (BSA) at a concentration of 1 mg/mL was used as standard and the protein content was calculated from the standard curve.

2.5. Extraction of proteins for Dot-Blot analysis

Total protein extractions were performed to use subsequently the soluble fractions for Dot-Blot analysis as recently reported by (Gagaoua, Terlouw, Boudjellal, et al., 2015). Briefly, 80 mg of muscle were homogenized using a Polytron (x22 000) in a denaturation/extraction buffer containing 8.3M urea, 2M thiourea, 1% DTT and 2% CHAPS. After 30 min of centrifugation at 10 000g at 8°C, the supernatant was stored at -20°C until use for protein assay and biomarkers quantification by Dot-Blot.

2.6. Immunological protein quantification by Dot-Blot

The abundance of 18 protein biomarkers (including the entire proteins, their isoforms and protein fragments) belonging to five different biological pathways (**Table 1**): heat shock

proteins (α B-crystallin, Hsp20, Hsp27, Hsp40, Hsp70-1A/B, Hsp70-8, and Hsp70-Grp75); muscle fiber structure (α -actin, MyLC-1F, CapZ- β and MyBP-H); metabolism (ENO3, LDH-B and MDH1); oxidative resistance (DJ-1, Prdx6 and SOD1) and proteolysis (μ -calpain) were determined according to the protocol described by (Guillemin et al., 2009). The conditions for use and specificity of primary antibodies against these 18 proteins in bovine muscle were previously checked by western-blot (Gagaoua, Terlouw, Boudjellal, et al., 2015). An antibody was considered specific against the studied protein when only one band at the expected molecular weight was detected by western blot. Optimal dilution ratios for each of the 18 antibodies were determined at the same time, using the conditions indicated by the supplier of the reactant and adapted to bovine muscle samples. Conditions retained and suppliers for all primary antibodies dilutions are reported in (Table 1).

For the Dot-Blot analysis, the protein samples (15 μ g) were spotted in quadruplicate on a nitrocellulose membrane with the Minifold I Dot-Blot apparatus from Schleicher & Schuell Biosciences (Germany) in a random order on the 96-spot membrane. In addition, a mixed standard sample (15 μ g) was deposited for data normalization. The Dot-Blot membranes were air-dried for 5 min, blocked in 10% PBS milk buffer at 37 °C for 20 min, and then incubated to be hybridized with the specific antibody of each protein in the conditions reported in Table 1. Subsequently, the membranes were incubated at 37°C for 30 min with the anti-mouse secondary fluorochrome-conjugated LICOR-antibody IRDye 800CW (1 mg/mL) supplied by LI-COR Biosciences (Lincoln, NE, USA) and used at a dilution of 1/20000. Infrared fluorescence detection was used for quantification of the relative protein abundances. Subsequently, the membranes were scanned using the Odyssey NIR imager (LI-COR Biosciences), with an 800 nm laser, a 169 μ m spatial resolution and a fixed gain of 5. Finally, the Dot-Blot images were quantified with GenePix PRO v6.0 (Axon). Each dot volume was calculated as the total dot intensity from which the median local background value multiplied by the dot area was subtracted. Because Dot-Blot offers the possibility of replicates, a data-prefiltering approach was implemented to eliminate outlier values mainly due to dust. The exclusion technique of outliers was based on the Medium Absolute Difference (MAD) and applied before repeated values were averaged. Finally, to make the data comparable between assays, the data were normalized using a regression-approach based on the used mix standard specific for each muscle. Thus, relative protein abundances were based on the normalized volume and expressed in arbitrary units.

2.7. Electrophoresis separation of Myosin Heavy Chain (MyHC) isoforms

The abundance of the three other proteins corresponding to myosin heavy chain (MyHC) isoforms was quantified by SDS-PAGE. The muscle fiber types were identified by separating MyHC isoforms using an adequate and high-resolution mini-gel electrophoresis as described by (Picard, Barboiron, Chadeyron, & Jurie, 2011).

For that, the myofibrillar proteins were extracted using a buffer containing 0.5 M NaCl, 20 mM Na Pyrophosphate, 50 mM Tris, 1 mM EDTA and 1 mM Dithiothreitol. After homogenization of approximately 100 mg of frozen muscle in 5 mL of the extraction buffer using a Polytron (x22 000) and an incubation time of 5 min at 4°C on ice, the sample was subjected to a centrifugation at 5000 x g for 10 min at 4°C. Following centrifugation, the supernatant was diluted 1:1 (v/w) with glycerol at 87% and stored at -20°C until use. The samples were then mixed with an equal volume of loading buffer (basic 2 x Laemmli) containing 4% SDS (w/v), 125 mM Tris (pH 6.8), 20% glycerol (v/v), 10% β -mercaptoethanol (v/v) and 0.02% pyronin Y (w/v), incubated at room temperature for 10 min and then heated (70°C) in a water bath for 10 min. The protein extracts (10 μ g) were loaded per well onto 0.75-mm-thick gels and were separated using 9.2% polyacrylamide gels. The lower running buffer consisted of 50mM Tris (base), 75mM glycine and 0.05% w/v SDS. The upper running buffer was at 2x the concentration of the lower running buffer and β -mercaptoethanol (0.07% v/v) was added. Ten micrograms of protein extracts were loaded per well onto 0.75-mm-thick gels mounted on a Mini-Protean II Dual Slab Cell electrophoretic system (Bio-Rad). The migration was carried out at a constant voltage of 70 V for 30 hours at 4°C. Controls of bovine muscle containing three (MyHC-I, Ila and IIx) or four (MyHC-I, Ila, IIx and I Ib) muscle fibers were added at the extremities of each gel. After migration, the gels were fixed in 30% (v/v) ethanol and 5% acetic acid (v/v) and then stained with colloidal Coomassie Blue R250 for 24 h. Gels were destained in a 30% ethanol (v/v) and 5 % acetic acid (v/v) solution until the background was sufficiently cleared. After staining in a Coomassie Blue dye solution, the gels were scanned and the proportions of the different MyHCs bands were quantified by densitometry with ImageQuant Software 5500 (Amersham Biosciences/GE Healthcare) and expressed in percentage.

The quantification of the bands revealed the existence of MyHC-I Ib isoform in only some animals (4 animals of 42 (AA, n = 1 and LIM, n = 3). Consequently MyHC-I Ib percentages were totaled with those of MyHC-I Ix creating a new variable "MyHC-I Ix+b" (fast glycolytic fibers).

2.8. Statistical analysis

Statistical analyses were conducted using the SAS software (SAS 9.1, SAS Institute INC, Cary, NC, USA). To determine the significance of differences between the least squares means of color coordinates and the 21 biomarkers between the two breeds (Aberdeen Angus vs. Limousin), PROC GLM procedure was performed. Significant differences were identified using Tukey's test at a significance level of $P < 0.05$. The PROC CORR of SAS was used to compute Pearson correlation coefficients. Correlation values were considered significant at $P < 0.05$. Principal components analyses (PCAs) were carried out using the PROC PRINCOMP procedure. Multiple regression analyses were performed using PROC REG to create best models for the color coordinates (as dependent variables, x) using the 21 protein biomarkers (as independent variables, y) for each breed. Partial R-squares, regression coefficients, t -values and significance of each retained variable are given for the models. The absence of collinearity was systematically verified for each regression equation (model), by producing condition indices and variance proportions using the COLLIN procedure. Variables were identified as collinear if they possessed both a high condition index > 10 and a proportion of variation > 0.5 for two or more traits (Gagaoua, Terlouw, Micol, et al., 2016).

3. Results and Discussion

3.1. Comparisons of color parameters between Aberdeen Angus and Limousin breeds

There was no significant difference for Lightness or Redness between Aberdeen Angus (AA) and Limousin (LIM) breeds, but LIM had more yellow meat (**Table 2**). The increased Chroma of LIM is explained by their increased yellowness. Overall, the color coordinates were within the range of values reported for LT muscles from pure breeds producing relatively light meat (Cuvelier, Cabaraux, et al., 2006; Gil et al., 2001). A study by (Chambaz, Scheeder, Kreuzer, & Dufey, 2003) reported approximately similar L^* -values (38.1) for Limousin steers. Other studies reported L^* -values of 34.6 and of 37.4 for young Limousin (Raes et al., 2003) and Aberdeen Angus bulls (Cuvelier, Cabaraux, et al., 2006), respectively. The small variations in L^* -values obtained in the various studies may be explained by the different finishing diets. For example, (Priolo, Micol, & Agabriel, 2001) found that animals raised on pasture have darker meat compared to those raised on concentrates (L^* is 5% higher). Moreover and in agreement with the findings of the present study, young animals produce slightly lighter meat (Priolo et al., 2001). a^* -values were similar to those described by (Chambaz et al., 2003) for Angus and Charolais (14.2), Simmental (14.3) and Limousin

(14.7) cattle. The b^* -values in this study are in agreement to those reported (15.0 – 15.3) for young Limousin bulls by (Cuvelier, Cabaraux, et al., 2006) but higher than those found by (Chambaz et al., 2003).

3.2. Comparisons of biomarker abundances between Aberdeen Angus and Limousin breeds

The two breeds were selected on the basis of their well-documented differences, first in their physiological maturation rates (early vs. late) and second, in intramuscular fat accumulation and perceived meat quality differences (Picard et al., 2014; Sadkowski, Jank, Zwierzchowski, Oprządek, & Motyl, 2009). Twelve of the 21 quantified biomarkers were significantly different between the AA and LIM bulls (**Table 3** and **Figure 1**). Protein differences between breeds or animal types were reported in earlier studies indicating for example an effect of animal type (breed and gender) on 15 proteins of the 24 analyzed by Dot-Blot (Guillemin et al., 2011). Within young bulls, breeds (Charolais, Limousin and Blond d'Aquitaine) differed in protein abundances in the LT muscle (Chaze et al., 2013).

Among the quantified structural proteins, CapZ- β and all the isoforms of myosin heavy chains MyHC-I, -IIa and -IIx/b (**Figure 1**) were significantly different between the two breeds. The proportion of MyHC-IIx/b was highest in LIM breed and those of the three other proteins were highest in AA. The proportions of the different MyHC fibers isoforms and also LDH levels, an indicator of fiber type composition (Gagaoua, Terlouw, Micol, et al., 2016), allowed earlier to identify the LT muscle of these AA bulls as relatively oxidative and that of LIM bulls as more glycolytic oriented. MyHC isoforms are primary determinants of muscle fiber characteristics such as metabolic, contractile and morphological properties (Picard, Lefaucheur, Berri, & Duclos, 2002). The characteristics of energy metabolism may influence the *post-mortem* conversion of muscle into meat, hence affecting final meat quality traits (Choi & Kim, 2009). For example, greater proportions of type I fibers are associated with a slower rate and extent of *post-mortem* pH decline (Choi, Ryu, & Kim, 2007), which may at least in part explain the lower pHu values observed in the LIM compared to the AA breed. Finally, although greater μ -calpain activity has been reported in fast glycolytic muscles (O'Halloran, Troy, Buckley, & Reville, 1997), no significant difference was obtained for this proteolytic enzyme between the two breeds. The abundances of α -actin, MyBP-H and MyHC-IIa were not different between the two breeds. CapZ- β or F-actin-capping protein subunit β is the most abundant barbed-end-capping protein which anchors the barbed ends of sarcomeric actin to the Z-disc (Maiti & Bamberg, 2013). Using proteomics, CapZ- β was shown to have

different levels of expression between Large White and Meishan pigs (Xu et al., 2009) and was suggested to be involved in the pathways affecting beef (Chaze et al., 2013) and pork (Ponsuksili et al., 2009) quality traits.

In the present study, the abundances of 5 of the 7 Hsp proteins were significantly different between the two breeds. The small Hsp proteins (α B-crystallin, Hsp20 and Hsp27) and Hsp40 were higher abundant in the AA breed. The abundances of the large Hsp70-1A/B and Hsp70-8 were not different but the abundance of Hsp70-Grp75 was higher in the LIM than in the AA breed. Differences in the expression of Hsp proteins between breeds were earlier reported in pigs and sheep (Hamelin et al., 2007). These differences may be due to muscle contractile and metabolic properties (Picard et al., 2014; Xu et al., 2009). Specifically, the LT muscle of AA was characterized by more slow oxidative activity compared to the LT of LIM, and slow oxidative muscles are known to contain more sHsp (Folkesson et al., 2013). The higher levels of Hsp70-Grp75 in the LIM compared to the AA breed may be related to higher activity levels of this breed during the slaughter period. Using these breeds, separate measurements on the bulls of this study showed that the LIM breed was slightly more reactive than the AA breed in various stress reactivity tests carried out during the rearing period, as well as at slaughter as shown by faster heart rates (Bourguet, Deiss, Boissy, & Terlouw, 2015). Increased stress status is often associated with increased physical reactions, and consequently, metabolic rate. As increases in Hsp70-Grp75 were related to enhanced oxidative and mitochondrial activity (Gagaoua et al., 2017; Gagaoua, Terlouw, Boudjellal, et al., 2015), higher Hsp70-Grp75 levels in LIM bulls may partly reflect higher activity levels at slaughter, possibly due to stress.

Two of the three metabolic proteins were significantly different; LDH-B was highest in the LIM breed and ENO3 was highest in the AA breed. The higher abundance of LDH-B in LIM breed is in line with the earlier studies describing higher activity of glycolytic enzymes in this breed (Cuvelier, Clinquart, et al., 2006). LDH reflects the permeability and the damage extent of the sarcolemma, consequently the extracellular appearance of LDH may be used to detect tissue damage (Z. Liu et al., 2016). This is coherent with the possible greater activity levels of the LIM compared to the AA breed. ENO3 is a glycolytic enzyme catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the penultimate step of glycolysis and described to be more abundant in striated muscles. Its higher level in the AA breed is unexpected, since the LT muscle of this breed is less glycolytic compared to the LIM breed. However, the AA breed is relatively fat and the result is coherent with a recent finding

showing that fatter pigs had a greater abundance of ENO3 (J. Liu et al., 2009). In addition, recent findings suggest that ENO3 may have other functions, involving cellular protection during hypoxia (Wulff, Jokumsen, Højrup, & Jessen, 2012). This is in coherence with the high levels of small Hsp proteins for the AA breed (Keady et al., 2013).

Of the antioxidant enzymes, only Prdx6 was significantly different (**Table 3**) with higher abundances in AA breed. Prdx6 is expressed in nearly all tissues including skeletal muscle, and protects cells against oxidative stress (Wang et al., 2003). The Prdx6 PLA₂ activity catalyzes hydrolysis of the acyl group at the sn-2 position of glycerophospholipids, with specific affinity for phosphatidylcholine, to produce free fatty acids and a lysophospholipid (Fisher, 2017). The higher levels in AA may be thus explained by their greater fat content compared to LIM breed (Keady et al., 2013) and also by greater proportions of type I fibers rich in mitochondria, known as a reservoir of peroxiredoxins (Lee et al., 2014).

3.3. Relationships between the studied variables

The first two axes of the principal component analysis using the instrumental color parameters and the protein biomarkers allowed efficient discrimination between the two breeds and explained 49.5% of the variability (**Figure 2**). The first principal component accounting for 32% of variability was positively correlated with MyHC-IIx/b, Hsp70-Grp75, LDH-B and CIE-*a**, -*b**, -*C** and negatively with MyHC-I, -IIa, α B-crystallin, ENO3, Hsp20, 27, 40 and CapZ- β . The second principal component accounting for 17.5% of the variability was positively correlated with μ -calpain, Prdx6, DJ-1, MyLC-1F, Hsp70-8 and MDH1 and negatively with CIE-*L** alone. Thus, these proteins can be used for beef traceability to identify the breed at the origin of the meat. Earlier studies proposed proteomic markers to discriminate between pig breeds (Kwasiborski, Sayd, et al., 2009; Mach et al., 2010). Further, discriminative markers (single nucleotide polymorphism) were identified to distinguish between Japanese domestic and imported beef (Sasazaki et al., 2011).

3.4. Relationship between biomarker abundances in the early post-mortem period and meat color coordinates

The correlation analyses showed that certain protein biomarkers were correlated with CIE-*L**, *a**, *b** and *C** color coordinates and that these correlations differed between the two studied breeds (**Figure 3**). For the AA breed, seven correlations were found. *L** was correlated with MyHC-IIa ($r = -0.38$) and ENO3 ($r = -0.44$); *a** with DJ-1 ($r = -0.49$) and α B-

crystallin ($r = -0.45$); b^* with Prdx6 ($r = -0.41$), Hsp27 ($r = +0.39$) and Hsp70-8 ($r = -0.43$) and C^* with Prdx6 ($r = -0.44$). For the LIM breed also, seven correlations were found. L^* was correlated with Prdx6 ($r = -0.47$) and Hsp70-8 ($r = -0.39$); a^* with Hsp70-1A/B ($r = +0.48$); b^* with MyHC-IIa ($r = -0.52$), MyHC-IIx/b ($r = +0.45$), MyLC-1F ($r = +0.39$), Prdx6 ($r = +0.46$) and μ -calpain ($r = +0.41$) and C^* with MyHC-IIa ($r = -0.49$), MyHC-IIx/b ($r = +0.45$), Prdx6 ($r = +0.46$) and Hsp70-1A/B ($r = +0.40$). The correlations showed certain similarities. For example, Prdx6 was correlated with b^* and C^* (**Figure 4**) for both breeds, however, the direction of the correlations are inverted (negatively for AA and positively for LIM).

The regression models obtained using color coordinates as dependent, and biomarker proteins abundances as independent variables, are given per breed in **Tables 4** and **5**. They explained from 31 to 56% of the variability using a maximum of three explanatory variables. The regression equations were trait and breed-dependent and no universal model was found across breeds. Nonetheless, some consistencies exist, for example, CapZ- β was retained in several regression models (L^* , a^* and C^*) of the AA breed (**Table 4**). Other proteins were common for both breeds and entered in the regression equations of the same color coordinate, but the direction differed. Particularly, LDH-B was retained differently (positive vs. negative) in the models of a^* for the AA and LIM breed, respectively.

The involvement in meat color of several proteins belonging to different but partly related biological pathways involved in muscle contraction, metabolism, heat stress, signaling, and apoptosis is in agreement with recent omic studies on pork (Sayd et al., 2006), beef (Gagaoua, Terlouw, Micol, et al., 2015b; Joseph et al., 2012), lamb (Gao, Wu, Ma, Li, & Dai, 2015), poultry (Desai et al., 2016) and fish (Desai et al., 2014).

The common relationship in the present study between Prdx6 (an antioxidant protein) and b^* and C^* color parameters for both breeds and with L^* of the LIM breed is of interest. The relationships of the different color traits with Prdx6 were opposite between the two breeds. The oxidation of polyunsaturated membrane lipids in muscle foods may cause a loss of fresh meat color and the addition of plant antioxidant may counteract the phenomena (Mancini & Hunt, 2005). Hence, Prdx6 may improve meat color in AA as LT muscles with higher Prdx6 abundances were less yellow, in part related to the status of myoglobin in muscle. Prdx6 belongs to the peroxiredoxins family which helps reduce oxidative stress and is also implicated in a variety of cellular processes, including metabolism, apoptosis and ageing (Fisher, 2017; Gagaoua, Terlouw, Micol, et al., 2015b). Members of this superfamily were

reported to help maintain meat color stability in other beef breeds (Gagaoua et al., 2017; Gagaoua, Terlouw, Micol, et al., 2015b; Joseph et al., 2012; Wu et al., 2015). Negative correlations were reported between Prdx6 with a^* -values and metmyoglobin reducing activity of Chinese *Luxi* yellow cattle (Wu et al., 2015). Prdx6 may counteract processes underlying meat discoloration (Gagaoua, Terlouw, Micol, et al., 2015b; Joseph et al., 2012) by competing with myoglobin for oxygen (Mb) or products of oxidative stress reactions (Prdx6).

DJ-1 is another protein related to oxidative stress and shows a negative relationship with a^* and C^* of the AA breed. Negative relationships between DJ-1 and a^* were found earlier by proteomic studies in pork (Sayd et al., 2006) and cattle (Wu et al., 2015). DJ-1 correlated further negatively with L^* -values of pork meat (Kwasiborski et al., 2008). Thus, these three studies as well as our results found negative correlations between DJ-1 and meat color parameters in different muscles, breeds and species. DJ-1 has the capacity to oxidize and translocate to mitochondria in response to oxidative stress, thus serving a role as sensor and protector against toxicity arising from oxidative stress and limiting mitochondrial fragmentation (Sayd et al., 2006; Thomas et al., 2011). It was suggested that its overrepresentation in light samples may be due to its property to protect against exposure to further oxidative stress (Sayd et al., 2006).

Hsps prevent aggregation and denaturation of proteins and were also related to meat color in several studies including different species. Small (α B-crystallin, Hsp27) and large (Hsp70-8, Hsp70-1A/B) Hsp proteins were correlated with the color traits in both breeds (**Figure 3**). Hsp70-Grp75 and Hsp40 were retained (**Table 4** and **5**), both negatively, in the regression equations of b^* (AA breed) and L^* (LIM breed), respectively. The involvement of Hsp proteins in beef quality traits including color was earlier reported (Gagaoua, Terlouw, Micol, et al., 2015b; Joseph et al., 2012; Picard & Gagaoua, 2017; Picard et al., 2017). For example, we have recently found that α B-crystallin, Hsp40, Hsp70-8 and Hsp70-1A/B were correlated with the L^* , a^* , b^* color traits of young Blond d'Aquitaine bulls (Gagaoua, Terlouw, Micol, et al., 2015b). The study found further that Hsp70-1A/B and μ -calpain were strongly correlated together as well as with the LT L^* , a^* , b^* color traits of these bulls. The present study found also that they were positively correlated and retained in the model of b^* -values of LIM. We have described a biological mechanism underlying these relationships involving apoptosis and proteolytic pathways as well as stress and oxidative mechanisms (Gagaoua, Terlouw, Micol, et al., 2015b). In pork, several Hsp proteins were reported to be related with meat color parameters. Among them, Hsp27 and α B-crystallin negatively correlated with L^*

(Hwang, Park, Kim, Cho, & Lee, 2005); Hsp72 negatively and positively correlated with L^* and a^* , respectively (Kwasiborski, Rocha, & Terlouw, 2009) and Hsp90 negatively correlated with both L^* and b^* (Zhang et al., 2014). The involvement of Hsps and oxidative proteins may be explained by their protective functions, thereby preserving metabolism and retarding proteolysis and apoptosis onset. Antioxidants stabilize further the overall intracellular redox state, thus stabilizing myoglobin and the light penetrating or scattering of structural proteins.

The large Hsp proteins allowed further explaining meat color using regression analyses. Hsp70-Grp75 and Hsp70-8 chaperones explained together 36% of the variability of AA b^* -values. For the LIM breed, Hsp70-1A/B and 70-8 explained together 38% of the variability of a^* -values. One hypothesis is that the large Hsp70-Grp75 plays a central role in color and sensory meat qualities (Gagaoua et al., 2017; Gagaoua, Terlouw, Boudjellal, et al., 2015; Gagaoua, Terlouw, & Picard, 2015). Hsp70-Grp75 may further explain the link between mitochondria and endoplasmic reticulum via Inositol 1,4,5-triphosphate receptor type 1 (IP3R1) (Kaufman & Malhotra, 2014; Mammucari, Patron, Granatiero, & Rizzuto, 2011; Ouali et al., 2013; Ozaki, Yamashita, & Ishiguro, 2011), which is also involved in tenderness (N. K. Kim, Cho, et al., 2008; N. K. Kim, Lim, et al., 2008). Hsp70-Grp75 was also related to meat color coordinates in LT muscle of young Blond d'Aquitaine bulls (Gagaoua, Terlouw, Micol, et al., 2015b) and LT and *Rectus abdominis* muscle of PDO Maine-Anjou cull cows (Gagaoua et al., 2017). Opposite relationships between Hsp90 contents and L^* and b^* were reported for early *post-mortem* muscle of pigs (Zhang et al., 2014). After animal bleeding, apoptosis is induced and in response, Hsp proteins are recruited to impede the onset of apoptosis and to chaperone the unfolding of muscle proteins (Gagaoua, Hafid, et al., 2015; Ouali et al., 2013). Thus, the involvement of Hsps in meat color may be in part related to their protective role against protein denaturation.

In the present study, two of the three metabolic enzymes were related with meat color coordinates. ENO3 was significantly and negatively correlated with L^* of the AA breed and was also retained as an explanatory variable in its regression model, explaining alone 20% of the variability. Lower early *post-mortem* pH resulting from faster metabolic rate may explain the relationship between decreased lightness and increased metabolic enzymes. Further, LDH-B was positive in the model of a^* for the AA breed and negative for the LIM breed, and explained 5% and 10% of the variability, respectively. Relationships between metabolic enzymes and meat color have been earlier reported in beef, pork, poultry and lamb (Canto et al., 2015; Desai et al., 2016; Gao et al., 2015; Joseph et al., 2012; Sayd et al., 2006). For

example, an earlier study by (Canto et al., 2015) classified steaks according to color stability and identified nine proteins showing differential abundance among which three glycolytic enzymes (phosphoglucosmutase-1, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase M2). LDH was further negatively correlated with L^* in pork (Kwasiborski et al., 2008). Cuvelier and co-workers reported positive and negative correlations with L^* and a^* respectively for young Belgian Blue, Limousin and Aberdeen Angus bulls (Cuvelier, Cabaraux, et al., 2006). All these metabolic enzymes are directly involved in ATP production during metabolism. Today we know that greater capacity for glycolytic metabolism are associated with increased color-life, presumably from greater stores of NADH or greater ability to regenerate NADH to fuel metmyoglobin reduction (Joseph et al., 2015; Mancini & Hunt, 2005). Thus, LDH may help to replenish the pool of *post-mortem* NADH (by reduction of NAD^+) by the lactate-LDH system which may be used for metmyoglobin reduction (Joseph et al., 2015) and thus affecting beef (Gagaoua et al., 2017; Y. H. Kim, Keeton, Smith, Berghman, & Savell, 2009) and pork color (Żelechowska, Przybylski, Jaworska, & Santé-Lhoutellier, 2012). In agreement with our findings, Gil *et al.* reported that the relationships are meat color parameter and breed-dependent (Gil et al., 2001). In their study, LDH was positively correlated with L^* -values in three (Asturiana de los Valles, Pirenaica and Retinta) out of seven Spanish beef cattle breeds, and negatively with a^* -values in two breeds (Asturiana de los Valles, Pirenaica) and with b^* -values in another breed (Bruna dels Pirineus). Thus, proteins related to glycolysis play a significant role in meat color.

The enzymatic equipment of the cell is directly related to the type of the muscle fibers. Muscles with greater proportion of red fibers have greater concentrations of mitochondria. The enzyme systems responsible for oxygen consumption and metmyoglobin reducing activity are part of the electron transport chain located in the mitochondria, recently suggested to be involved in beef color (McKeith et al., 2014). Muscle fiber type proportions are known to differ considerably across muscles and breeds and these differences may therefore be directly associated with differences in color due to differences in pigment, glycogen and lipid content, enzymatic equipment related to energy metabolism, mitochondrial oxygen consumption, and reducing capacity through enzymatic and non-enzymatic mechanisms (Klont, Brocks, & Eikelenboom, 1998). For example, muscles containing low proportion of slow twitch oxidative fibers (type I) have greater L^* -values (Gagaoua, Terlouw, Micol, et al., 2015b; Gil et al., 2008), possibly due to their greater myoglobin content and glycolytic potential. Furthermore, these fibers have greater contents of MyLC-1F, a member of the

myosin light chain family (Bouley et al., 2005; Hailstones & Gunning, 1990). MyLC-1F is prone to phosphorylation, a reaction playing a significant role in meat color development (Li et al., 2017). In accordance with this, MyLC-1F and α -actin were negatively correlated with L^* in pig LT muscle (Hwang et al., 2005). MyLC-1F was further negatively correlated with b^* in pig LT muscle (Żelechowska et al., 2012). These specific characteristics of different fiber types probably explain at least part of the various correlations between myofibrillar proteins and meat color coordinates and the differences already found between beef and pork.

More relationships with the color parameters were found for the LIM compared to the AA breed. In the regression models, MyHC-I explained alone 20% of L^* of the LIM breed. MyHC-IIa explained 26 and 24% of the variability of b^* and C^* , respectively. For the AA breed, CapZ- β was the only retained explanatory variable for L^* (positive), a^* and C^* (both negative) and the explained variability was weak, ranging from 10 to 12%. *Post-mortem* muscle pH decline is accompanied by myofibrillar structural changes modifying the degree of light penetrating or scattering by the structural elements (Hughes, Kearney, & Warner, 2014).

The specific relationships between CapZ- β (more abundant in the AA than the LIM breed) and the L^* and a^* color parameters of AA meat have not been reported elsewhere. CapZ- β , a capping protein of the thin actin filament at the barbed and pointed ends, has a role in the organization of the thin filament and its anchor to the Z-line (Maiti & Bamburg, 2013). This protein plays a significant role in pH decline and beef and pork tenderness (Chaze et al., 2013; Gagaoua, Terlouw, Micol, et al., 2015b; Hwang et al., 2005). Muscle fiber type proportions may further influence the amount of free water and structural modifications caused by apoptosis and proteolysis and consequently, light reflectance and scattering properties of the meat. In several proteomic studies, the presence of fragments from cytoskeleton proteins was observed early *post-mortem* (Paredi, Raboni, Bendixen, de Almeida, & Mozzarelli, 2012) and relationships between *post-mortem* degradation of structural proteins and meat quality traits have been reported (Morzel, Terlouw, Chambon, Micol, & Picard, 2008).

Conclusion

This study showed that the use of semi-quantitative techniques like Dot-Blot offers the possibility of characterizing the proteome of several animals at the same time and helps to understand better the process of conversion of muscle into meat and its effect on meat color quality. The relationships between selected protein biomarkers and meat color were demonstrated and allowed to propose metabolic processes underlying meat color that appear

to differ between the breeds to a certain extent. These differences may be at least partly caused by the different contractile and metabolic properties of the muscles between the two breeds. Differences in lipid content and composition may also have contributed. Overall, the biological mechanisms involved in meat color involve biomarkers earlier found to be related to tenderness.

Conflict of interest

The authors have declared that no competing interests exist.

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Figure Captions

Figure 1. Electrophoretic separation (A) of MyHC isoforms in m. *Longissimus thoracis* of young Limousin and Aberdeen Angus bulls. The different wells correspond to a mixt of animals (n = 2) selected randomly. Samples of bovine *Longissimus thoracis* with three (C1) and four (C2) types of MyHC were used as controls. Scattergrams of the proportion (B) and variance analysis of the different MyHC isoforms (I, Iia, Iix+b) detected in the two muscles after densitometry analysis. MyHC-I (AA = $28.51 \pm 7.43\%$; LIM = $22.70 \pm 2.03\%$); MyHC-Iia (AA = $54.28 \pm 6.74\%$; LIM = $29.91 \pm 4.83\%$); MyHC-Iix+b (AA = $17.21 \pm 10.26\%$; LIM = $47.40 \pm 5.19\%$). ** $P < 0.01$; *** $P < 0.001$.

Fiber types: I, slow oxidative fiber; Iia fast oxido-glycolytic fiber; Iix, fast glycolytic fiber. Fiber types Iib were detected in 4 animals of 42 (AA, n = 1 and LIM, n = 3). Consequently MyHC-Iib percentages were totaled with those of MyHC-Iix creating a new variable “MyHC-Iix+b” (fast glycolytic fibers). (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 of the projection of the protein biomarker abundances and instrumental meat color coordinates for both AA and LIM young bulls. The bi-plot showing the distribution of the two breeds (AA in gray and LIM in orange color) on the first two principal axes is given on the corner at the bottom right of the PCA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure 3. Venn diagrams depicting the biomarkers found significantly ($P < 0.05$) correlated with the instrumental meat color coordinates (L^* , a^* , b^* and C^*) for both AA (left) and LIM (right) young bulls. The directions and coefficients of the correlations are given in the text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Figure 4. Example of common correlations (Pearson correlation analysis) found for both breeds (AA, gray circles and LIM, orange circles) between Prdx6 (in arbitrary units) and Chroma (C^*) but the direction is different. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 1. List of the 18 protein biomarkers quantified using the Dot-Blot technique. The suppliers and conditions for each primary antibody used in this study are given as described in Gagaoua *et al.* (2015b).

Protein biomarker name (<i>gene</i>)	UniProtKB ID	Monoclonal (Mo) or Polyclonal (Po) antibodies references	Antibody dilutions
Heat Shock Proteins			
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>DNAJA1</i>)	P31689	Mo. anti-human Santa Cruz HSP40-4 (SPM251):SC-56400	1/250
Hsp70-1A/B (<i>HSPA1B</i>)	P08107	Mo. anti-human Abnova HSPA1B (M02), clone 3B7	1/2000
Hsp70-8 (<i>HSPA8</i>)	P11142	Mo. anti-bovine Santa Cruz HSC70 (BRM22):SC-59572	1/250
Hsp70-Grp75 (<i>HSPA9</i>)	P38646	Mo. anti-human RD Systems Clone 419612	1/250
Metabolism			
Enolase 3 (<i>ENO3</i>)	P13929	Mo. anti-human Abnova Eno3 (M01), clone 5D1	1/45000
LDH-B (<i>LDHB</i>)	P07195	Mo. anti-human Novus LDHB NB110-57160	1/50000
MDH1 (<i>MDH1</i>)	P40925	Mo. anti-pig Rockland 100-601-145	1/1000
Structure			
CapZ- β (<i>CAPZB</i>)	P47756	Mo. anti-human Abnova CAPZB (M03), clone 4H8	1/250
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
Oxidative resistance			
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
Proteolysis			
μ -calpain (<i>CAPN1</i>)	P07384	Mo. anti-bovine Alexis μ -calpain 9A4H8D3	1/1000

Abbreviations: LDH-B: Lactate dehydrogenase chain B ; MDH1: Malate dehydrogenase 1 ; CapZ- β : F-actin-capping protein subunit β ; MyLC-1F: Myosin Light chain 1F; MyBP-H: Myosin Binding protein H; DJ-1: Parkinson diseaseprotein 7; Prdx6: Cis-peroxiredoxin; SOD1: Superoxide dismutase Cu/Zn

Table 2. Least-square means of color coordinates in the two breeds (Aberdeen Angus vs. Limousin young bulls).

Variables	Aberdeen Angus	Limousin	SEM	<i>P</i> -value
<i>Lightness (L*)</i>	36.08	34.95	0.51	ns
<i>Redness (a*)</i>	14.32	14.85	0.35	ns
<i>Yellowness (b*)</i>	13.06	16.00	0.51	**
<i>Chroma (C*)</i>	19.44	21.92	0.53	*

Significances: ns: not significant; * $P < 0.05$; ** $P < 0.01$

Table 3. Least-square means of the protein biomarkers quantified by Dot-Blot (18) or SDS-PAGE (three Myosin heavy chains isoforms (MyHC) in the two breeds (Aberdeen Angus vs. Limousin young bulls).

Protein biomarkers	Aberdeen Angus	Limousin	SEM	<i>P</i> -value
<i>Heat shock proteins</i>				
α B-crystallin	26.55	17.32	1.21	***
Hsp20	20.26	17.09	0.72	*
Hsp27	28.68	19.36	1.56	***
Hsp40	18.08	15.60	0.34	***
Hsp70-1A/B	17.60	19.38	0.68	ns
Hsp70-8	15.81	16.59	0.26	ns
Hsp70-Grp75	9.12	12.68	0.41	***
<i>Metabolism</i>				
Enolase 3	17.49	11.82	0.60	***
LDH-B	10.18	13.81	0.50	***
MDH1	12.34	13.92	0.59	ns
<i>Oxidative resistance</i>				
DJ-1	17.06	16.17	0.44	ns
Prdx6	15.48	13.04	0.32	***
SOD1	17.65	15.38	0.70	ns
<i>Proteolysis</i>				
μ -calpain	14.19	13.77	0.34	ns
<i>Structure</i>				
CapZ- β	19.36	14.36	0.61	***
α -actin	16.95	16.37	0.45	ns
MyBP-H	13.28	12.40	0.80	ns
MyLC-1F	14.93	14.12	0.27	ns

Significances: ns: not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 4. Regression equations of meat color coordinates of AA breed samples using protein biomarkers.

Dependent variable	R-squared ¹	S.E	Entered independent variable ²	Partial R-squared	Regression coefficient	t-value	P-value
<i>Lightness</i> (<i>L*</i>)	0.31*	0.20	ENO3	0.20	-0.53	-2.62	0.017
		0.20	CapZ-β	0.11	+0.35	+1.72	0.103
<i>Redness</i> (<i>a*</i>)	0.41**	0.21	DJ-1	0.24	-0.68	-3.27	0.004
		0.24	LDH-B	0.05	+0.49	+2.00	0.050
		0.23	CapZ-β	0.12	-0.43	-1.88	0.070
<i>Yellowness</i> (<i>b*</i>)	0.56***	0.18	Hsp70-8	0.24	-0.68	-3.85	0.001
		0.18	SOD1	0.19	-0.53	-2.91	0.009
		0.17	Hsp70-Grp75	0.12	-0.36	-2.12	0.040
<i>Chroma</i> (<i>C*</i>)	0.35*	0.20	DJ-1	0.13	-0.44	-2.22	0.004
		0.20	Hsp27	0.12	+0.38	+1.88	0.017
		0.20	CapZ-β	0.10	-0.32	-1.61	0.012

¹Significance of the models: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

²Variables are shown in order of their entrance in the regression models.

Table 5. Regression equations of meat color coordinates of LIM breed samples using protein biomarkers.

Dependent variable	R-square ¹	S.E	Entered independent variable ²	Partial R-squared	Regression coefficient	t-value	P-value
<i>Lightness</i> (<i>L*</i>)	0.51***	0.20	Prdx6	0.22	-0.62	-3.09	0.007
		0.20	MyHC-I	0.20	-0.54	-2.75	0.004
		0.18	Hsp40	0.09	-0.32	-1.73	0.030
<i>Redness</i> (<i>a*</i>)	0.48**	0.26	Hsp70-1A/B	0.23	+0.95	+3.63	0.002
		0.26	Hsp70-8	0.15	-0.51	-1.96	0.006
		0.19	LDH-B	0.10	-0.34	-1.77	0.009
<i>Yellowness</i> (<i>b*</i>)	0.40*	0.19	MyHC-IIa	0.26	-0.48	-2.58	0.019
		0.19	μ -calpain	0.14	+0.37	+1.99	0.040
<i>Chroma</i> (<i>C*</i>)	0.36*	0.19	MyHC-IIa	0.24	-0.45	-2.32	0.030
		0.19	Hsp70-1A/B	0.12	+0.35	+1.79	0.019

¹Significance of the models: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

²Variables are shown in order of their entrance in the regression models.

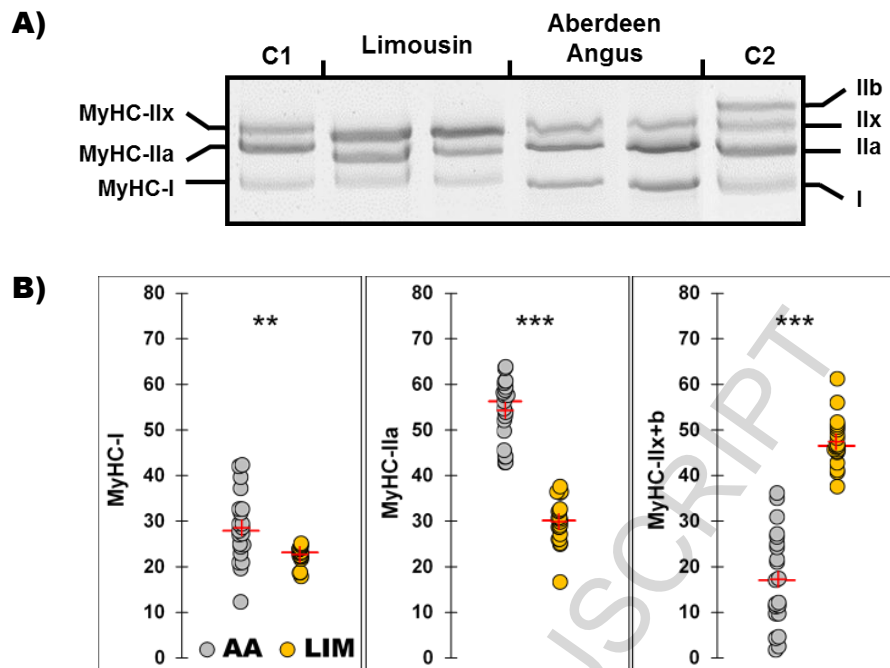


Figure 1.

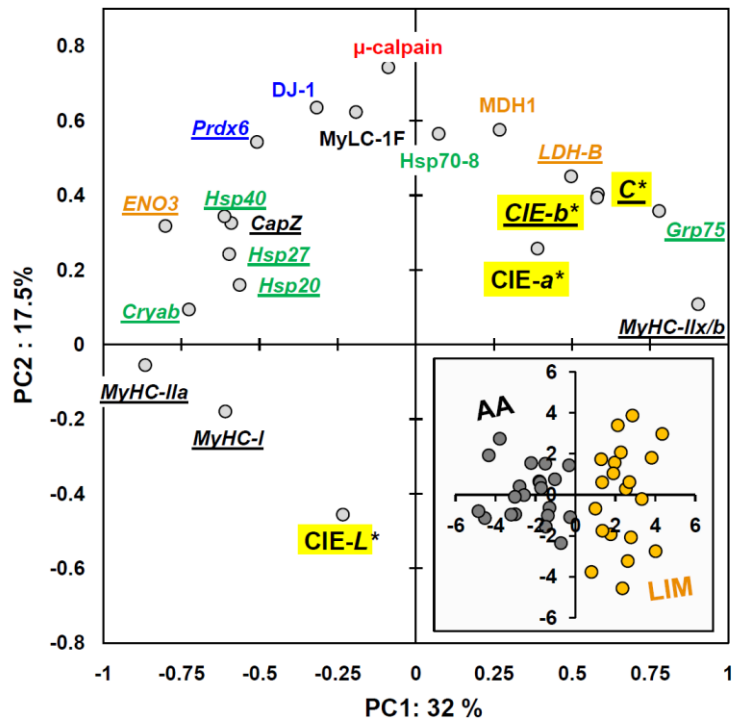
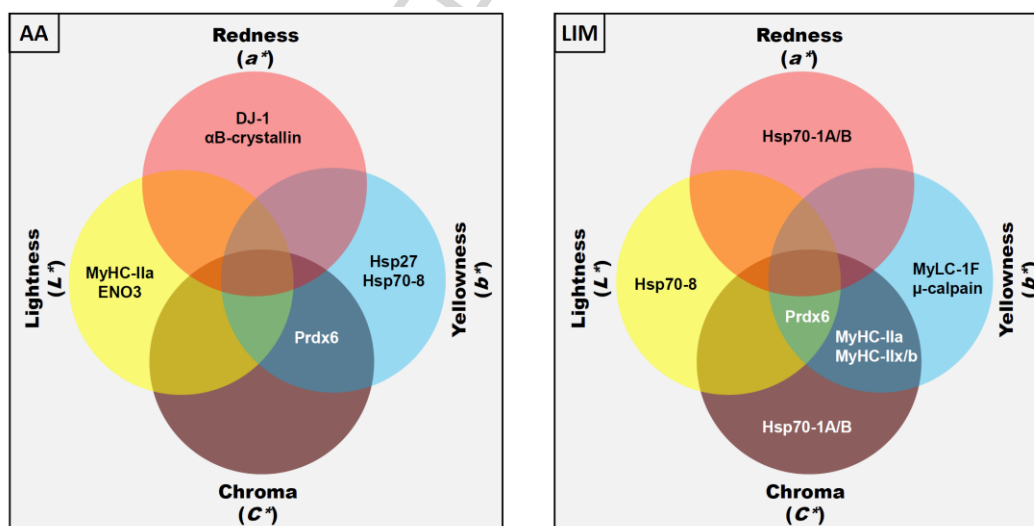


Figure 2.

Figure 3.



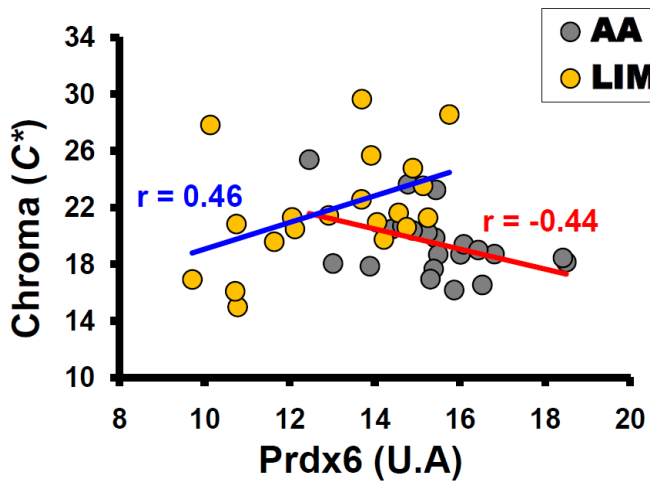


Figure 4.

Highlights

- Protein biomarkers for meat tenderness are also related with meat color
- The correlations between biomarkers and meat color are muscle and color parameter-dependent
- Color of different muscles showed consistent relationships involving similar biological pathways
- The biomarkers discriminated efficiently between the two breeds

ACCEPTED MANUSCRIPT