Differences in light scattering between pale and dark beef *longissimus thoracis* muscles are primarily caused by differences in the myofilament lattice, myofibril and muscle fibre transverse spacings.

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Abstract

Beef colour is essential to consumer acceptability with dark muscle colours being problematic. Dark meat has less light scattering but the mechanisms are unknown. We hypothesise that three mechanisms are responsible for decreased light scattering in dark meat, namely (i) larger lateral separation of myofilaments, (ii) decreased optical protein density in the I-band and (iii) decreased denaturation of sarcoplasmic proteins. Nineteen beef *longissimus thoracis* muscles, divided into 'light', 'medium' and 'dark' colour groups, were assessed for light scattering by reflectance confocal microscopy, sarcomere length, and myofilament lattice spacing by small-angle X-ray diffraction. Dark muscles had a longer lattice spacing, shorter sarcomeres and wider muscle fibre diameters compared to lighter colour groups, indicating that the transverse spacing of muscle fibres that occurs post-mortem, with pH decline, is central to light scattering development. Dark muscles also had more degradation of the Z-disc and higher sarcoplasmic protein activities, which could impact on the optical density and contribute to lower light scattering.

Keywords

Meat colour, skeletal muscle structure, myofilament lattice spacing, cytoskeleton, sarcoplasmic protein, sarcomere.

1. Introduction

The appearance of meat is largely dependent on the colour of the muscle and is a key attribute of consumer acceptability. Within beef muscle, the red pigments, myoglobin and haemoglobin, are the primary absorbers of certain wavelengths of light and so give rise to red meat colour. In addition, structural elements of the muscle (myofibrillar, cytoskeletal and interaction with other sarcoplasmic proteins) that both absorb and scatter the light (Macdougall, 1970) affect the paleness/darkness of the muscle. Light scattering by diffusion or deflection by particles or interfaces is thought to occur between various structures of the muscle that have different refractive properties, such as changes in the optical density that occur in the gaps between adjacent myofibrils and/or at the A-I interfaces of the sarcomere (Offer et al., 1989), or even the intersection between the I-band and the Z-line, but the exact components responsible for light scattering are yet to be defined.

The structure and light scattering properties of dark and pale meat are very different and are associated with the pH of the muscle. Previously, beef *longissimus* muscles classified as dark, with a high pH (pH > 5.8), have been shown to have muscle fibres that are swollen, with less light scattering compared to light muscles with a low pH (pH = 5.4) (Hughes, Clarke, Purslow, & Warner, 2017). Dark, high pH meat has a 'swollen' structure (larger diameter muscle fibres) due to reduced shrinkage and is accompanied by more transmittance and less light scattering and reflectance (Irving, Swatland, & Millman, 1989; Swatland, 2008). These muscles also typically have a shorter sarcomere length compared to lighter muscles (Hughes et al., 2017; Irving et al., 1989; Warner, Kauffman, & Greaser, 1997), and in pork *longissimus* have been shown to have a larger spacing between the myofilaments (Irving et al., 1989). In comparison, pale, low pH meat, undergoes greater transverse shrinkage of the lattice, which is accompanied by greater muscle fibre shrinkage and greater fluid loss, classically giving rise to pale, soft, exudative (PSE) meat. These findings have mainly been observed in pork and chicken, and also in beef (Tarrant & Mothersill, 1977), although the opposite condition of dark, firm dry (DFD) meat with a high ultimate pH is more common.

Previously we have shown that reducing the pH environment of a muscle fibre induces fibre shrinkage and is associated with increased global brightness or more light scattering within that fibre (Hughes et al., 2017; Hughes, Clarke, Purslow, & Warner, 2018). However, not all of the changes in light scatter that occur in low pH meat can be reversed by increasing the pH post-rigor, which suggests that some irreversible change such as protein denaturation at low pH may either contribute to changes in myofilament lattice spacing (e.g. by denaturation of myosin heads) or induce further light scattering by precipitation of particles in the sarcoplasmic fluid, or increase scattering from certain structures by decorating them with aggregated protein. In pale pork, the shrinkage of the myofilament lattice has been linked to both myosin head and sarcoplasmic protein precipitation (Liu, Arner, Puolanne, & Ertbjerg, 2016). So, it is likely these events do not occur in dark meat and consequently there is less development of the light scattering. Therefore, we hypothesise that three mechanisms are responsible for decreased light scattering in dark meat, namely (i) a larger lateral separation of thick and thin myofilaments (ii) decreased optical protein density in the I-band and (iii) decreased denaturation of sarcoplasmic proteins.

The aim of this current investigation is to test this hypothesis. Whereas pH differences in previous work were assumed to drive changes in the myofilament lattice spacing, we aim to make a direct correlation between light scattering, colorimetric measurements and myofilament lattice dimensions measured by small angle X-ray diffraction (SAXS). In addition, as the optical protein density of the sarcomere could be impacted by the integrity and location of the sarcoplasmic proteins, the degree of denaturation or aggregation of some sarcoplasmic proteins will be monitored by measuring the activity of two of the most abundant sarcoplasmic proteins enzymes, namely aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).. Mass spectroscopy is also used to identify proteins or fragments produced by post-mortem proteolysis that are more prominent either in light or dark muscles.

Few visualisation techniques have been employed to identify the specific proteins, components or regions of the cell that are involved in light scattering. More recently, we have used reflectance confocal laser scanning microscopy (rCLSM) as a useful tool to visualise the overall global brightness

as a quantitative measure of light scattering intensity (Hughes et al., 2017). By using rCLSM we can obtain measurements of the structural dimensions in the muscle cell, but also the periodic spacing (distance between global brightness peaks) of the scattering elements involved. This technique will be employed to facilitate the identification of these scattering elements and possible sites of increased or decreased optical density contrast in pale versus dark meat samples.

2. Methods

2.1 Carcass assessment, sample collection and storage

Beef *longissimus thoracis* muscles from the left side of the carcass were collected 72 to 96 h post-mortem (PM) from a commercial meat processor. Samples were collected 3 to 4 d PM to maximise the time from slaughter to grading, thus ensuring the final pH had been reached and meat colour compliance was more likely (Hughes, Kearney, & Warner, 2014). Carcass sides were quartered between the 10th and 11th ribs, and the exposed muscle surface colour was developed (bloomed) for 1 h at 10 °C. Muscles were visually assessed for meat colour using a standard set of colour chips, by a qualified AUS-MEAT (Authority for Uniform Assessment of Meat; standard Australian beef grading system) assessor. A total of 19 carcasses were allocated to one of 3 meat colour groups (light, medium or dark) as defined by AUS-MEAT colour scores: 1C (light, n = 7); 2 (medium, n = 7); >3 (dark, n = 5), respectively, with a meat colour >3 considered to be unacceptably dark (AUS-MEAT, 2005). Unfortunately, due to a lack of dark meat coloured carcasses, only 5 replicates from this group could be collected. Using the assessor data, we were advised that the dark meat carcasses also had an unacceptably high pH (≥ 5.71), as per AUS-MEAT guidelines and all of which had pH values ≥ 6.00 . These carcasses were from pasture fed, male bovine animals (<320 kg) that were up to 36 months old, with 0 to 4 permanent incisor teeth. The remaining 14 carcasses from the light and medium colour groups were from 100 d grain fed, female bovine animals (<320 kg), that were up to 30 months old, with 0 to 2 permanent incisor teeth. For all colour groups, fat colour scores were all at the lighter/whiter end of the scale (<3), with the subcutaneous fat depth ranging from 8 to 27 mm.

All muscles were vacuum packed and transported back to the laboratory using insulated containers and stored at 5 °C. Further processing was conducted at 10 °C, whereby a thick slice (40 mm thick) was removed from the caudal end and stored at -1 °C for rCLSM. A second slice (40 mm thick) was cut and used for small angle X-ray scattering (SAXS) and colour measurements. A third slice (25 mm thick) was trimmed of subcutaneous fat and connective tissue and cut into small cubes and placed in 4 separate 25 ml plastic tubes, where they were subsequently stored at -80 °C and these samples were used for sarcoplasmic activity assays, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and light microscopy analysis. A fourth slice (40 mm thick) was cut and used for drip loss measurement. The remaining primal was vacuum packed and stored at -20 °C.

2.2 Muscle analysis

2.2.1 Drip loss

At 4 to 5 d PM, drip loss was measured using the method of Honikel, Kim, Hamm, and Roncales (1986). Briefly, muscle was cut into 50 g cubes (~30 x 30 x 40 mm), suspended using thread at 5 °C and covered in a plastic bag (ensuring no direct contact made with meat surface). Samples were weighed before and after 48 h, with the difference considered drip loss.

2.2.2 Colour and pH measurements

At 5 to 6 d PM, colour measurements were made on the SAXS slice after trimming 5 mm from the surface and blooming at 4 °C for 60 min. A Minolta chromameter CR-400 (Minolta Pty Ltd, Japan, light source D65, observer angle 2°, light projection tube CR-A33d, with ϕ 22mm disc) was used to measure lightness, redness and yellowness; L*, a* and b* values, respectively. Calibration was at the same temperature, using white and black calibration tiles. The pH was measured prior to SAXS measurement using a TPS WP-80 pH meter with a polypropylene spear-type gel electrode (Ionode IJ 44) and temperature probe (TPS Pty Ltd, Springwood, QLD, Australia). Calibration of the pH equipment was conducted at 5 - 10 °C, using pH 4 and 7 buffers (TPS Pty Ltd, product no. 121382 and 121388, respectively).

2.2.3 Small angle X-ray scattering (SAXS) measurements

At 5 to 6 d PM, samples were prepared for SAXS analysis by slicing steaks in half (dorsal to ventral slice), then using a scalpel to cut a 10 mm diameter (2-3 mm depth) sub-sample adjacent to the seam of the muscle (near to the dorsal side) whilst avoiding intramuscular fat. The sub-samples were cut in longitudinal orientations, so that the axis of the diffraction pattern was perpendicular to the muscle fibre axis (equatorial diffraction). Sub-samples were mounted onto the plate using Kapton tape and a blank (kapton tape only), was also fixed to the plate.

Transmission measurements irradiated the sub-samples in an orthogonal grid, at a minimum meat volume size of 0.250 x 0.12 x 1 mm. This was conducted over 110 shots x 1 s exposures, with a camera length of 7.025 m at 27 ± 1 °C. The detector was a Pilatus2- 1M with 2.5×10^{12} photons/s and a momentum of 10 keV. Calibrations were conducted using silver behenate for the d-spacing and glassy carbon for the sample thickness. Image analysis was conducted using the Scatterbrain program (Australian Synchrotron Facility, 2016). For one sample, any outliers were removed (beam clipping edge of plate) and shots were summed. Peak fitting of the data was mostly between $q = 0.11$ and $0.045 \text{ \AA}^{-1}$, using simultaneous fitting of a power-law model (mostly with exponent between -2 and -1) and two Gaussian peaks, using starting values adequate to guarantee convergence upon a viable solution. Data of the peak position (centre), peak width (sigma), peak height (amplitude) was used to calculate d-spacing ($d = 2 \times \pi / q$), peak width (in q units) and integrated peak area. The Bragg reflections $d_{1,0}$ and $d_{1,1}$ were considered to be the lattice spacings between myosin filaments and myosin and actin filaments respectively, as reviewed by Millman (1998) and schematically explained in supplemental Figure S1.

2.2.4 rCLSM and image analysis

An Olympus FluoviewTM 1000 CLSM was used in the reflectance mode for measurement of light scattering properties expressed as global brightness values (mean greyscale pixel intensity) as described previously for isolated muscle fibres (Hughes et al., 2017). The internal surface of the 40 mm thick slice was cut (10 x 10 x 3 mm) using a scalpel, both longitudinally and transverse to the muscle fibre axis and placed on a coverslip for viewing. Images were captured at a depth of 30 μm ,

with a total of 6 images collected in each orientation for any one sample. Image analysis was conducted using polygon sections around the muscle fibre perimeter, as described previously for isolated muscle fibres.

2.3 Isolated muscle fibre fragments and myofibrils analysis

2.3.1 Isolation procedure

Isolated muscle fibre fragments and myofibrils were used for sarcomere length measurement, enzyme activity assays and SDS-PAGE sample preparation. The method of isolation has been described previously (Hughes et al., 2017), with 1 ± 0.05 g muscles extracted in 10 ml of ice-cold 380 mM mannitol + 50 mM potassium acetate (KAM) adjusted to pH 5.40, 5.60 or 6.10 for light, medium and dark meat colours, respectively. Fractions from each step of the procedure were collected in the following buffers: supernatants from the first (S1) and second (S2) washes were both in KAM, and the third wash (S3) was in 0.1 M sodium phosphate buffer, 0.5 mM EDTA, 2 mM dithiothreitol at pH 7.4. When supernatants were removed, a small aliquot (20 μ l) was reserved for SDS-PAGE, the total volume of the supernatant was measured, and then pellets were replenished with exactly the same volume of the appropriate buffer.

2.3.2 Aldolase and GAPDH assays

Aldolase was assayed using a modified method of R. Wu and Racker (1959). Supernatants from the extraction were diluted (1:10) and 20 μ l was pipetted in duplicate into a 96 well plate. The assays were started by adding 200 μ l of the aldolase assay mixture which contained 50 mM Tris- HCl, pH 7.5, 50 mM potassium chloride, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 3 mM magnesium acetate, 1 mM fructose biphosphate and 16.6 μ g/ml α -glycerol-3-phosphate dehydrogenase/triosephosphate isomerase (GmbH, catalogue no 737.259). The rate of oxidation of nicotinamide adenine dinucleotide (NADH) was monitored at 340 nm over 5 min using a Biostrategy SpectraMax M3 plate reader. Using the $V_{\max}$ and the NADH extinction coefficient of 6220 M/ cm⁻¹ the enzyme in each fraction was calculate. One unit (U) being defined as 1 μ mol of NAD oxidised/ min. The activity of each of the 3 extracts (S1, S2, S3) are presented as a percentage of the total activity of the extracts.

GAPDH was assayed using a modification of the method of Bass, Brdiczka, Eyer, Hofer, and Pette (1969). Supernatants were diluted at 1:20 and 20 μ l was pipetted in duplicate into a 96 well plate. The assays were initiated by adding 200 μ l of the GAPDH assay mixture which contained 0.05 M Tris-HCl, pH 8.0, 0.05 M potassium chloride, 0.2 mM EDTA, 3 mM magnesium acetate, 10 mM ATP, 5 mM 3-phosphoglyceric acid, 3.3 μ g/ml phosphoglycerate kinase, 0.15 mM NADH. The rate of oxidation of NADH was monitored at 340 nm over 5 minutes using the plate reader as described above. The activity of each of the 3 extracts (S1, S2, S3) were presented as a percentage of the total activity of the extracts.

2.3.3 SDS-PAGE

The S1, S2 and S3 fractions from one light and one dark muscle was selected for SDS-PAGE analysis based on the following criteria: the light muscle was selected based on having a high lightness and low pH value ($L^* = 34.79$, pH = 5.45) and dark muscle having a low lightness and high pH value ($L^* = 29.01$, pH = 6.31). Samples were loaded on an equal volume basis (to gauge the quantity of individual proteins within each fraction), whereby 80 μ l of sample was added to 20 μ l 5x SDS reducing buffer and 6 μ l was added per lane. Mini-protean TGX 10 well gels (Bio-rad, cat no 456-9033) were used for protein separation, with a low molecular weight pre-stained standard in SDS reducing buffer (Bio-rad, cat no 161-0373) applied. Gels were immersed in a gel tank containing tris-glycine running buffer at 40 mA for 40 min, and subsequently stained in quick Coomassie blue (Protein Ark, product code: Gen-QC-Stain-1L) for 30 min (room temperature) and stored in milli Q water overnight (4 $^{\circ}$ C). Images of gels were captured and viewed the following day using a Bio-Rad EZ Gel Doc and Image LabTM software (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia).

2.3.4 Nano liquid chromatography tandem mass spectrometry (NanoLC ESI MS/MS) analysis

The mass spectrometer used was a TripleTOF 5600 (SCIEX, Redwood City, USA) with a nanoflow HPLC system (Eksigent Ultra nanoLC). The protein band of interest from the dark muscles (S3 fraction ~35 kDa) was destained with acetonitrile (ACN): ammonium bicarbonate, reduced (25 mM dithiothreitol), alkylated (55 mM iodoacetamide), washed and dried. Proteins were digested with 150 μ g trypsin for 18 h. Peptides were extracted, then dried. The sample was reconstituted in 12 μ L

loading buffer prior to analysis and 10 μ L was injected onto a peptide trap (OptimizeTech peptide Captrap) for pre-concentration and desalted with 0.1% formic acid, 2% ACN, at 10 μ L/min for 5 min. The peptide trap was then switched into line with the analytical column (Halo C18, 160 Å, 2.7 μ m, 150 μ m x 10 cm). Peptides were eluted from the column using a linear solvent gradient, with steps, from H₂O:CH₃CN (95:5; + 0.1% formic acid) to H₂O:CH₃CN (5:95; + 0.1% formic acid) with constant flow (500 nL/min) over an 80 min period. The LC eluent was subjected to positive ion nanoelectrospray MS analysis in information dependant acquisition (IDA) mode. In IDA mode a TOF-MS survey scan was acquired (m/z 350-1500, 0.25 s), with the ten most intense multiply charged ions (with counts >150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 200 ms (m/z 100-1500) with rolling collision energy dependent on the charge and size of the precursor ion. ProteinPilotTM 5.0 software (SCIEX) was used to search the MS/MS data against the UniProt database (*Bos taurus*: version 20170920: 107,784 proteins) allowing the identification of proteins in the gel band. The peptides (95%) is defined as the number of distinct peptides having at least 95% confidence. The sequence coverage (%) is the percentage of matching amino acids from identified peptides having confidence greater than 0, divided by the total number of amino acids in the sequence. The total score is the total amount of evidence for a detected protein. To check the consistency in the appearance of the protein band of interest, a secondary set of samples (one muscle from light and dark groups) were run on SDS-PAGE which confirmed the presence of the band.

2.3.5 Sarcomere length

Sarcomere length measurements were made on myofibrils resuspended in KAM (after collection of S1 and resuspension in a second wash). Bright field images were taken on a Leica DM6000 microscope (Leica Microsystems, Germany) at 100x magnification. Ten images were taken and at least 50 myofibrils were measured per sample. Measurements were made using Image J software (Rasband, 2014). Each myofibril had at least three sarcomere measurements conducted along its length and the average distance between the anisotropic regions was calculated (μ m).

2.4 Statistical analysis

One way analysis of variance (ANOVA) was used to examine the effects of treatments on individual (univariate) traits, using Genstat 16th edition (GenStat, 2013). For multiple correlations, a multivariate analysis of variance fitting a generalized linear model, was applied to generate correlation coefficients of multiple variables. The SAS GLM model with the option MANOVA (version 9.4, 2002-2012 by SAS Institute Inc., Cary, NC, USA) was used for the analysis. In the model, the response variables included 19 measurements (lightness, redness, yellowness, pH, drip loss, sarcomere length, myosin-myosin spacing, myosin-actin spacing, confocal global brightness (longitudinal and transverse), fibre diameter, aldolase activity in S1, S2, and S3 fractions, GAPDH activity in S1, S2, transverse and longitudinal peak distance for confocal global brightness measurements). Eleven of these variates were plotted in the correlation table. In order to take into account of trait variations due to different color groups, animals with different weights from different breed types, we fitted colour group, breed and hot carcass weight as fixed effects in the model.

3. Results

3.1 Characterisation of muscle colour groups

Table 1 shows the colorimetric measurements on the three colour groups of muscle samples studied. Muscles that were classified as non-compliant by AUS-MEAT standards, had a dark colour in both orientations and lower lightness in the muscle. The dark colour group had a lower lightness, redness and yellowness value, regardless of measurements made longitudinal or transversely to the muscle fibre axis ($P < 0.001$). The confocal micrographs in Figure 1 show the differences in the intensity of scattered light from samples in the light, medium and dark classes of muscles; as expected, light samples showed the highest level of back-scattered light and dark samples the least. In addition, as shown in Table 2, dark muscles also had a higher pH and a lower drip loss ($P < 0.001$) compared to the other colour groups. The light and medium colour groups had similar values for all colorimetric, pH

and drip loss measurements. Therefore, dark muscles showed a distinct separation of colour, pH and drip loss compared to the light and medium colour groups.

3.2 Light scattering and microscopy

Table 2 also shows that dark muscles had lower global brightness values (overall intensity of back-scattered light in reflection confocal microscopy) and longer periodic spacings in the reflected images, both in the longitudinal and transverse orientations ($P < 0.05$). The periodic spacings in both orientations were strongly positively correlated with each other ($R^2 = 0.72$, $P < 0.001$, data not shown). Table 2 also demonstrates that dark muscles had a shorter sarcomere length than light or medium muscles and that dark muscles had the largest muscle fibre diameter whereas the light muscles had the smallest ($P < 0.05$). Light microscopy images of myofibrils from dark muscles revealed distinct A- and I-bands but with no appearance of a Z-line, whereas in myofibrils from light muscles, the appearance of the Z-line was more distinct (images not shown).

3.3 SAXS and myofilament lattice spacing

Figure 2 shows line plots of the intensity of equatorial small-angle diffraction from representative samples of light, medium and dark muscles. As explained in the legend of supplementary Figure. S1, the x-axis in these plots is calibrated in reciprocal values of Ångströms ($\text{\AA}^{-1}$). Two differences are clearly apparent from the X-ray diffraction data. Firstly, as reported in Table 2, dark muscles had greater transverse myofilament spacings (both myosin-myosin and myosin-actin) compared to the other colour groups ($P < 0.05$). Secondly, the relative intensity (area under the curve above the fitted baseline) of the 1,0 and 1,1 peaks was different in dark versus light or medium samples. Table 2 shows that the mean value of the intensity ratio between the 1,1 and 1,0 peaks for the dark samples was significantly higher than for light and medium samples ($P = 0.015$).

3.4 Correlations among Multiple Variables

Lightness was strongly negatively correlated ($P < 0.05$) to the myosin-myosin and myosin-actin spacing, (as shown in Table 3, -0.62 and -0.67, respectively). The myosin-myosin spacing was also strongly negatively correlated ($P < 0.05$) to both yellowness (-0.79) and drip loss (-0.64). Lightness

and drip loss showed similar trends for sarcoplasmic protein activities, with positive correlations observed with aldolase and GAPDH activities in S1 fractions and negative correlations with GAPDH activity in S3 fractions (drip loss also had a negative correlation for aldolase activity in the S3 fraction). The aldolase activity in S1 and S3 fractions were also positively correlated to the GAPDH activities in S1 and S3 fractions, respectively. Redness, pH, sarcomere length and fibre diameter measurements were not correlated ($P>0.05$) to any lattice spacing or sarcoplasmic protein activity measurements (data not shown).

In both orientations of global brightness, when analysing the data across three colour groups, there were no overall significant correlations to any variate ($P>0.05$). However, Figure 3 shows the correlation scatter plots between lightness and global brightness in both orientations, with associated trend lines.

3.5 Sarcoplasmic protein activities and mass spectrometry

Table 4 shows the activities of GAPDH and aldolase in extracts from light, medium and dark muscles. Sarcoplasmic extracts from light muscles had lower GAPDH activities ($P<0.05$) in the third supernatant fractions (S3), after extraction in the sodium phosphate buffer at pH 7.4. The first supernatant (S1) from these light muscles had a higher activity of GAPDH ($P<0.05$), after extraction in the mannitol buffer at pH similar to the muscle (pH 5.4). No differences were observed for aldolase activity in the S1 and S3 fractions ($P>0.05$), but these extracts did have larger SEDs. When values were calculated as U, similar trends in mean and P -values were observed between colour groups, although all the GAPDH fractions were different ($P<0.05$) and all the aldolase fractions were similar ($P>0.05$) between colour groups. Figure 4 shows the separation by SDS-PAGE of sarcoplasmic proteins from light and dark muscles. A duplicate fractionation procedure was completed without dithiothreitol (S3.2) to test the effect of disulphide bond formation and showed no differences. When sarcoplasmic proteins from light and dark muscles were extracted close to the pH of the muscle (pH 5.4 and 6.1, respectively), an extra band (red arrow) was observed in the dark S3.1 and S3.2 fractions at ~35 kDa that was not observed in light fractions. This band was used for mass spectrometry analysis.

Table 5 shows the results of analysis of the 35 kDa gel band by nanoflow liquid chromatography/tandem mass spectrometry. This revealed a number of protein identifications, and the top 13 matches are listed: The Four and a half LIM domains 1 (FHL1) protein had the highest total score.

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4. Discussion

4.1 In light muscles, the myofilament lattice had shorter spacings between myofilaments thus promoting an increased A-band protein density, which combined with the integrity of the Z-line, would increase the contrast or refractive index mismatch both along the sarcomere length and between adjacent myofibrils.

Muscle lightness had a strong correlation with the transverse lattice spacing between myofilaments, with dark samples of beef *longissimus thoracis* having a longer distance between myosin-myosin and myosin-actin filaments than paler samples. This compliments previous observations made in pale, soft, exudative (PSE) and dark, firm, dry (DFD) pork loins, where filament separation was correlated with 400/700 nm reflectance (Irving et al., 1989).

The correlation coefficients between light scattering measurements, and lightness or myofilament lattice spacing were not significant in this study ($P>0.05$), and was likely a result of an inadequate number of observations as large error variances were observed in the analysis. In addition, the different positive and negative relationships that occurred between light scattering and lightness for the different colour groups, as shown in Figure 3, were also generated with similar insufficiencies. However, dark muscles consistently showed low lightness and light scattering values, whereas light muscles had more light scattering ($P<0.01$).

Theoretical analyses of light scatter from muscle fibres (Baskin, Roos, & Yeh, 1979; Ranasinghesagara & Yao, 2007; Xia, Weaver, Gerrard, & Yao, 2006; Yeh, Baskin, Lieber, & Roos, 1980; Yeh, Corcoran, Baskin, & Lieber, 1983) consider both diffraction from the transverse myofilament lattice and from the regular longitudinal spacing of A- and I-bands, superimposed with multiple scattering from the edges of cylindrical myofibrils and muscle fibres, and the gaps between them. The longitudinal and transverse periodicity of peaks in the scattered light intensity measured by rCLSM reported in Table 2, were in the order of 2 μm , in both orientations. Whilst this was similar in magnitude to sarcomeric structures in the longitudinal direction, there was clearly an interplay

between light scatter from the myofibrillar lattice (spacings in the order of 20 – 40 nm) and scatter from gaps between adjacent myofibrils to create this periodicity in the transverse orientation. As Irving et al. (1989) pointed out, a reduction of the lateral spacing of the myofilament lattice with decreasing pH caused an increased protein density in the A-band, and hence an increased refractive index. A greater difference in refractive index between the A-band and the I-band, combined with a more intact Z-line and further spacing between adjacent myofibrils could cause greater light scattering in the structure. Thus, pH-dependent changes in the lateral (transverse) spacing of myofilaments could contribute to changes in both the lateral and the longitudinal back-scatter of light that has been measured in reflectance spectroscopy or, in our case, reflectance confocal microscopy.

Prior to rigor, sarcomeres are known to behave isovolumetrically, whereby active muscle shortening generates more overlap of actin and myosin filaments and a larger myofibril diameter, and this is dependent upon the pH of the surrounding medium (Rome, 1967). Due to this isovolumetric behaviour of living muscle at physiological pH, the transverse separation of the thick and thin myofilaments in the A-band of myofibrils is greater at short sarcomere lengths and smaller at longer sarcomere lengths. In addition to the effects on the lateral separation of thick and thin filaments in the A-band, a shorter sarcomere length and hence wider myofibril diameter pre-rigor is expected to reduce or eliminate the lateral spaces between adjacent myofibrils, as shown schematically by Offer et al. (1989). If sufficient shrinkage of the myofilament lattice does occur, and myofibrils are further apart, additional light scattering could occur from the refractive index mismatch between the high protein density A-bands of adjacent myofibrils and the lower sarcoplasmic protein density in gaps between adjacent myofibrils. We believe this is the dominant light scattering region in the light muscle fibres but not in dark muscle fibres.

It was interesting that the longitudinal periodicity values were longer in the darker muscles (with shorter sarcomere lengths), but there was a lack of relationship between sarcomere length and global brightness in either the transverse or longitudinal directions ($P > 0.05$). Longitudinal periodicities were in general longer than the length of the A-band (1.6 μm), suggesting that the change in protein density at the A-band/I-band junction at each end of the A-band did not dominate the periodicity in the

longitudinal direction and these bright peaks of reflection could be aliasing between a variety of structures with different periodicity. In the lighter muscles, the increased contrast between the A-band region of one myofibril compared to its surrounding sarcoplasm, combined with differences in optical protein density of the I-band relative to the A-band region and the stronger appearance of the Z-disc structures may be implicated in this. Our results clearly showed light muscle fibres with a low pH had a smaller muscle fibre diameter, with a global brightness pattern in the muscle fibre that was not just more intense, but had shorter periodic spacing, indicating myofibrils were further apart creating more opportunity for light scattering to occur between them, as we have described in previous work (Hughes et al., 2017). This research further substantiates this theory, with evidence that the lighter muscle myofilaments are also closer together compared to dark muscle myofilaments.

Dark muscles had shorter sarcomeres compared to their lighter muscle counterparts in our experiments, and this agrees with the findings of (Irving et al., 1989), but the reason for this relationship is still unexplained. However, there was no significant correlations ($P>0.05$) observed between sarcomere length and global brightness in either orientation and confirms our previous findings (Hughes et al., 2018), which suggests that it is not the actual sarcomere length that is critical to light scattering, but most likely the optical protein density that exists in the A and I-bands and also the gaps between adjacent myofibrils (especially for paler muscles).

4.2 Dark muscles had extra mass on the myofilaments that contribute to the refractive index mismatch and were likely caused from a different organisation of sarcoplasmic proteins around the actin filament and/or excess of proteins or peptides from more proteolytic degradation of the Z-line. .

As noted in the results section, dark muscles in our experiments had a higher ratio of intensity between the 1,1 and 1,0 equatorial diffraction peaks. The relative areas under the two 1,1, and 1,0 peaks (peak intensities) provide information about the relative amount of mass associated with the thick and thin filaments. In classic X-ray studies of muscle contraction and muscle going onto rigor (Elliott, Lowy, & Worthington, 1963; Huxley, 1968), the ratio of 1,1 to 1,0 peak intensities was interpreted as a measure of the binding of myosin heads to the thin filaments, i.e., as myosin heads attach to the thin filaments the intensity of the 1,0 peak increased. However, in post-rigor muscle

nearly all the myosin heads are bound to the actin filaments, and so the differences in the intensity ratio seen in Figure 2 and quantified in Table 2 were unlikely to arise from different amounts of cross bridge attachment. Although denaturation of the myosin heads was associated with the low pH-high temperature conditions causative of PSE pork (Offer, 1991), binding of the myosin heads to actin was assumed to have occurred before this denaturation, and this is borne out by the fact that shortening of the myosin heads upon denaturation was linked with further reductions in the myofilament lattice spacing. Irving et al. (1989) found a relationship between subjective ranking of pork meat paleness and a non-parametric ranking of the 1,1:1,0 intensity (using method of Elliott et al., 1963), again with higher intensity ratios associated with darker samples. Irving et al. (1989) raised the possibility that proteolysis in post-rigor muscle may cleave some of the myosin heads (heavy meromyosin subfragment 1) so that the mass of this S1 fragment was more widely, and less regularly distributed. However, if proteolysis of S1 fragments was more advanced at a higher pH, this would mean that there would be less mass regularly centred on the thin filament positions, so decreasing the 1,1 intensity. A more likely explanation was that there is some addition in mass to the thin filaments by the attachment of other proteins or peptides in the darker samples, at higher pH.

To identify the specific proteins which may be involved in creating this extra mass on the thin filaments, some investigations into sarcoplasmic protein activity and SDS-PAGE electrophoresis patterns of the sarcoplasmic proteins were conducted. It should be noted that measurements from regular peaks in these X-ray diffraction experiments only give information about the relative mass centred on the thin filament position in the A-band, as it is only in the A-band that thin filaments take up a regular hexagonal arrangement between the thick filaments, so giving rise to a distinct diffraction peak due to their regular arrangement. In the I-band, the thin filaments are less ordered (Craig & Padron, 2004) and so the thin filaments in the I-band, and any proteins adhering to them in this region of the sarcomere, did not contribute to peaks in the SAXS diffraction pattern but just to amorphous background scatter. Glycolytic enzymes such as fructose biphosphate aldolase are known to bind to troponin on the thin filaments and affect the ratio of intensities between the (1,1) and (1,0) spacings (Stuart, Morton & Clarke, 1979). These findings showed that the binding of this enzyme occurs in the

A-Band (the only region of the sarcomere where the (1,1) spacing, which corresponds to the distance between overlapping thick and thin filaments exists). Although there is every reason to hypothesise that decoration of thin filaments by such enzymes will extend into the I-band, the lack of contributions to thin filaments in the I-band to the (1,1) peak means that the X-ray diffraction peak intensity ratios reported here cannot confirm this hypothesis. However, the similarities between the correlation coefficients for lightness and drip loss with structural attributes (both lattice spacing and sarcoplasmic protein activities) suggest a dual mechanism involving both of these components is involved.

Hughes et al (2017) demonstrated that some, but not all, of the increases in reflected light intensity caused by irrigating muscle fibres with a low pH solution could be reversed by resuspending the same fibres in a high pH solution. This led to speculation that there may be an additional contribution to light scattering in low pH muscle that was irreversible, and increased sarcoplasmic protein denaturation at low pH was one possible mechanism proposed. While the current finding of a high correlation between reduced myofilament lattice spacing and increased lightness demonstrates the strong contribution of pH-dependent myofilament spacing to light scattering, the observations from SAXS of an increased mass decorating the thin filaments in dark muscles also points to an additional mechanism other than simple thick and thin filament spacing. It is for these reasons that we included the analysis of sarcoplasmic proteins that could be extracted from dark versus light muscles at the pH of the post-rigor samples (light samples at pH 5.4, medium at pH 5.6 and dark muscles at pH 6.1) and subsequently at a higher pH (7.4) in the presence of EDTA and dithiothreitol.

As many of the soluble sarcoplasmic proteins are enzymes, the current study included measurements of two prominent enzymes involved in muscle glycolysis, namely aldolase and GAPDH. The great majority of the active enzymes were extracted in the first washing (S1) of the muscles with an isotonic solution buffered to the appropriate ultimate pH of each group, but no differences were observed between light, medium and dark muscle sarcoplasmic activities in the S1 fractions. This argues that these sarcoplasmic proteins were not preferentially denatured in the lower pH (lighter) beef muscle samples. However, the correlation coefficients indicate these enzyme activities were related to both lightness and drip loss, indicating these meat quality indicators have some integrated mechanistic

drivers involving these proteins. The lightness and drip loss were also correlated to the myofilament spacing structural aspects of the muscle, although no direct correlation was observed between lattice spacing and sarcoplasmic activity.

This lack of sarcoplasmic denaturation in the lighter muscle contrasts with the lower sarcoplasmic protein solubility seen in PSE pork (Joo, Kauffman, Kim, & Park, 1999; Warner et al., 1997) or in pork incubated at 40 °C to simulate PSE conditions (Liu, Ruusunen, Puolanne, & Ertbjerg, 2014). The fact that dark (DFD) meat is more common in beef and pale (PSE) meat is more common in pork points to differences in post-mortem changes between these species. Perhaps the involvement of sarcoplasmic proteins in lightness and drip loss in beef muscles of a different colour, relies on the specific relocation of the protein, with more sarcoplasmic proteins being “lost” via higher quantities of drip, as outlined by Savage, Warriss, and Jolley (1990).

Very little aldolase or GAPDH activity could be detected in the second washing of muscle samples with solutions buffered to the appropriate pH for each colour group (S2) indicating that most, if not all, of the free enzymes in the muscle had been extracted. However, on subsequent washing of the muscle samples with a higher ionic strength solution at pH 7.4 (S3), additional GAPDH and aldolase activity could be recovered, indicating that enzymes in a native/active condition were likely bound to non-soluble (myofilament) proteins. There were significantly greater amounts of activity in the S3 extraction GAPDH in the dark muscles, and this coincides with the added mass of protein decorating the thin filaments in dark muscles as shown by the SAXS intensity ratio results. Precipitation and/or aggregation of denatured sarcoplasmic protein particles could contribute to altered light scattering properties. Sarcoplasmic proteins such as carbonic anhydrase, aldolase, PGM 2 and GAPDH can be found in the I-band/ Z-line region, either bound directly or indirectly to actin filaments (aldolase, PGM 2 and GAPDH) as a glycolytic multi-enzyme complex, and suggests these proteins were involved in structural differences between light and dark colour groups, and warrants further investigation via targeted immunolabelling visualisation. PGM and GAPDH have been implicated in beef *longissimus* colour development previously (Canto et al., 2015). In addition, these two proteins and aldolase are also known to generate lateral associations or cross links between adjacent actin

filaments (Morton, Weidemann, & Clarke, 1988). The presence of these sarcoplasmic proteins in the dark muscle extracts in conjunction with the higher activity of GAPDH, suggests they were still in their native condition, which suggests they were still in a glycolytic complex associated with the thin filaments and could contribute to differences in protein density. However, the SAXS evidence suggests that this association is not limited to the I-band, as added mass can be seen attached to the thin filaments overlapping the thick filaments in the A-band. Further investigations on the link between lightness, drip loss, myofibril lattice spacing and enzyme activities are required to elucidate the underlying causative mechanisms.

From the SDS-PAGE analysis (Figure 4) the S3 sarcoplasmic extracts from dark muscle contained some proteins or protein fragments that appeared at circa 35 kDa, which suggests some additional degradation had occurred in these dark muscles compared to the lighter muscles. This band also appeared in a secondary supernatant S3 (S3.2- similar buffer without dithiothreitol) indicating disulphide bridges were not involved. The mass spectrometry data in Table 5 identifies the major components of this 35 kDa band as the cytoskeletal proteins: Four and a half LIM domains 1 (FHL1), titin, myotilin and 14-3-3 protein epsilon, all of which are associated with the I-band/Z-line region, and are involved in assembly of the cytoskeletal framework of the cell. Similarly, these proteins bind directly or indirectly to actin and are involved in the stability of the Z-line. High pH beef *longissimus* muscles are known to have more degradation of cytoskeletal proteins, such as titin, nebulin and filamin with an associated loss in integrity of the Z-line (Lomiwes, Farouk, Wu, & Young, 2014; Yu & Lee, 1986) and our light microscopy images showed a loss in integrity of the Z-line within the dark muscle sarcomeres. We acknowledge that some biomarkers for meat colour traits maybe different with the breed variation that exists between the meat colour groups in this study (Gagaoua, Terlouw, & Picard, 2017) and hence breed was included in the analysis, but still warrants further investigation. However, the current experiment indicated there was an increased disruption of these scaffolding proteins in dark muscle.

A possible hypothesis for the role of these proteins in determining the lack of Z-line integrity, cytoskeletal framework modification and consequently the sarcomeric refractive index mismatch in

dark, high pH muscles is explained. In the I-band, titin contains an extensible elastic region which runs obliquely between the end of the myosin filament and the Z-line, which generates force in both orientations of the sarcomere (Fukuda, Granzier, Ishiwata, & Kurihara, 2008; Higuchi & Umazume, 1986). Within this elastic region, there is an N2A region, where FHL1 binds. FHL1 has been shown as a negative regulator of titin N2B (equivalent of N2A region in human cardiac muscle), suggesting that a loss of FHL1 reveals phosphorylation sites on titin which extends the range in which sarcomeres are able to stretch under physiological conditions (Raskin et al., 2012). Within the dark muscle S3 (pH 7.4) extracts, the presence of FHL1 protein and the titin fragment suggests these proteins had undergone some degradation. High pH muscles are known to have an earlier degradation of titin, with lighter, low pH muscles showing more intact titin (Warner et al., 1997; G. Wu, Farouk, Clerens, & Rosenvold, 2014). So, the presence of these proteins in the dark muscle extracts indicates that degradation of the titin 'spring' had occurred, either pre-rigor or post-rigor. In comparison, the absence of this degradation band in light muscle extracts, suggested these proteins were still intact. This could be a result of altered proteolysis, as the N2A segment of skeletal titin is known to interact with calpain proteases (Sorimachi et al., 1995) and high pH muscles have been previously implicated in early autolysis of μ -calpain (Lomiwes et al., 2014). Calpain proteolysis was not measured in the current experiment and would require further investigation, but if it occurred, it would result in a permanent modification to the cytoskeleton and a reduction in the protein density in the I-band. The fact that darker muscles had shorter sarcomere lengths means that any degradation of titin post-rigor would occur in titin molecules shorter than their elastic rest length, i.e. unstretched and somewhat folded or buckled. By removing this titin 'spring' in dark sarcomeres, there would be the partial removal of the cytoskeletal framework. This may have some involvement in the extent of longitudinal and transverse shrinkage that can occur within the sarcomere as rigor progresses, but of course still requires further validation..

In dark muscle sarcomeres, the combination of further myofilament spacing, more mass on the thin filaments, the lack of integrity of the Z-line are all likely to impact on the both the intensity and periodic spacing of the light scattering elements. The larger diameter muscle fibres with longer

periodic spacing of light scattering elements in both orientations of the muscle fibre suggests the lack of spacing between adjacent myofibrils and muscle fibres probably somewhat reduces the quantity of light scattering that can occur in the muscle. We have yet to prove this is due to the lack of shrinkage that occurs in the structure as determined by the shortfall of the pH decline.

Finally, due to the nature of the samples collected from each colour group at the meat processor, there were some limitations to this study. Muscles from light and medium colour groups were from female 100 d grain fed Angus cattle, whereas muscles from the dark colour group came from male, pasture fed cattle, where the breed was unknown. Various on farm factors are known to impact the occurrence of dark meat, e.g. pasture fed animals typically have a higher incidence of dark meat compared to grain fed animals (Ponnampalam et al., 2017), and this was reflected in the samples available from the meat processor on the day of collection. We did include breed and hot carcass weight as fixed effects in the analysis model to minimize the effects on the analysis results. Nonetheless, this is a limitation of the study.

5. Conclusion

Increased light scattering in muscle fibres with lower ultimate pH post-rigor has contributions from mechanisms that are both reversible, on reverting the pH to higher values, and from irreversible mechanisms. Myofilament lattice spacing appears to be central not only to lightness but also to drip loss in post-rigor meat. Within the muscle cells of dark meat, the wider lateral spacing of myofilaments, shorter sarcomere lengths, lack of gaps between myofibrils, loss in integrity of the Z-line and added mass on the thin filaments all created less opportunity for light scattering to occur, compared to paler muscles with a lower ultimate pH. In addition, some other irreversible changes in the muscle cells must occur in paler, low pH samples due to the difference observed in sarcoplasmic protein activity. These can include increased sarcoplasmic denaturation or relocation, less complexing of undenatured sarcoplasmic proteins to myofilament proteins, or different degrees of proteolysis. In dark muscles, an increased abundance of degradation products of the cytoskeleton (especially proteins

FHL-1 and titin) and the proposed complexing of a higher amount of undenatured sarcoplasmic proteins such as GAPDH to actin filaments may also be other potential contributing factors to light scattering due to variations in protein density, but requires further experimentation. In addition, the underlying mechanisms that exist between myofilament lattice spacing, sarcoplasmic protein activity, lightness and drip loss require further exploration, especially when considering animal factors such as feeding regime, breed, gender and age of the animal.

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8. Tables and Figures

Table 1: Colorimetric values of beef *longissimus thoracis* muscle in the longitudinal and transverse orientations of light, medium and dark colour groups, as defined by AUS-MEAT colour scores: light 1C; medium 2; dark >3, respectively. Measurements were made on a 30-35 mm thick slice after blooming at 4 °C for 60 mins.

	Longitudinal					Transverse				
	Light	Medium	Dark	SED	P-value	Light	Medium	Dark	SED	P-value
Lightness (L*)	35.3	34.5	28.2	0.90	<0.001	34.8	34.0	27.4	0.45	<0.001
Redness (a*)	15.9	15.4	10.0	0.59	<0.001	16.6	16.5	10.5	0.36	<0.001
Yellowness (b*)	1.0	0.4	-4.4	0.50	<0.001	1.8	1.1	-3.9	0.31	<0.001

Table 2: Effect of light, medium and dark colour groups, as defined by AUS-MEAT colour scores: light 1C; medium 2; dark >3, respectively on beef *longissimus thoracis* muscle. Myofilament spacing was conducted at 5-6 d PM using small angle x-ray scattering (SAXS). Measurements for pH were also made at 5-6 d PM. Global brightness (long-longitudinal or trans-transversely to muscle fibre axis) and muscle fibre diameter measurements were conducted at 7-8 d PM, using reflectance confocal laser scanning microscopy (rCLSM).

	Light	Medium	Dark	SED	P-value
pH	5.47	5.52	6.15	0.085	<0.001
Drip loss (%)	5.2	5.7	1.7	0.63	<0.001
rCLSM global brightness ¹ (long)	121.0	102.3	78.4	11.66	0.008
rCLSM global brightness ¹ (trans)	105.8	95.3	61.9	10.58	0.002
rCLSM longitudinal periodicity (distance between peaks, μm)	1.89	2.08	2.13	0.102	0.006
rCLSM transverse periodicity (distance between peaks, μm)	1.79	1.90	2.15	0.088	0.007
Sarcomere length (μm)	2.13	2.11	1.89	0.076	0.015
Muscle fibre diameter (μm)	65.1	71.0	75.6	2.96	0.009
Myosin- myosin spacing (nm)	36.3	36.7	38.6	0.55	0.002
Myosin- actin spacing (nm)	21.4	21.3	22.6	0.38	0.006
Intensity ratio (1,1 peak to 1,0 peak)	2.41	2.85	3.65	0.356	0.015

¹ Mean pixel intensity

Table 3: Correlation coefficients (r) between beef *longissimus thoracis* meat quality attributes (transverse colour and drip), structural myofilament spacings, global brightness light scattering values (longitudinal and transverse) and enzyme activities in the S1 and S3 fractions. In each cell, a significant partial correlation value is indicated with * or **, corresponding $P<0.05$ or $P<0.001$, respectively. Boxed areas indicate key areas of interest. Global brightness values expressed as a mean pixel intensity, sarcoplasmic fraction activities were measured in enzymatic U and then expressed as a percentage (%) of the total for all washes within an enzyme.

Lightness (L*)	1						
Yellowness (b*)	2	0.324					
Drip loss (%)	3	0.687*	0.401				
Myosin- myosin spacing (nm)	4	-0.617*	-0.79*	-0.635*			
Myosin-actin spacing (nm)	5	-0.673*	-0.46	-0.209	0.591		
rCLSM global brightness (longitudinal)	6	0.191	0.203	0.03	-0.386	-0.288	
rCLSM global brightness (transverse)	7	0.516	-0.119	0.326	-0.237	-0.255	0.717*

Aldolase activity in S1 fraction	8	0.714*	0.294	0.578*	-0.397	-0.294	-0.184	0.044			
Aldolase activity in S3 fraction	9	-0.532	0.18	-0.624*	0.018	0.076	0.357	-0.134	-0.604*		
GAPDH activity in S1 fraction	10	0.679*	0.178	0.616*	-0.411	-0.354	0.14	0.246	0.781*	-0.641*	
GAPDH activity in S3 fraction	11	-0.606*	0.191	-0.592*	0.101	0.214	-0.071	-0.413	-0.523	0.841**	-0.815*
		1	2	3	4	5	6	7	8	9	10

Table 4: Effect of light, medium and dark colour groups, as defined by AUS-MEAT colour scores: light 1C; medium 2; dark >3 respectively on the proportion of sarcoplasmic enzyme activities (%), namely GAPDH and aldolase, in each fraction. Muscles were extracted and supernatant fractions S1, S2 and S3 from 1st, 2nd and 3rd washes were collected and assayed individually. Solutions used for the washes were S1 and S2: 380mM mannitol and 50mM potassium acetate buffered to muscle pH (light pH 5.4, medium pH 5.6 & dark pH 6.1) and for S3: 0.1 M sodium phosphate buffer, 0.5 mM EDTA, 2 mM dithiothreitol at pH 7.4. All activities were measured in enzymatic U and then expressed as a percentage (%) of the total for all washes within an enzyme.

Sarcoplasmic enzyme	Fraction	Light	Medium	Dark	SED	<i>P</i> -value
GAPDH	S1	90	87	86	1.7	0.053
	S2	0.09	0.09	0.03	0.018	0.011
	S3	8	11	13	1.7	0.018
Aldolase	S1	84.2	83.7	74.8	4.85	0.135
	S2	1.5	0.9	1.1	0.22	0.017
	S3	14.3	15.4	24.2	4.91	0.132

Table 5: Identifications resulting from in-gel trypsin digestion of the 35 kDa protein band isolated from dark meat colour group of beef *longissimus thoracis* muscles. The excised band taken from the S3 fraction was analysed using nanoflow liquid chromatography tandem mass spectrometry (NanoLC MS/ MS) and proteins were identified by searching the MS/MS data against the UniProt database.

Accession no.	Name	Species	Peptides (95%)	Sequence coverage (%)	Total score
F1MR86	Four and a half LIM domains 1	<i>Bos taurus</i>	77	85.81	50.56
F1N757	Titin	<i>Bos taurus</i>	7	0.23	11.66
Q3SZX4	Carbonic anhydrase 3	<i>Bos taurus</i>	7	26.92	11.10
P68252	14-3-3 protein gamma	<i>Bos taurus</i>	8	21.05	9.94
P63243	Receptor of activated protein C kinase 1	<i>Bos taurus</i>	5	15.77	8.27
Q3T169	40S ribosomal protein S3	<i>Bos taurus</i>	4	21.81	8.06
P62261	14-3-3 protein epsilon	<i>Bos taurus</i>	7	25.49	9.84
Q0VCX9	Myotilin	<i>Bos taurus</i>	3	7.62	6.05
A6QLL8	Fructose-bisphosphate aldolase	<i>Bos taurus</i>	3	17.03	4.64
P26452	40S ribosomal protein SA	<i>Bos taurus</i>	3	13.90	4.49
G3N3C9	LIM domain binding 3	<i>Bos taurus</i>	2	4.25	4.42

Q32KV0	Phosphoglycerate mutase 2	<i>Bos taurus</i>	2	11.86	4.42
P10096	Glyceraldehyde-3-phosphate dehydrogenase	<i>Bos taurus</i>	3	12.91	4.14

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Light

(a)

(b)

(c)

Medium

(d)

(e)

(f)

Dark

(g)

(h)

(i)

Figure 1: Effect of colour group ('light', a, b, c; 'medium', d, e, f and 'dark' g, h, i) on reflection confocal laser scanning microscopy (rCLSM) images of beef *longissimus thoracis* muscle fibres in the transverse orientation. Colour group was defined by AUS-MEAT colour scores: 1C; 2; >3, respectively. Each image is taken from a sample of muscle from a different carcass. Scale bar = 40 μm .

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(a) Light, (pH 5.46)

(b) Medium, (pH 5.47)

(c) Dark, (pH 6.09)

Figure 2: Small angle X-ray scattering (SAXS) example of peak fitting output plots for (a) light, (b) medium or (c) dark meat colour of beef *longissimus thoracis*. Units along the x-axis are q ($\text{\AA}^{-1}$) and

the y-axis represents the intensity of the signal. Figures in parentheses are the mean pH of the samples in each group.

Figure 3 Correlation scatterplots between beef *longissimus thoracis* colour (lightness measured on 35 mm steak in transverse orientation) and the global brightness light scattering values, as measured using reflectance confocal laser scanning microscopy (rCLSM) from images taken of muscle fibre fragments in either (a) longitudinal or (b) transverse orientation. The effect of light (circles, dashed trend line), medium (squares, dotted trend line) and dark (triangles, solid trend line) colour groups, as defined by AUS-MEAT colour scores: light 1C; medium 2; dark >3, respectively, on the corresponding linear trend lines was noticeably distinct between colour groups. The r and P -values for each trend line for light, medium and dark muscles are as follows (a) light: $r = -0.25$, $P = 0.5883$; medium: $r = 0.47$, $P = 0.2884$; dark: $r = -0.68$, $P = 0.2099$ and (b) light: $r = -0.43$, $P = 0.3372$; medium: $r = 0.54$, $P = 0.2110$; dark: $r = 0.61$, $P = 0.2756$.

(a) Light

(b) Dark

Figure 4: SDS-PAGE separation protein patterns showing the differences in protein bands between sarcoplasmic extracts from either (a) 'light' or (b) 'dark' meat colours of beef *longissimus thoracis* muscles. Muscles were extracted and supernatant fractions S1, S2 and S3 from 1st, 2nd and 3rd washes were collected and prepared individually. Solutions used for the washes were S1 and S2: 380 mM mannitol and 50 mM potassium acetate buffered to muscle pH (light pH 5.4 & dark pH 6.1) and for S3 (lane label S3.1): 0.1 M sodium phosphate buffer, 0.5 mM EDTA, 2 mM dithiothreitol at pH 7.4. Supernatant S3 was also tested without dithiothreitol (S3.2) and showed no difference in electrophoretic profiles. Red arrow indicates ~35kDa band removed for mass spectrometry.

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Difference in light scattering between pale and dark beef *longissimus thoracis* muscles are primarily caused by differences in the myofilament lattice, myofibril and muscle fibre transverse spacings.

Highlights

- Dark muscles have a longer myofilament lattice spacing compared to light muscles
- Dark muscles have short sarcomere lengths compared to light muscles
- Myosin-myosin myofilament spacing is central to colour and light scattering
- Dark muscles have a higher sarcoplasmic enzyme activity
- Titin and four and a half LIM domains (FHL1) are implicated in dark meat colour

Light

(a)

(b)

(c)

Medium

(d)

(e)

(f)

Dark

(g)

(h)

(i)

Figure 1

(a) Light, (pH 5.46)

(b) Medium, (pH 5.47)

(c) Dark, (pH 6.09)

Figure 2

(a)

(b)

Figure 3

(a) Light

(b) Dark

Figure 4