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Abstract

The development of proteomic biomarkers for meat tenderness remains an important challenge. The present study used *Longissimus thoracis* (LT) and *Semitendinosus* (ST) muscles of young bulls of three continental breeds (Aberdeen Angus, Blond d'Aquitaine and Limousin) to i) identify cellular pathways robustly related with meat tenderness, using potential protein biomarkers and ii) describe biochemical mechanisms underlying muscle to meat conversion. Correlation networks reveal robust correlations, *i.e*. present for at least two breeds, between potential meat tenderness biomarkers. For the two muscles of the three breeds, DJ-1 and Peroxiredoxin 6 were consistently correlated with Hsp20 and µ-calpain, respectively. For the three breeds, µ-calpain was related to Hsp70-8 in the LT muscle. Various correlations were muscle specific. For the three breeds, DJ-1 was correlated with Hsp27 for the ST, and with ENO3 and LDH-B for the LT muscle. Overall, in the LT, more correlations were found between proteins related to the glycolytic pathway and in the ST, with the small Hsp's (Hsp20, 27 and αB-crystallin). Hsp70-Grp75 appeared involved in several relevant biological pathways. At the scientific level, results give insights in biological functions involved in meat tenderness. Further studies are needed to confirm the possible use of these biomarkers in the meat industry to improve assurance of good meat qualities.

Keywords: Biomarkers; beef; muscle; breed; meat tenderness; correlation networks.

1. Introduction

In recent years, there has been a growing interest in the relationship between proteins and related genes, and meat quality traits. Functional proteomics aim to elucidate the biological function of proteins combining electrophoretic and protein sequencing technologies. They may be used to identify molecular markers, or biomarkers, that predict meat sensory qualities, including tenderness [1-9]. Such biomarkers are quantifiable indicators of biological processes and may help increasing our understanding of the biochemical processes related to various meat qualities [6, 10]. Better knowledge of muscle to meat conversion would i) facilitate genetic selection, ii), help to evaluate the potential sensory quality of future meat products of existing animals, and iii) orient rearing systems and genetic choice to obtain desired meat qualities.

Today, a substantial amount of data exists on proteins that are related to various meat quality aspects. Studies show good coherence in the biological pathways involved in the development of meat quality. Generally, the proteins involved in meat quality are related to protective functions, glycolytic metabolism, mitochondrial activity and apoptosis, proteolysis, and cell structure [2]. However, the relative impacts of these biological pathways on meat quality development differ considerably between studies and even between breeds and muscles [3]. Similarly, although these studies find associations between proteins of similar or different biochemical pathways, the exact proteins that are correlated often differ. These differences may be explained by differences in the physical and physiological characteristics of the animals studied [11]. We need to get further insight in the functioning of proteins of similar and different biological pathways and ultimately, their relationship with meat quality development. One step would be to identify proteins showing robust correlations, that is, proteins that are correlated irrespectively of their physiological or physical environment.

The aim of the present study was to identify correlations between proteomic markers, existing in more than one breed or muscle and to discuss the underlying biological pathways. Therefore, we evaluated levels of Heat shock proteins (Hsp's), and proteins involved in metabolism, structure, oxidative resistance and proteolysis in two muscles (*Longissimus thoracis* and *Semitendinosus*) of three continental beef breeds (Aberdeen Angus, Blond d'Aquitaine, and Limousin). These breeds differ in leanness and earliness and the muscles in metabolic and contractile properties. The proteins studied are potential biomarkers of bovine meat tenderness [3, 12-14].

2. Material and Methods

2.1. Animals and sample collection

The study was part of the larger European ProSafeBeef project (FOOD-CT-2006-36241) and organized in two replicates (2 consecutive years, during the spring/summer seasons) in a balanced experimental design. It used 71 young bulls: Aberdeen Angus (AA; n =21), Blond d'Aquitaine (BA; n =25) and Limousin (Li; n =25). At 12 months of age, they were subjected to a 105 day finishing period until slaughter. They were housed in 6m x 6m straw-bedded pens with 4 animals of a same breed to a pen. Diets consisted of concentrate (75%) and straw (25%). Before slaughter, all animals were food deprived for 24 h to limit the risk of carcass contamination by microbes in the digestive tract during evisceration, but had free access to water. At a live weight around 665 kg, the animals were slaughtered at the experimental abattoir of the INRA Research centre in compliance with the current ethical guidelines for animal welfare. Bulls were directly transported (4.5 ± 0.1 min) in a lorry (3 x 2 m livestock compartment) from the experimental farm to the experimental abattoir situated at 2 km from the rearing building, with 2 bulls of the same home pen per transport to avoid social isolation stress. After unloading, they were slaughtered within 3 min using standard industry procedures and respecting EU regulations. Slaughter procedures from the loading at the farm to bleeding took 10.6 ± 0.1 min. Slaughter took place between 08.00 h and 10.00 h am. Bulls were stunned by penetrative captive bolt prior to exsanguination [15]. The carcasses were not electrically stimulated and they were chilled and stored at 4°C from 1 h until 24 h *post-mortem*. *Longissimus thoracis* (LT, mixed fast oxido-glycolytic) and *Semitendinosus* (ST, mixed fast glycolytic) muscle samples were excised 30 min *post-mortem* and frozen in liquid nitrogen before storage at –80°C until protein extractions for Dot-Blot analysis or Myosin Heavy chains isoforms (MyHC) quantification.

2.2. Extraction of proteins for Dot Blot

Total protein extractions were performed to use subsequently the soluble fractions for Dot-Blot analysis according to Bouley *et al.* [16]. Briefly, 80 mg of muscle was homogenised in a denaturation/extraction buffer containing 8.3 M urea, 2 M 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. The protein concentrations of the extracts were determined according to the Bradford method [17] using the Bio-Rad Protein Assay. Bovine serum albumin (BSA) at a concentration of 1 mg/mL was used as standard.

2.3. Immunological protein quantification

The abundances of the 18 biomarkers (including intact proteins, their fragments and complexes) listed in **Table 1** were quantified by the Dot-Blot technique according to the protocol described by Guillemin *et al.* [18] using specific antibodies previously validated by western-blot. Briefly, western blots were used in order to check the specificity of all the antibodies. An antibody was considered specific against the studied protein when only one band at the expected molecular weight was detected by western blot. Western blots with the 18 primary antibodies show that all the antibodies bound specifically to the bovine protein with the expected theoretical molecular weight.

Proteins evaluated belong to five different biological pathways (Table 1): muscle fibre structure (Actin, MyBP-H, CapZ-β and MyLC-1F); metabolism (ENO3, LDH-B and MDH1); proteolysis (μ-calpain); oxidative resistance (DJ-1, Prdx6 and SOD1); and Heat shock proteins (αB-crystallin, Hsp20, 27, 40, Hsp70-1A/B, 70-8 and 70-Grp75) were determined according to [11, 12]. After quantification, a ratio corresponding to small Hsp per Hsp70s was calculated using the following equation: s/70 = (Hsp20 + Hsp27 + αB-crystallin) / (Hsp70-1A/B + Hsp70-8 + Hsp70-Grp75).

Compared to Western Blot, Dot-Blot is a rapid technique, but with a similar coefficient of variation inter and intra assay (10%). Optimal dilution ratios of the antibodies were determined at the same time, using the conditions indicated by the supplier of the reactant and adapted to bovine muscle samples [11]. Conditions retained and suppliers for all primary antibodies are reported in **Table 2**. Protein extracts (15 μg) of each of the 140 muscle samples were spotted (four replications per muscle sample) on a nitrocellulose membrane with the Minifold I Dot-Blot apparatus from Schleicher & Schuell Biosciences (Germany) in a random order on the 96-spots membrane. In addition, a mixed standard sample (15 μg) was deposited for data normalization as reported by Guillemin *et al.* [18]. 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 primary antibody of each protein (**Table 2**). Subsequently, the membranes were incubated at 37°C for 30 minutes with the anti-mouse fluorochrome-conjugated LICOR-antibody IRDye 800CW (1 mg/mL).

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 a 800 nm laser, a 169 μm spatial resolution and a fixed gain of 5.

Dot-Blot images were quantified with GenePix PRO v6.0 (Axon) [18]. 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.

2.4. Electrophoresis and quantification of Myosin Heavy Chain (MyHC) isoforms

The abundance of the three other proteins corresponding to MyHC isoforms was quantified by an appropriate SDS-PAGE technique [19]. One hundred mg of frozen muscle was ground using a Polytron in 5ml of extraction buffer solution containing 0.5 M NaCl, 20 mM Na Pyrophosphate, 50 mM Tris, 1 mM EDTA and 1 mM Dithiothreitol. The sample was kept 10 min at 4°C on ice, and then centrifuged for 5 min at 5000g. Following centrifugation, the supernatant was diluted 1:1 (v/w) with glycerol at 87% and stored at –20°C until used. The samples were then mixed with an equal volume of loading buffer 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) for 10 min. The proteins were separated by SDS-PAGE electrophoresis according to Picard *et al.* [19] 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. 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, the gels were scanned and the proportions of the different MyHC bands were quantified by densitometry with ImageQuant Software 5500 (Amersham Biosciences/GE Healthcare). The quantification of the bands revealed the existence of MyHC-IIb isoform [20] in only some animals (8 animals of 71). Consequently

MyHC-IIb percentages were totalled with those of MyHC-IIx creating a new variable "MyHC-IIx+b" (fast glycolytic fibres).

2.5. Statistical analysis and construction of the correlation networks

Raw data from the sensory meat quality biomarkers were analysed using the GLM procedure of SAS (SAS 9.1, SAS Institute INC, Cary, NC, USA) to evaluate effects of breed and replicate and their interactions, all introduced as fixed effects. For subsequent analyses, data were standardized to remove the effects of replicate, by calculating Z-scores using the PROC STANDARD. More precisely, the standard score of a raw score x was calculated using the following formula: $\boldsymbol{z} = \frac{\boldsymbol{x} - \mu}{\boldsymbol{\sigma}}$ where: μ is the mean of the population of each breed and σ is the standard deviation of the population [11]. Pearson correlation coefficients were calculated using Z-scores using the PROC CORR of SAS. If correlations between two biomarkers were significant ($P < 0.05$) for more than one breed, data of the different breeds were combined to calculate an overall correlation coefficient on Z-scores. Final results are presented as correlation networks which represent all the correlated biomarkers for a given muscle, combining two (BA-Li; BA-AA or AA-Li) or three breeds (AA, BA and Li).

3. Results

Tables 3 and **4** show that nearly all the breed effects found for the biomarkers abundances in the ST were also found in the LT muscle. For both muscles, AA had higher levels of small Hsp's, s/70, ENO3, MyHC-I and MyHC-IIa, and lower levels of MyHC-IIx+b and LDH-B compared to the other breeds. BA had higher levels of µ-calpain, and to a lesser extent, Hsp70-8, than the other breeds. Specifically in the LT muscle, breed effects were further found for Hsp70-Grp75, Hsp40, MDH1, CapZ-β, α-actin and MyLC-1F. Specifically, LT Hsp70-Grp75 levels of AA were lower than in Li while BA had highest levels and LT CapZ-β levels were higher in AA compared to Li and BA. DJ-1 had a breed effect only in the ST muscle. Some effects of replicate or breed x replicate interactions were also found. Most of these were minor, with some exceptions. For example, for ST Hsp20 levels, Li breed had the lowest levels during the first and the highest during the second replicate, compared to the other groups (**Table 4**). Similarly, LT MDH1 levels of AA and Li for the first replicate were lower than those obtained in the other groups (**Table 3**). In contrast to the LT, in the ST muscle, no breed effects were found for the structural proteins (CapZ-β and α-actin).

The correlation networks based on all breeds showed also a number of similarities between the LT and ST muscles (**Fig. 1**). For the LT and ST, respectively, 17 and 13 biomarkers were correlated. The correlations were organised in a single network for the LT muscle (**Fig. 1a)** and two networks for the ST muscle (**Fig. 1b**). Almost all correlations were positive. Eleven (Hsp20, Hsp27, αB-crystallin, s/70, DJ-1, μ-calpain, Prdx6, MyHC-IIa, MyHC-IIx+b, MyLC-1F and Hsp70-Grp75) of the 13 biomarkers retained for the ST muscle were also for the LT muscle. For both the LT and ST muscles, correlations were found between Hsp27 and s/70, between Prdx6 and μ-calpain, between Hsp20 and DJ-1 and between MyHC-IIa and MyHC-IIx/b (**Fig. 1**).

The networks based on common correlations in the BA and Li breeds found 21 and 19 biomarkers, organised in a single network for both LT and ST muscles, respectively (**Fig. 2a,b**). Of the correlated biomarkers, most of them were found to be shared by the two muscles. First, DJ-1 was correlated with Hsp27, LDH-B and MDH1. Second, Hsp70-8 was correlated with Prdx6, MyLC-1F and Hsp70-1A/B. Last, Hsp70-1A/B was correlated with αB-crystallin.

The networks based on common correlations in the AA and Li breeds found 4 and 8 additional biomarkers with respect to Fig. 1, organised in a single network for the LT and two networks for ST muscles (**Fig. 3a,b**). Of the retained biomarkers, four (LDH-B, μ-calpain, s/70, and CapZ-β) were common for the LT and ST muscles. Only one correlation was common for both muscles: the one between LDH-B and μ-calpain.

The networks based on common correlations in the AA and BA breeds found 4 and 3 additional biomarkers with respect to Fig. 1, organised in two networks for the LT and ST muscles, respectively (**Fig. 3c,d**). Of the retained biomarkers, three (Hsp70-1A/B, αB-crystallin and ENO3) were common for both muscles. No common correlation was found between the two muscles.

The networks based on pairs of breeds contained mostly positive correlations, with the exception of five correlations which were negative. Most of the correlation coefficients were between about 0.4 and 0.6 ($P < 0.05$) for both muscles. Among the robust relationships found for the three breeds (AA + BA+ Li) and two muscles (LT and ST), the correlation between Peroxiredoxin 6 and μ-calpain ($P < 0.01$) is presented in **Fig. 4** as an example.

4. Discussion

The aim of this study was to identify robust relationships between meat quality biomarkers of meat tenderness [2, 12, 13] using correlation network analyses. A correlation between biomarkers was considered robust if it existed for more than one breed or muscle studied. The interpretation of these consistent correlations may improve our understanding of the underlying biological pathways and interactions in different muscles or breeds.

For the two muscles of the three breeds, DJ-1 was positively correlated with Hsp20. For the three breeds, DJ-1 was further correlated with Hsp27 for the ST and with ENO3 and LDH-B for the LT muscle. For the BA and Li breeds, DJ-1 was correlated with Hsp27, MDH1 and LDH-B of the two muscles. DJ-1 is a highly conserved protein of 189 amino acids present in the cytoplasm as well as in intracellular organelles and protects against oxidative stress [21]. For example, it promotes the expression of a number of mitochondrial enzymes involved in reactive oxygen species (ROS) removal [22]. It was further reported to play an anti-apoptotic role, by the involvement of its anti-oxidant activities. It was reported that a deficiency in DJ-1 leads to increased apoptosis, possibly by decreasing Bax expression (a pro-apoptotic protein regulator) and inhibiting caspase activation [23].

The correlations between DJ-1 and ENO3, LDH-B and MDH1 may be indirectly related to energy metabolism. ENO3 catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate, and LDHB catalyses the inter-conversion of pyruvate and lactate with concomitant inter-conversion of NADH and NAD^+. MDH1 plays a pivotal role in the malate-aspartate shuttle operating between cytosol and mitochondria [24]. MDH1 also uses the reduction of NAD^+ to NADH to catalyse reversibly the oxidation of malate to oxaloacetate. This NADH may be used in the electron transport chain for ATP production. Thus, high MDH1 content may be indicative of an increase in the oxidative phosphorylation capacity of the muscle due to cell stress.

The associations found between these enzymes and DJ-1 may be related to the production of ROS associated with increased metabolic activity. During oxidative stress, DJ-1 is re-localized to the mitochondria, where it has a key role in scavenging mitochondrial H_2O_2 and limiting mitochondrial fragmentation [25]. MDH1 is involved in the final steps of glycolysis before malate enters the mitochondrion, hence the relationship between DJ-1 and MDH-1 may be relatively direct. The other enzymes, ENO3 and LDH-B are more closely related to the glycolytic pathway and are not directly involved in the production of ROS. Their

association with DJ-1 may thus be indirect, as increased levels of these enzymes may express increased energy metabolism, including ROS producing pathways.

The correlations between DJ-1 and Hsp20 were remarkably consistent across muscles and breeds as they included also the AA breed. DJ-1 was further correlated with Hsp27 levels in both muscles of the Li and BA breeds. DJ-1 and these small Hsp's were correlated with several other proteins evaluated in this study, mostly involved in glycolysis and oxidative stress. Hsp20 and 27 are simultaneously expressed at high levels in many mammalian tissues including skeletal muscle [26]. Members of the small Hsp family are known to act as molecular chaperones preventing unfolded proteins from aggregation or facilitating refolding of unfolded proteins [27]. Particularly, these small Hsp's protect proteins of the cytoskeleton [28]. In addition to these activities, Hsp20 and 27 have the ability to collaborate to control the redox status [29]. The positive correlations between small Hsp's and DJ-1 may be explained by increased ROS production. For example, in exercising muscles, increased levels of Hsp27 were associated with lower TBARS levels, suggesting that these Hsp's may lower oxidative stress status [30]. In conclusion, increased ROS production may have induced simultaneously higher levels of DJ-1 and small Hsp's.

For the two muscles of the three breeds, µ-calpain was consistently correlated with Prdx6 (Figure 4). Calpains are a class of proteins that belong to the Ca^{2+}-dependent, non-lysosomal cysteine proteases involved in proteolysis. µ-calpain requires micromolar concentrations of Ca^{2+} for its activation [31]. Prdx6 is a bifunctional protein with both glutathione peroxidase and phospholipase A2 (PLA2) activities [32]. Prdxs are known as antioxidant enzymes which convert hydrogen peroxide to water. They can be regulated by oxidation, phosphorylation and proteolysis [33]. The positive correlation with µ-calpain may be explained by earlier observations indicating that antioxidant enzymes, such as Prdx6, may protect proteases, including µ-calpain [34]. Other studies reported that phosphorylation is likely to decrease Prdx activity, while proteolysis makes the protein more resistant to inactivation by overoxidation in response to rising levels of peroxide [33].

A second possible mechanism explaining the relationship between Prdx6 and µ-calpain may be related to the PLA2 group of Prdx6. This group is able to hydrolyse phosphatidylcholine, which are basic phospholipids normally present on the outer leaflet of the cell membranes [35]. At the first stages of apoptosis, considered to be central in muscle to meat conversion [36], the phospholipids distribution in the cell membranes is inverted:

phosphatidylserine switches to the outer and phosphatidylcholine to the inner leaflet of the membrane by a flip-flop process [36, 37]. Inversion of membrane polarity may cause modifications in the membrane fluidity which will become more permeable to ions such as Ca^{2+}, thereby increasing µ-calpain activity. However, it seems today difficult to take conclusions about the functions of Prdx6 or other antioxidant proteins in *post-mortem* muscle and thus more detailed investigations will be needed in order to clarify the exact nature of the relationship between increased oxidative enzymes activities and ultimate meat quality.

Other robust correlations involve part of the proteins mentioned above and members of the Hsp70 family. µ-calpain was positively correlated with Hsp70-8 in LT muscle for the three breeds. In addition, µ-calpain was positively correlated with Hsp70-Grp75 and Hsp70-1A/B in the LT muscle of the Li and BA bulls. Hsp70-8 was positively correlated with Prdx6, MyLC-1F and Hsp70-1A/B for both muscles of BA and Li breeds. Hsp70-1A/B was further positively correlated with αB-crystallin.

The Hsp70 kDa family proteins are among the most highly conserved protein families found in a wide array of organisms [38, 39]. Hsp proteins are categorized into several families that are named on the basis of their approximate molecular weight. Among the best known and investigated Hsp is the Hsp70 family. They were reported by our group to play an important role in meat tenderness in different breeds and muscles [2, 3, 10]. Their functions include folding of nascent polypeptides, protein translocation across membranes, chaperone-mediated autophagy, and prevention of protein aggregation under stress conditions [39]. Hypoxic and ischemic conditions and increased production of ROS induce Hsp70 expression, helping to maintain the mitochondrial membrane potential and ATP levels [39]. Hsp70 proteins are further involved in apoptotic pathways as they sequester pro-apoptotic factors such as Bcl-2 [40]. Hsp70-1A/B is abundantly induced in response to cellular stress, possibly due to its function to preserve proteins [38]. Hsp70-8 is constitutively expressed in most tissues. Hsp70-1A/B and Hsp70-8 are highly related chaperones and are often suggested to have similar physiological functions. They were both reported to collaborate with Hsp40 to ensure a good functioning of the muscle under oxidative stress conditions [38]. This is coherent with results in the present study, showing that both chaperone proteins were correlated with the co-chaperone Hsp40 (*DNAJA1*) in the LT muscle of BA and Li breeds. Like Hsp70-1A/B and Hsp70-8, Hsp40 is related to beef tenderness [41]. An emerging hypothesis is that Hsp40 may decrease apoptosis and, therefore, meat aging and its tenderization during meat storage.

The positive relationship between Prdx6 and Hsp70-8 can also be related to the activity of the PLA2 group of Prdx6. Hsp70-8 binds to membrane phospholipids and interacts with PLA2 activity [42, 43] and may thus interfere in reactions between the PLA2 group of Prdx6 and phospholipids.

The association of Hsp70-1A/B with αB-crystallin in the LT muscle of the three breeds and in the ST muscle for BA and Li breeds may be explained by their role in the regulation of apoptosis. Hsp70-1A/B and αB-crystallin were reported to regulate interactively apoptosis by binding members of the apoptotic cascade, thereby slowing the process [44]. The association of Hsp70-1A/B with μ-calpain in the LT muscle agrees with results of other studies [11, 45]. This positive relationship may be explained by oxidative stress occurring during the *ante* and *post-mortem* periods, increasing simultaneously levels of Hsp70-1A/B and μ-calpain.

The relationship between Hsp70-8 and MyLC-1F in both muscles for BA and Li breeds may be explained by the protective action of members of the Hsp70 family on damaged structural proteins [46]. MyLC-1F is associated with the head region of the myosin molecule [47] and was reported to be released from the myofibrillar fraction during *post-mortem* ageing [48]. From this relationship, we can suggest that heat-shock response may enhance *post-mortem* contractility through a modulation of thick-filament regulation.

Hsp70-Grp75 was linked to various proteins involved in different biological pathways, including glycolytic metabolism (LDH-B and MDH1), proteolysis (μ-calpain), structure (MyLC-1F) and oxidative stress (DJ-1 and Prdx6). Hsp70-Grp75 is a multifunctional member of the Hsp70 family. It is the only known mitochondrial Hsp70 chaperone, and serves as a unique scavenger of toxic protein aggregates [38]. It plays a key role in the folding of matrix-localized mitochondrial proteins and is central in the transport of proteins into the mitochondrion [49]. Its relationship with μ-calpain agrees with earlier studies reporting that mitochondrial calpain activity is influenced by Hsp70-Grp75 activity [50]. More specifically, Ca^{2+} exerts regulatory effects on calpains [2, 51] and Hsp70-Grp75 may enhance Ca^{2+} trafficking from the ER by linking the inositol 1,4,5-trisphosphate receptor of the endoplasmic reticulum (ER) to the mitochondrial voltage-dependent anion channels [2, 52]. The correlation between Hsp70-Grp75 and DJ-1 suggests that the former was further linked to the oxidative pathway, which is coherent with an earlier indication that DJ-1 translocates to the mitochondria after oxidative stress, via Hsp70-Grp75 [53]. The role of Hsp70-Grp75 on Ca^{2+} trafficking from the ER to the sarcoplasm may also explain the relationship between Hsp70-

Grp75 and the enzymes of the glycolytic pathway in the present study. In addition, Hsp70-Grp75 is known to be induced by glucose deprivation and agents perturbing glycolysis [38], which may further explain this correlation. We suggest that Grp75 may be involved in the regulation of cell response to variations in glucose levels.

Overall, various correlations were muscle specific. Considering the BA and Li breeds, Hsp70-Grp75 was correlated to proteins belonging to many different biological pathways in the LT muscle, which is coherent with its multifunctional role. For example, in the LT muscle, DJ-1 and µ-calpain showed also many correlations, but these did not involve as many different biological pathways as the proteins correlated with Hsp70-Grp75. In the ST muscle, Hsp70-Grp75 was correlated with small Hsp's, but not in the LT muscle. Similarly, in the LT, but not the ST muscle, Hsp40 was correlated with proteins of various biological pathways. Overall, in the LT, more correlations were found between proteins related to the glycolytic pathway and in the ST muscle, more correlations were found with the three small Hsp's. These differences may be related to differences in metabolic functioning between the muscles and need further investigation. The proteins in the present study were selected for their known relationship with tenderness development. The anti-apoptotic function of small Hsp's is known to have negative consequences for meat tenderness. The larger amount of correlations between these Hsp's and other proteins in the ST muscle may explain the generally lower tenderness of this muscle of the Li and BA breeds [3].

5. Conclusion

The present study demonstrates that several robust relationships exist between proteins belonging to similar or different biological pathways. Particularly, DJ-1 and Prdx6 were correlated with Hsp20 and µ-calpain, respectively, irrespective of muscle and breed. Considering BA and Li bulls only, Hsp70-Grp75, DJ-1 and µ-calpain were correlated with proteins related to glycolytic, oxidative and Hsp's. Results suggest that of the many cellular functions involved in development of tenderness, mitochondrial properties and oxidative stress play a major role and possibly initiating a cascade of reactions involved in the development of tenderness. Proteins with cell protective functions, particularly anti-oxidative proteins and Hsp's seem to play key roles. A better understanding of these aspects would increase our knowledge of the mechanisms underlying tenderness according to muscle, breed and gender.

Conflict of interest

The authors have declared that no competing interests exist.

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Table 1. List of the 18 protein biomarkers of beef tenderness investigated using the Dot-Blot technique in this study.

Protein name	Gene	UniProtKB ID
Heat Shock Proteins		
αB-Crystallin	*CRYAB*	P02511
Hsp20	*HSPB6*	O14558
Hsp27	*HSPB1*	P04792
Hsp40	*DNAJA1*	P31689
Hsp70-1A/B	*HSPA1B*	P08107
Hsp70-8	*HSPA8*	P11142
Hsp70-Grp75	*HSPA9*	P38646
Metabolism		
ENO3 (Enolase 3)	*ENO3*	P13929
LDH-B (Lactate Dehydrogenase Chain B)	*LDHB*	P07195
MDH1 (Malate Dehydrogenase 1)	*MDH1*	P40925
Structure		
CapZ-β (F-actin-capping protein subunit β)	*CAPZB*	P47756
α-actin	*ACTA1*	P68133
MyLC-1F (Myosin Light Chain 1F)	*MYL1*	P05976
MyBP-H (Myosin Binding Protein H)	*MYBPH*	Q13203
Oxidative resistance		
DJ-1 (Parkinson disease protein 7)	*PARK7*	Q99497
Prdx6 (Cis-Peroxiredoxin)	*PRDX6*	P30041
SOD1 (Superoxide Dismutase Cu/Zn)	*SOD1*	P00441
Proteolysis		
μ-calpain	*CAPN1*	P07384

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

Target protein	Antibody references	Dilutions[1]
Heat Shock Proteins		
αB-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
Metabolism		
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
Structure		
CapZ-β	Monoclonal anti-human Abnova CAPZB (M03), clone 4H8	1/250
α-actin	Monoclonal anti-Rabbit Santa Cruz α-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
Oxidative resistance		
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
Proteolysis		
μ-calpain	Monoclonal anti-bovine Alexis μ-calpain 9A4H8D3	1/1000

[1] Dilutions of each antibody are as described in Gagaoua *et al.* (2015)

Table 3: Effects of breed, replicate and breed x replicate interaction on beef tenderness biomarkers in the *Longissimus thoracis* muscle.

Variables[1]	Breed[2] (B) x Replicate[3] (R)						SEM[4]	*P-value*[5]		
	AA1	AA2	BA1	BA2	Li1	Li2		(B)	(R)	B x R
Heat shock proteins										
αB-crystallin	24.4b	29.4a	12.4d	19.0c	16.1d,c	20.3b,c	0.84	***	***	ns
Hsp20	22.3a	17.4b	17.1b	17.8b	19.4a,b	16.8b	0.51	ns	*	t
Hsp27	28.4a	29.1a	18.9b	19.0b	22.0b	20.9b	1.08	***	ns	ns
Hsp40	18.4a	17.6a,b	15.6b	18.3a	16.2b	15.9b	0.26	**	ns	**
Hsp70-1A/B	16.8b	18.7a,b	15.7b	19.4a,b	16.9b	21.3a	0.46	ns	***	ns
Hsp70-8	16.3a,b	15.1b	16.8a,b	18.1a	16.0b	17.0a,b	0.22	**	ns	*
Hsp70-Grp75	9.0c	9.2c	17.0a	16.2a	11.9b	13.2b	0.44	***	ns	ns
s/70 [6]	1.81a	1.85a	0.98b	1.05b	1.30b	1.13b	0.05	***	ns	ns
Metabolism										
ENO3	17.1	17.9	17.5	12.8	13.9	13.0	0.56	*	ns	ns
LDH-B	10.1c	10.2c	18.9a	19.2a	13.2b,c	14.9b	0.59	***	ns	ns
MDH1	10.5b	14.7a	14.9a	15.8a	10.6b	16.8a	0.45	*	***	*
Structure										
CapZ-β	19.6a	18.9a	14.4b	17.1a,b	15.5b	15.3b	0.42	***	ns	ns
α-actin	17.9a,b	15.6b	17.9a,b	20.8a	15.3b	17.0b	0.40	***	ns	*
MyLC-1F	15.0a,b	14.8a,b	15.0a,b	15.9a	13.6b	14.8a,b	0.2	*	ns	ns
MyBP-H	13.4	13.0	19.1	11.6	12.8	14.4	0.84	ns	ns	ns
MyHC-I	29.5a	27.1a,b	15.6d	21.8c	23.3c,b	23.9c,b	0.73	***	ns	*
MyHC-IIa	52.2b	57.2a	26.6d	21.8e	32.1c	29.7c,d	1.64	***	ns	*
MyHC-IIx+b	18.3c	15.7c	57.7a	56.3a	44.6b	46.4a	2.13	***	ns	ns
Oxidative resistance										
DJ-1	16.6	17.6	16.0	16.0	15.4	17.6	0.33	ns	ns	ns
Prdx6	15.8a	15.0a,b	13.1c	13.6c	12.5c	14.0c,b	0.22	***	ns	t
SOD1	17.0	18.6	15.7	15.9	15.1	15.9	0.42	ns	ns	ns
Proteolysis										
μ-calpain	14.4b	13.8b	14.9a,b	16.7a	12.8b	15.0a,b	0.28	**	*	ns

[1] All variables were expressed in arbitrary units except MyHC which were in percentage.
[2] Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, Li: Limousin
[3] Replicate: 1 and 2 for first and second year
[4] Standard error of the mean
[5] Significance level: ns: not significant, t $P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$
[6] s/70: ratio of small Heat shock proteins to Hsp70s calculated using the following formula: s/70 = (Hsp20 + Hsp27 + αB-crystallin / Hsp70-1A/B + Hsp70-8 + Hsp70-Grp75)

Table 4: Effects of breed, replicate and breed x replicate interaction on beef tenderness biomarkers in the *Semitendinosus* muscle.

Variables[1]	Breed[2] (B) x Replicate[3] (R)						SEM[4]	*P-value*[5]		
	AA1	AA2	BA1	BA2	Li1	Li2		(B)	(R)	B x R
Heat shock proteins										
αB-crystallin	11.4b	14.8a	5.8c	7.9c	6.9c	8.8c	0.46	***	**	ns
Hsp20	13.9a,b	13.0a,b,c	11.6b,c	13.8a,b	10.7c	15.4a	0.35	ns	**	**
Hsp27	20.2a	20.3a	12.7b	14.4b	12.8b	15.6b	0.60	***	*	ns
Hsp40	14.1	13.4	12.3	13.1	12.9	12.4	0.22	ns	ns	ns
Hsp70-1A/B	11.2b	13.3a,b	12.4a,b	12.9a,b	11.1b	14.3a	0.30	ns	***	ns
Hsp70-8	15.5a,b	14.1b	16.3a	15.8a,b	15.3a,b	16.1a	0.19	*	ns	t
Hsp70-Grp75	13.1	13.5	12.5	12.7	11.1	12.9	0.24	ns	ns	ns
s/70 [6]	1.15a	1.2a	0.74c	0.87b,c	0.81b,c	0.92b	0.02	***	*	ns
Metabolism										
ENO3	15.2a,b	15.9a	12.8b,c	11.9c	12.4b,c	13.3a,b,c	0.35	***	ns	*
LDH-B	10.6c	12.0b,c	16.0a	14.2a,b	12.6b,c	14.2a,b	0.33	***	ns	ns
MDH1	13.8b,c	17.7a	13.2c	16.6a	12.3c	15.9a,b	0.37	ns	***	ns
Structure										
CapZ-β	14.4	15.5	12.9	13.8	12.9	14.5	0.28	ns	ns	ns
α-actin	17.5	17.3	17.9	17.0	17.6	18.2	0.31	ns	ns	ns
MyLC-1F	14.9	15.0	15.5	15.9	14.6	15.3	0.18	ns	ns	ns
MyBP-H	13.8	13.9	13.0	12.9	13.1	13.6	0.36	ns	ns	ns
MyHC-I	11.5a	8.6b	6.6b,c	3.8d	8.2b	4.8c,d	0.43	***	***	ns
MyHC-IIa	30.4a	33.0a	20.6c	15.7d	26.1b	21.6c	0.88	***	t	*
MyHC-IIx+b	58.1d,e	58.4e	72.8b,c	80.5a	65.7c,d	73.6a,b	1.30	***	*	**
Oxidative resistance										
DJ-1	13.8b	15.9a	12.9b	13.2b	12.4b	13.4b	0.26	**	*	ns
Prdx6	17.5a	18.0a	15.5b	15.7b	14.0c	16.1b	0.23	***	*	ns
SOD1	16.4	15.4	15.3	15.6	14.5	16.2	1.22	ns	ns	ns
Proteolysis										
μ-calpain	13.3b,c	14.1a,b,c	14.5a,b,c	15.7a	12.5c	15.4a,b	0.26	*	***	ns

[1] All variables were expressed in arbitrary units except MyHC which were in percentage.
[2] Breed designation: AA: Aberdeen Angus, BA: Blond d'Aquitaine, Li: Limousin
[3] Replicate: 1 and 2 for first and second year
[4] Standard error of the mean
[5] Significance level: ns: not significant, t $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$
[6] s/70: ratio of small Heat shock proteins to Hsp70s calculated using the following formula: s/70 = (Hsp20 + Hsp27 + αB-crystallin / Hsp70-1A/B + Hsp70-8 + Hsp70-Grp75)

Figure captions

Fig. 1. Correlations networks within all breeds (AA + BA + Li) for *Longissimus thoracis* (**A**) and *Semitendinosus* muscles (**B**). The correlations values found using standardized data (Z-scores) are shown on each interaction line. The black edges connecting the red nodes are common for both muscles and three breeds.

Fig. 2. Correlation networks within breed interactions for BA and Li among *Longissimus thoracis* (**A**) and *Semitendinosus* (**B**) muscles. The correlation values found using standardized data (Z-scores) are shown on each new interaction line (black and dark gray edges).

Fig. 3. Correlation networks within breed interactions for AA and Li (**A, B**) and for AA and BA breeds (**C, D**) among *Longissimus thoracis* and *Semitendinosus* muscles respectively. The correlation values found using standardized data (Z-scores) are shown on each new interaction line (black and dark gray edges only).

Fig. 4. Example of robust correlations (Pearson correlation analysis on Z-scores) found for the three breeds (○) Aberdeen Angus; (Δ) Blond d'Aquitaine and (□) Limousin) between Peroxiredoxin 6 and μ-calpain ($P < 0.01$) for the **(A)** *Longissimus thoracis* and **(B)** *Semitendinosus* muscles

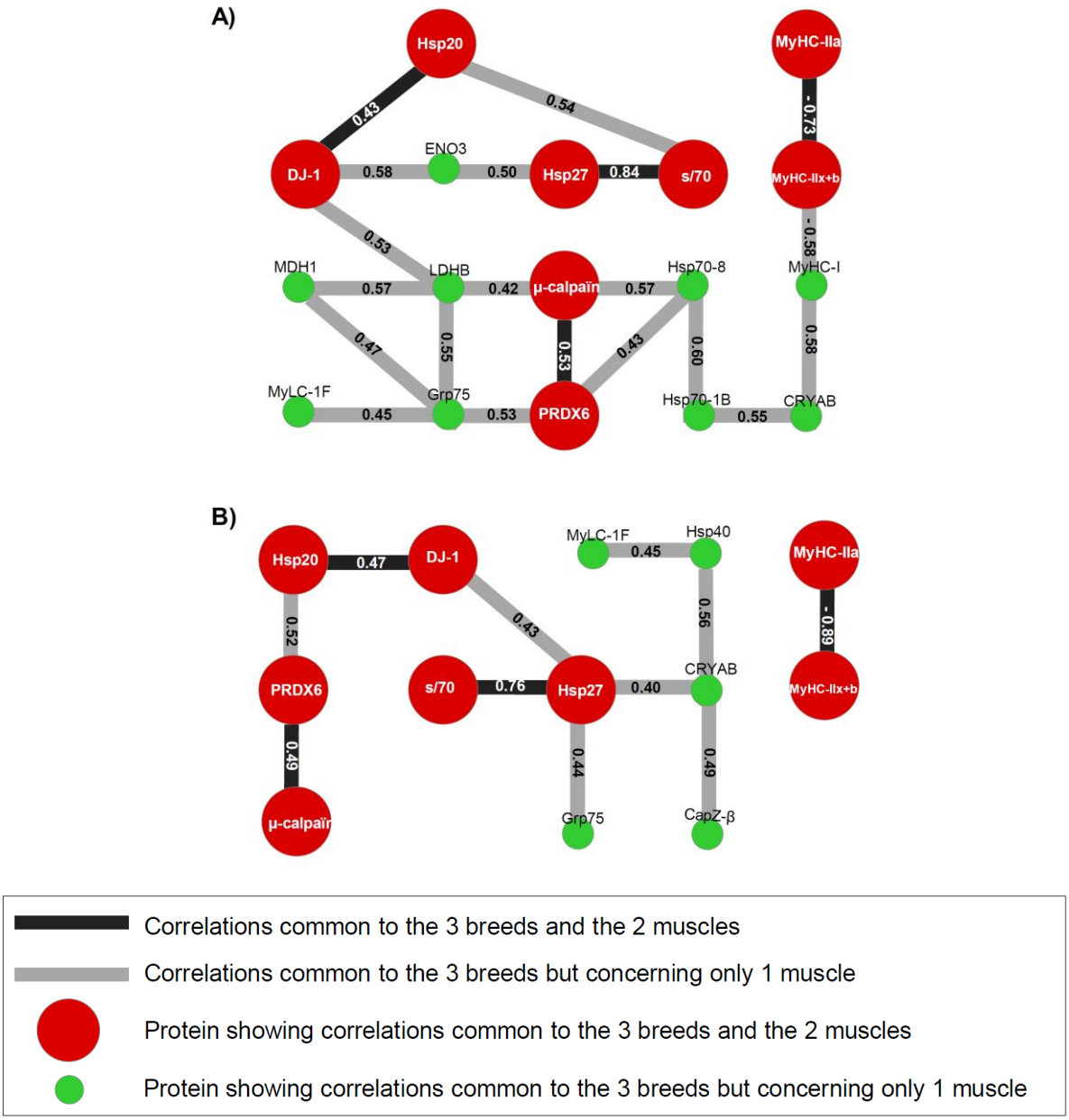
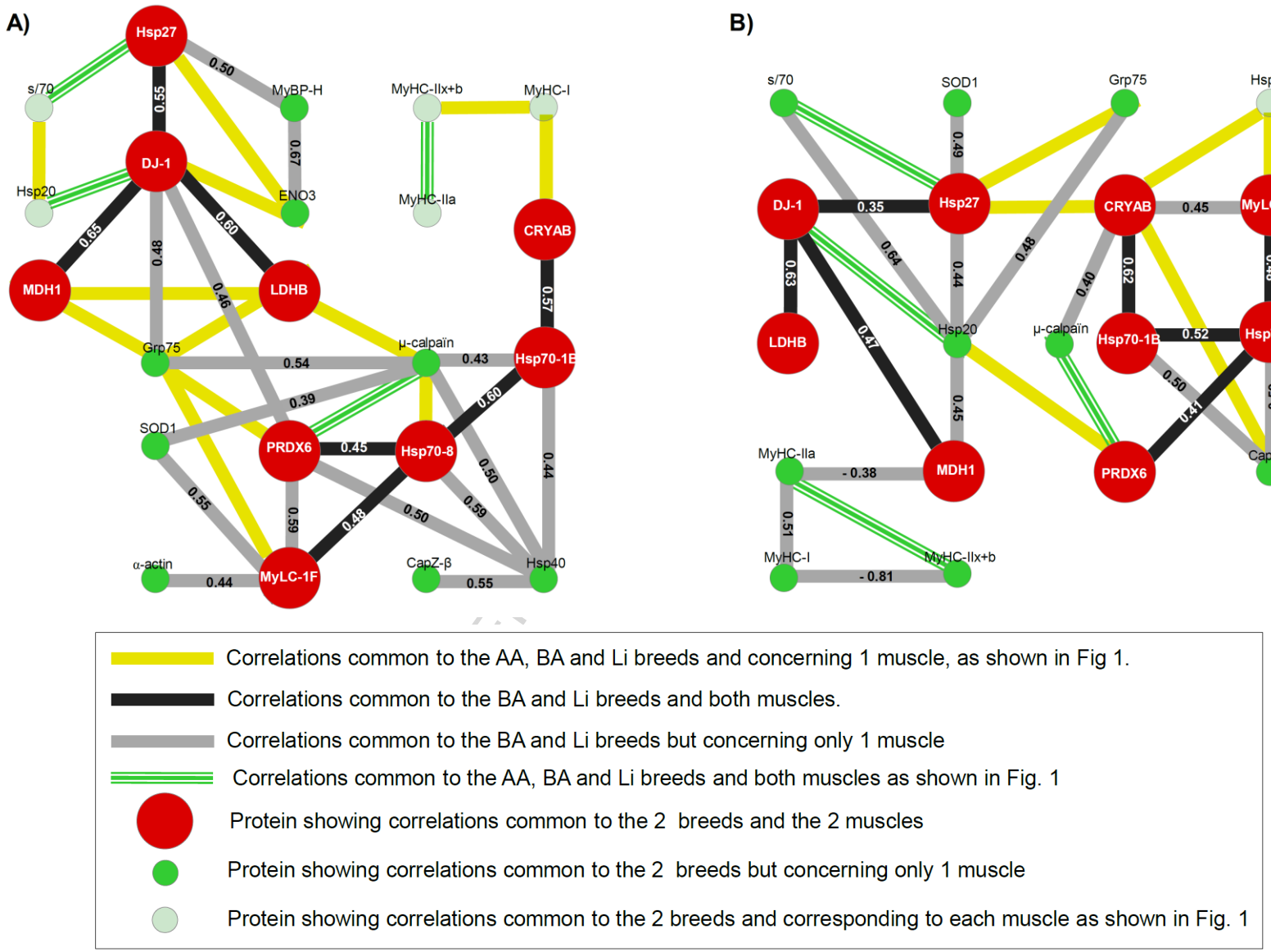


Fig. 1. Correlations networks within all breeds (AA + BA + Li) for *Longissimus thoracis* (**A**) and *Semitendinosus* muscles (**B**). The correlations values found using standardized data (Z-scores) are shown on each interaction line. The black edges connecting the red nodes are common for both muscles and three breeds.



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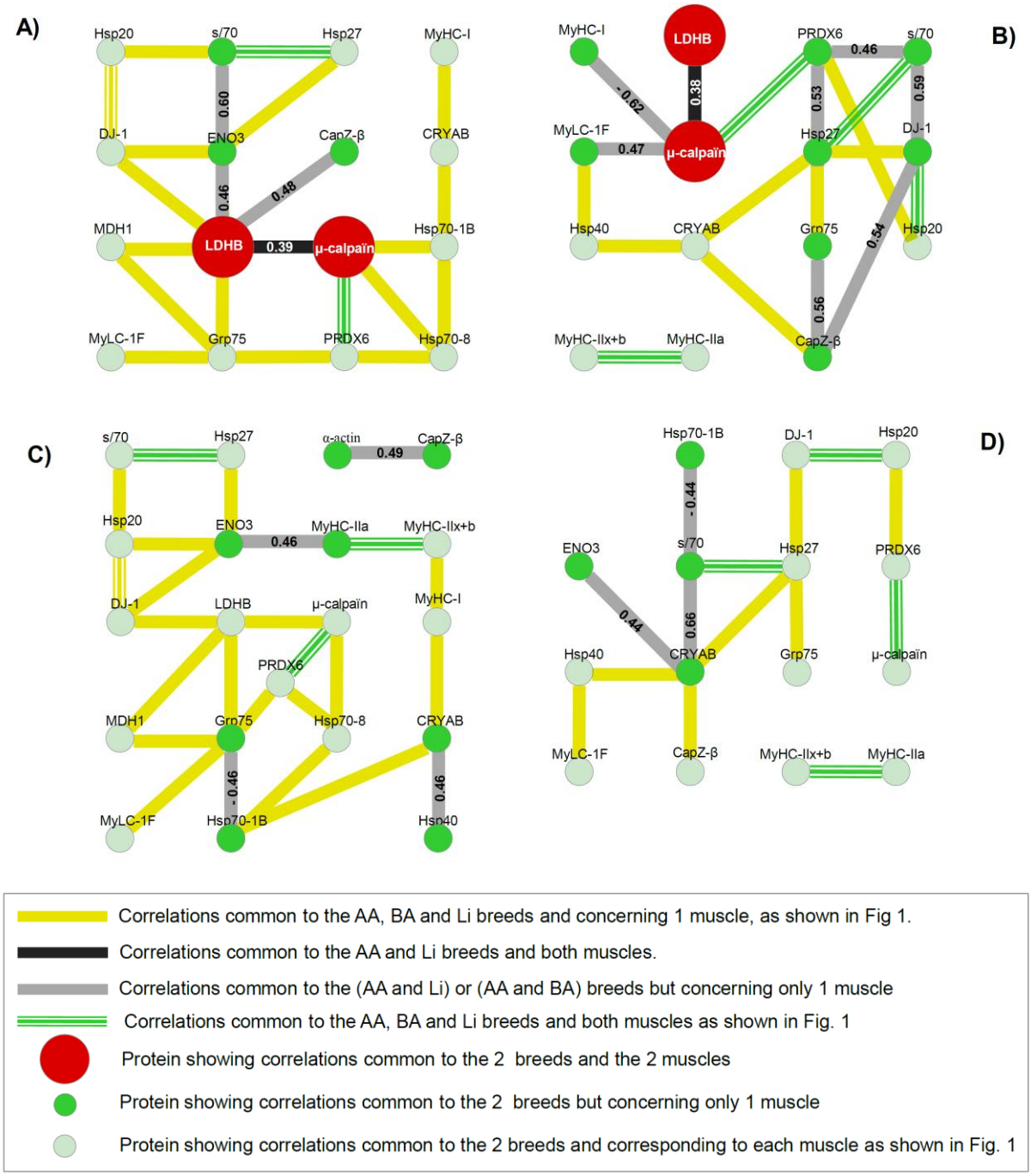

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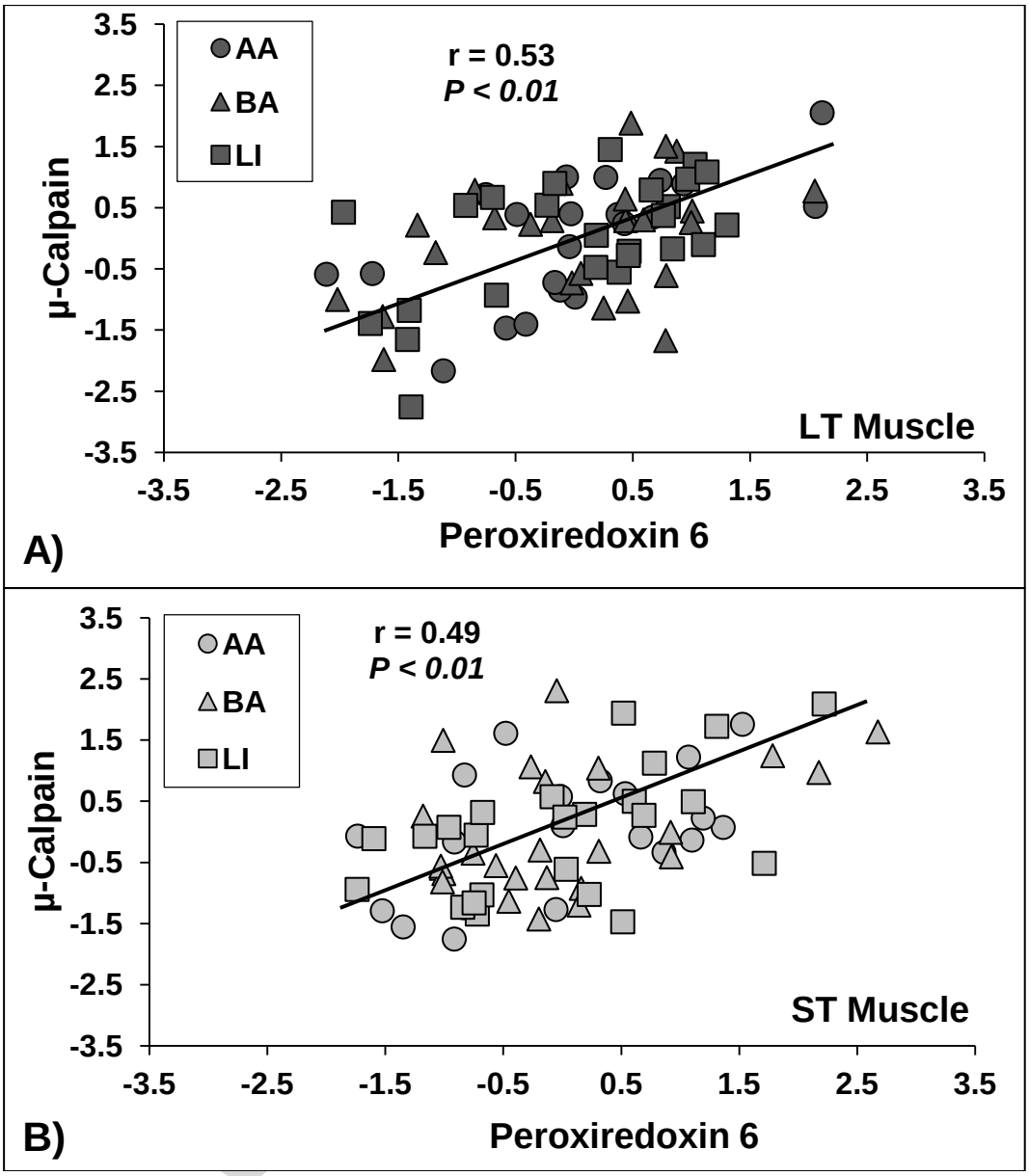

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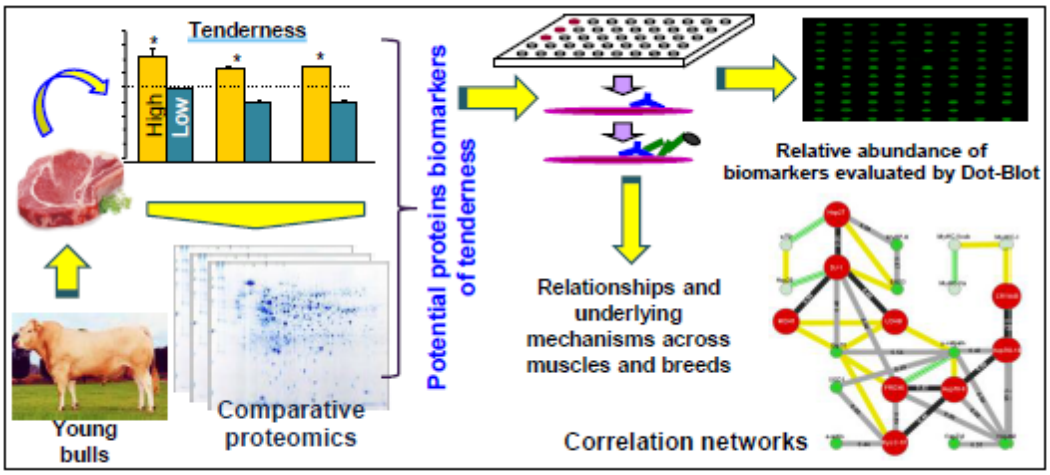
Graphical abstract



Biological significance

This study, using statistical correlation networks shows coherent relationships between meat tenderness protein markers earlier identified by our group using comparative proteomics. Studying three continental breeds (Aberdeen Angus, Blond d'Aquitaine and Limousin) and two different muscles (*Longissimus thoracis* and *Semitendinous*), this study reveals biological pathways involved in the process of meat tenderization. The development of reliable biomarkers related to meat tenderness represents an important challenge in meat science and to our knowledge; this study is a step forward for a better understanding of the main biological events leading to the conversion of muscle into tender or tough meat.

Highlights

- Biological pathways robustly related with meat tenderness were presented.
- Coherent relationships exist between biomarkers independently of breed and muscle.
- Correlation networks increase our knowledge on the mechanisms underlying tenderness
- In LT muscle, more correlations were found with proteins from glycolytic pathway
- In ST muscle, more correlations were found with the small heat shock proteins.