ACCEPTED MANUSCRIPT

A high rigor temperature, not sarcomere length, determines light scattering properties and muscle colour in beef M. *sternomandibularis* meat and muscle fibres

J. Hughes [A*], F. Clarke [B], P. Purslow [C], R. Warner [D]

[A] CSIRO Agriculture and Food, 39 Kessels Road, Coopers Plains, Qld, Australia

[B] School of Environment and Science, Griffith University, Nathan, Qld, Australia

[C] Departamento de Tecnologia de los Alimentos, Facultad de Ciencias Veterinarias, Universidad Nacional Del Centro de La Província de Buenos Aires, Tandil, Bs.As., Argentina

[D] Department of Veterinary and Agricultural Science, The University of Melbourne, Parkville, Vic, Australia

*Corresponding author. Email: joanne.hughes@csiro.au

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Abstract

Beef meat colour is impacted by both myoglobin status and the light scattering properties of the muscle, and the specific causative scattering elements of the latter are still unknown. We hypothesize that stretching muscles during rigor will generate a structure which favours light scattering, by increasing the length of the I-band (longer sarcomeres) and that a high rigor temperature will cause protein reconfiguration, changing the muscle structure and promoting light scattering. Muscle fibre fragments were isolated from four beef *M. sternomandibularis* and subjected to stretching (plus, minus) and three incubation temperatures (5, 15, 35 °C). Reflectance confocal laser scanning microscopy (rCLSM) revealed sarcomere stretching alone was not solely responsible for light scattering development. A high rigor temperature (35 °C) was more favourable for light scattering. Stretching and taking muscle into rigor at 35 °C promoted transverse shrinkage of muscle fibres and increased light scattering and could be applied post-mortem (PM) to reduce the occurrence of problematic dark meat.

Keywords

Meat colour; skeletal muscle structure; reflection confocal laser scanning microscopy (rCLSM); muscle fibre; sarcomere length; rigor temperature.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. Introduction

Meat colour is determined not only by the quantity and oxidative status of myoglobin, but also by the structural opacity and light scattering properties of the muscle fibres from which they are composed (MacDougall, 1982). One of the areas of the muscle that may impact on the light scattering properties of the meat is the sarcomere length, as determined primarily by the lengths of the isotropic (I-band), rather than the anisotropic area or A-band (Periasamy, Burns, Holdren, Pollack, & Trombitas, 1990). Currently there is no research relating the sarcomere length microscopic structural characteristics to the light scattering properties of post-rigor beef muscle, after the ultimate pH has been reached.

Some preliminary studies indicate there is an inverse relationship between sarcomere length and light scattering within muscle fibres at a high pH (~pH 7) or physiological conditions (Bozler, 1958; R. Jeacocke, 1984). Bozler (1958) measured light scattering using a spectrophotometer on glycerol-extracted rabbit muscle fibres and a chemical induced contraction (via addition of either ATP or $CaCl_2$), which caused an increase in light scattering by over 55 %. R. Jeacocke (1984) used various optical set-ups to investigate light scattering in beef *M. sternomandibularis* single muscle fibres and in the whole muscle. Using a rigor temperature of 22 °C in the single muscle fibre preparation, R. Jeacocke (1984) observed that an increase in scatter associated with rigor was proportional to the degree of filament overlap. Both of the Bozler and Jeacocke experiments were conducted close to physiological pH conditions (pH 7 and 6.9 respectively), and R. Jeacocke (1984) noted the diminished effect of rigor-induced light scattering as the pH of the bathing medium was lowered. Therefore, this experiment aims to further explore the generation of light scattering at various sarcomere lengths (via stretching) after the muscle has passed through rigor and the consequential impact of the associated lower ultimate pH (pH_u)..

In contrast to the above studies on stretched sarcomere lengths, shorter sarcomeres have been observed in dark, high pH meat (both pork *longissimus* muscles and beef *longissimus* muscle fibres) compared to paler, low pH meat (Hughes, Clarke, Purslow, & Warner, 2017; Irving, Swatland, & Millman, 1989; Warner, Kauffman, & Greaser, 1997). This may be indicative of a positive

ACCEPTED MANUSCRIPT

relationship between sarcomere length and light scattering, although it is conflated with pH effects. Sarcomeres are isovolumetric *in vivo* (see Millman (1998) for a review) and the shorter sarcomeres in dark pork loins are known to have myofilaments which are further apart giving rise to a lower red reflectance (Irving et al., 1989). A larger fibre diameter with increased myofilament lattice spacing is thought to contribute to decreased light scattering (Hughes et al., 2017). In addition, decreasing the pH of the surrounding medium in post-rigor beef *longissimus* muscle fibre fragments promotes fibre shrinkage and increased light scattering properties, but the reversibility of these structural changes is still under question as decreased pH may cause sarcomeric proteins to denature. Therefore, we aim to visually quantify the effect of pH cycling on the reversibility of the swelling/ shrinkage mechanism of muscle fibre fragments. Some of the above observations appear to conflict those reported at physiological pH and suggest either the muscle pH_u and/ or the rigor temperature to which the muscle is exposed, may override the increased light scattering during contraction at a physiological pH~7.

A high rigor temperature (35 – 40 °C) is known to generate structural alterations in the muscle, which could increase light scattering (Hughes, Oiseth, Purslow, & Warner, 2014; Kim, Warner, & Rosenvold, 2014). Elevated rigor temperatures in the muscle are associated with increased myosin and sarcoplasmic protein denaturation, both of which contribute to shrinkage of the myofilament lattice spacing (Liu, Arner, Puolanne, & Ertbjerg, 2016). Together, this effect may translate to shrinkage at larger length scales, producing more shrinkage of the myofibrils and muscle fibres and generating more opportunity for light scattering. Alternatively, it is possible that denatured sarcomeric proteins may deposit on myofilaments and change their optical protein density.

Currently the exact mechanism of light scattering is still unknown, but at the cellular level, the length of the I-band, the lateral distance between adjacent A-bands of different myofibrils and the A-I interface junction, could be involved (Offer et al., 1989), which would translate to structural differences at the muscle fibre and meat surface level. We hypothesize that (a) stretching muscles during rigor will generate a structure which favours light scattering, by increasing the length of the I-band, via longer sarcomeres; (b) a high rigor temperature would facilitate the protein reconfiguration and structural alterations which would promote light scattering; and (c) reducing the pH of immersion

ACCEPTED MANUSCRIPT

buffer will shrink the muscle fibre fragment whilst increasing light scattering but after exposing muscle fibres to low pH conditions, some permanent, irreversible structural modifications would occur.

2. Methods

2.1 Sample collection

All muscles were collected from carcasses derived from female yearlings (zero permanent incisor teeth) within the same group of cattle. The mean subcutaneous rib fat depth over the loin, at the $10^{th}/11^{th}$ rib, was 5 ± 0.8 mm, with a mean hot carcass weight of 165 ± 11.1 kg (average ± s.e.). From 4 carcasses, *M. sternomandibularis* (tongue root) muscles were collected from both sides at 10 min post-mortem (PM) and placed in individual plastic bags for transportation. These muscles were selected based on ease and speed of access (mainly for beef processor), their ability to be stretched mechanically quickly in a lab environment, small size and predominance in fibre type I or IIA (Totland & Kryvi, 1991). Muscles were transported from the beef processing facility to the laboratory for 50 mins in insulated containers and processed immediately on arrival. The muscles were maintained at a temperature of 20-25 °C in the insulated containers.

2.2 Treatments applied pre-rigor

Each muscle was cut perpendicular to the muscle fibre axis into 3 equal length sections, yielding a total of 6 individual sub-samples per carcass. These samples were randomly allocated to one of 6 treatments in a 2 x 3 design; pre-rigor stretching; stretched or unstretched and pre-rigor temperature, 5, 15 or 35 °C.

Muscles were attached across the width of a small plastic tray (175 x 120 mm) using bulldog clips to clamp each end of the muscle, so muscle fibres were stretched in the longitudinal orientation, from approximately 7 to 10 cm (Figure 1). Unstretched muscles were clamped to the same plastic tray at one end of the muscle only, allowing the free end to contract during rigor. Within 90 mins PM, all trays with muscles attached were then covered with plastic wrap to minimise moisture losses and

ACCEPTED MANUSCRIPT

placed in either the refrigerator (5 °C) or heated incubators (15 or 35 °C) for 16 to 20 h, after which colour, pH and reflectance confocal laser scanning microscopy (rCLSM) was measured. After 16 to 20 h, all samples were kept at 5 °C until ready for sample processing, except for the 5 °C treatment, which was kept at 5 °C until 30 h PM, in order to allow the muscle to reach final pH prior to processing.

2.3 pH and colour measurements and sample processing

Once the treatments were completed, the muscle was removed and the pH of the muscle was measured using a TPS WP-80 pH meter with a polypropylene spear-type gel electrode (Ionode IJ 44) and temperature probe (all from TPS Pty Ltd, Springwood, QLD, Australia). Calibration of the pH equipment was conducted at 15 - 20 °C, using pH 4 and 7 buffers (TPS Pty Ltd, product no. 121382 and 121388 respectively). For 15 or 35 °C treatments, the pH was measured at 18 to 19 h PM, with similar duplicate measures (~1 h apart) indicating muscles were in rigor, as indicated by the pH~5.5. For 5 °C treatment, the pH was also measured at 30 h PM, due to the slower pH decline at the colder temperature, with similar duplicate measures indicating muscles were in rigor later, at 29-30 h PM as indicated by the pH~5.5.

Muscles were unwrapped and the colour was developed (bloomed) at 5 °C for 60 min. Triplicate colour measurements (lightness, redness and yellowness; L*, a* and b* values) were made perpendicular to the muscle fibre axis using a Minolta CR400 chromameter (Minolta Pty Ltd, Japan, light source D65, observer angle 2°, light projection tube CR-A33d, with φ22 mm disc). Calibration was at 5 °C, using the white calibration tile as provided by the manufacturer.

A 1.0 ± 0.05 g sample was removed from each sub-sample for homogenisation, myofibril isolation for rCLSM and pH cycling. A sample of 10 – 20 g was removed from the remaining muscle, frozen and stored at -80 °C for subsequent measurement of sarcomere lengths.

2.4 Muscle fibre fragment preparation and myofibril isolation

A 1 ± 0.05 g sample was removed from the muscle and muscle fibre fragments were isolated using the method of Warner et al. (1997), for myofibrils, with modifications. Muscle samples were homogenised (Ultraturrax, 11,000 rpm, 3 x 4 second pulses) in 10 ml of ice-cold buffer (380 mM

ACCEPTED MANUSCRIPT

mannitol + 50 mM potassium acetate buffer) to minimise modifications to fibre fragments due to a change in osmolarity (Winger & Pope, 1981). This buffer was kept cold, on ice (~4 °C) throughout the entire fractionation procedure. Suspended washed muscle fibre fragments were subsequently viewed using rCLSM.

2.5 Sarcomere length measurement on myofibrils

Sub-samples were thawed (5 °C for 18 h) and the myofibrillar preparation procedure was repeated, using a 1 ml aliquot from resuspended myofibrils, during the second wash cycle. Bright field images of the preparation were taken using a Leica DM6000 microscope (Leica Microsystems, Germany) at 100x magnification. A minimum of 10 images were taken per sub-sample and at least 50 myofibrils were measured per homogenate. Measurements of sarcomere length were made using Image J software (Rasband, 2014). Each myofibril had at least three measurements conducted along its length and the average distance between A-bands were calculated (µm).

2.6 pH cycling irrigation treatments

Only one sample from a stretched muscle with a rigor temperature of 15 °C, having intermediate characteristics of all the muscles (pH_u = 5.52, L* = 28.28 and global brightness = 109.9), was used for the pH cycling movie. After homogenisation as described above, 100 µl of washed suspended muscle fibre fragments were pipetted onto a StarFrost superclean hydrophilic glass slide (cat no: G311SF-B, ProSciTech, Australia). Two strips of double sided tape were used to mount a coverslip (creating a 3 mm channel) and this was subsequently placed over the sample and attached to the glass slide. This allowed a small space between the slide and coverslip, as previously described by Wilding, Hedges, and Lillford (1986). An rCLSM image was captured before and after pH cycling at ⅓ of the depth into the muscle fibre, as described in Hughes et al. (2017). For pH cycling, 20 µl of 380 mM mannitol + 50 mM potassium acetate buffer (either pH 4.5 or 7.0, see below) was pipetted slowly into one end of the channel, whilst at the other end, a small piece of tissue was used to draw the solution across the slide. An Orion glass micro pH combination electrode (120mm tip, 1.3 x 37 mm long, cat no: ORI9810BNW, Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Victoria, Australia) attached to the TPS meter, was placed at the exit point of the solution in order to measure the pH. Initially, the pH

ACCEPTED MANUSCRIPT

was increased with addition of high pH buffer (pH 7.0), followed by a stabilization lag period of around 5 mins, followed by addition of a low pH buffer (pH 4.5) with a similar stabilization lag period, then the entire cycle process was repeated once. Throughout the pH cycling process, rCLSM images were captured in a movie format (xyzt), over a period of 24 mins, with 593 frames used (rate of 0.42 frames/ sec) at similar settings as described below. For the pH cycling procedure, 18 attempts were made (18 different sub-samples or 3 sub-samples from each different treatment). Some issues such as blockage, overflow around slide providing false pH readings were observed with the channel as well as washing off screen, large changes in depth and resolution with the muscle fibres. Thus, the final single sub-sample that is shown had none of these issues and was from a treatment that had dramatic changes in global brightness and fibre width.

2.7 Reflection confocal scanning laser microscopy (rCLSM)

A small drop of suspended muscle fibre fragment preparation was placed on a coverslip for viewing. A Leica TCS SP5 CLSM (Leica Microsystems, Germany) was used in the reflectance mode for measurement of light scattering properties in the longitudinal orientation of the muscle fibre fragments. A 63x magnification oil objective was used (pinhole set to 71.73 µm). An argon laser was used at 20 % power, (excitation 488 nm with emission slit width at 480 - 500 nm). Detection and amplification was conducted using the photomultiplier tube (PMT) at 550 V (0 % offset). For image acquisition, a high pixel resolution (512 x 512) at 400 Hz was used, with a Leica DFC450 C camera (Leica Microsystems, Germany). To minimise over exposure whilst maximising the resolution of the longitudinal fibre fragment, images were taken at ⅓ of the distance into the depth of the fragment and a total of 5 images per sample were taken. This reflectance mode collects back-scattered light (both reflected and scattered light deflected back in the direction of the incident illumination) and represents the intensity of the back-scatted light as brightness (greyscale intensity) at each point in the image.

2.8 Image analysis

From the images acquired, measurements were made for mean greyscale pixel intensity (global brightness as a measure of light scattering) on polygon selections around the fibre fragment perimeter and for fibre fragment widths (µm) perpendicular to the fibre axis using Image J software (Rasband,

ACCEPTED MANUSCRIPT

2014). The periodicity or peak distances in the longitudinal and transverse orientations of the fibres (3 measurements per image) were measured using Image Pro software (MediaCybernetics, 2012). Intensity profiles of the signal were generated and the mean distance from peak to peak was measured (μm) as described in Hughes et al. (2017). Unfortunately, after image processing, 3 sub-samples were identified as being measured incorrectly (namely one sub-sample for 15 °C and sub-samples from both stretched and unstretched for 35 °C, all from the same carcass) as the 488 nm laser was not warm, which negatively impacted on the intensity of images collected. Thus, these sample data were removed from the global brightness and periodicity analysis. The volumes reported were calculated using a formula for a cylinder (πr^2) multiplied by the sarcomere length and is a simplified assumption only. In reality, fibres are irregular polygons in cross sectional shape (Swatland, 2002).

2.9 Statistical analysis

Data analysis was conducted using Genstat 15th edition (VSN International Ltd, Hemel, Hempstead, U.K.). A linear mixed model of analysis of variance was used to examine the data, with the sample within carcase as a random effect and rigor temperature (5, 15 and 35 °C) and the muscle configuration (stretched and unstretched) as fixed effects.

3. Results and Discussion

Contrary to our hypothesis, the stretching treatment did not directly impact light scattering values. As shown in Table 1 and 2, with graphical displays in Figure 2 and 3, stretching increased both the sarcomere length ($P<0.001$) and the longitudinal peak distances ($P<0.05$); however, no change in either lightness or global brightness was observed ($P>0.05$). The stretching treatment was successful, as indicated by the increase in average sarcomere length from 2.06 for unstretched to 2.61 for stretched ($P<0.001$; LSD 0.091; Table 2). Stretching would have generated sarcomeres with longer I-bands compared to those of unstretched muscles and this suggests the length of the I-band was not solely involved in the development of scattered light. It also indicates the degree of overlap between the thick and thin myofilaments is not solely responsible for the development of light scattering. As a main effect, the stretching treatment had no effect on the muscle fibre width ($P>0.05$) although there was an interaction, which is discussed below. This leads us to reject hypothesis that (a); our results

ACCEPTED MANUSCRIPT

indicate that sarcomere length in post-rigor beef *M. sternomandibularis* muscle will have a direct effect on the light scattering properties of the muscle, through the increased length of the I-band.

The high rigor temperature (35 °C) muscles did have higher lightness ($P<0.001$) and global brightness ($P<0.05$), indicative of more light scattering compared to lower rigor temperature (5 and 15 °C) muscles (Table 1 and Figure 2). Representative images of the individual muscle fibres are shown in Figure 4. The advantage of the confocal technique is that, because light from all depths of the specimen other than the plane of focus is rejected, the intensity of the "reflected" or "back-scattered" light measured is exactly determined by the structures within the plane of focus, which in our experiments were the myofibrils within a muscle fibre. The 35 °C treatment images showed distinct bright bands perpendicular to the fibre axis across the whole width of the fibre, especially evident in the stretched configuration. In comparison, muscle fibres from lower rigor temperature treatments did not have such a distinct banding pattern that spanned across the whole fibre width, especially at 15 °C, indicative of a less regular light scattering pattern. This caused some inconsistencies in both transverse and longitudinal periodicity patterns during measurement and associated calculations (see Table 2), which generated no obvious differences between different temperatures ($P>0.05$) and suggests the lower rigor temperatures muscle fibres had a non-coherent structure compared to those from the high rigor temperature. This evidence supports hypothesis (b); that the high rigor temperature facilitated the development of structural characteristics which promoted light scattering and leads us to explore what these structural characteristics were and speculate on the possible causative scattering elements within the muscle.

In the stretched configuration at 35 °C, the increased amount of fibre width shrinkage was associated with more light scattering and lighter surface colour (Table 1, Table *2* and Figure 3b). Comparing all treatments, the stretched muscle taken into rigor at 35 °C had the smallest fibre width accompanied by the highest lightness values and global brightness values, suggesting transverse shrinkage of the muscle fibres promoted light scattering. Shrinkage of the muscle fibres would create larger spaces between neighbouring muscle fibres and create opportunity for more light to be diffused or deflected throughout the muscle structure, thus generating a lighter appearance at the surface. This is a similar

ACCEPTED MANUSCRIPT

mechanism to that observed with pH-induced shrinkage of beef *longissimus* muscle fibres (Hughes et al., 2017); however it is unlikely that the pH was the cause in this case due to the similarity in pH values at 15 °C (P>0.05), and is more likely to be caused by the higher rigor temperature. A reduction in post-rigor beef muscle fibre cross sectional area has been previously observed, even at mild heating/ cooking temperatures, between 25 to 40 °C, (Purslow, Hughes, & Warner, 2016), and these results confirm that a high rigor temperature of 35 °C in the stretched configuration was sufficient to promote fibre shrinkage and light scattering, as diagrammatically summarised in Figure 5 (a). This was not the case in the unstretched configuration, where fibre widths were similar (P>0.05) between temperature treatments, indicating longitudinal changes were more important in this configuration.

A high rigor temperature of 35 °C, when muscles were unstretched and free to shorten, generated a structure with longer sarcomeres (P<0.05) accompanied with a higher global brightness and longitudinal peak intensity, compared to muscles going into rigor at 5 °C (P<0.05). This is consistent with the findings of Honikel, Roncalés, and Hamm (1983) who showed that more shortening occurs in beef muscles when rigor temperatures are ≤15 °C. Some of the fibres (especially unstretched fibres) in the 5 °C treatment also had more undulations along their length indicative of a buckling previously described by Brown, Gonzalez-Serratos, and Huxley (1984). This was not evident in those fibres from high rigor temperature muscles. In summary, the muscles from the 35 °C unstretched treatment, had longer sarcomeres, increased lightness (P<0.001), higher longitudinal peak intensities (P<0.05) and higher global brightness values (P<0.05), compared to the 5 °C unstretched treatment. This provides further evidence, that light scattering occurs from more than one area and that both the transverse and longitudinal properties of the muscle fibres are involved. In summary, the lighter appearance of the unstretched, high rigor temperature muscle, was not caused by fibre width shrinkage, but was possibly a result of relocation and modification of sarcoplasmic or other structural proteins, remembering these muscles did have longer sarcomeres (as illustrated diagrammatically in Figure 5b), even though this was rejected in hypothesis (a). This could have generated a change in optical protein density along the length of the sarcomere, from some modification of these proteins.

ACCEPTED MANUSCRIPT

The I-band (rather than the A-band) of the sarcomere reduces during sarcomere length contraction (Periasamy et al., 1990) which suggests that in the 35 °C unstretched muscle, the length of the I-band may have played a role in the development of light scattering, likely in a co-dependent mechanism with sarcoplasmic proteins. In both electrically-stimulated beef and pale pork, the denaturation of sarcoplasmic proteins is associated with precipitation and some of the proteins binding to the I-band of the sarcomere and/ or the surface of myofibrils (Clarke, Shaw, & Morton, 1980; Liu et al., 2016). As sarcoplasmic proteins normally bind to proteins within the I-band, we propose this could impact on the distinct A/I banding pattern we observed along the length of the sarcomere (Figure 4). We postulate that this binding or reconfiguration of structural proteins could prevent further shortening from occurring, keeping the sarcomere in a longer configuration, as seen in the 35 °C unstretched treatment.

A high rigor temperature is also known to denature myosin heads (Stabursvik, Fretheim, & Frøystein, 1984) and it is likely this may has occurred during or prior to full rigor, prematurely interfering with actin and myosin cross bridge formation, thus preventing sarcomere shortening. It is likely that a combination of relocation of denatured sarcoplasmic proteins from the sarcoplasm to the sarcomere (leading to increased brightness and/or perceived length of the I-band) and less shortening/ fewer undulations (possibly due to early myosin denaturation) was responsible for increased lightness in the 35 °C unstretched muscle, although these theories still need to be tested. In addition, these results should be transferrable to other muscles on the carcass, but the effect of fibre type on light scattering has still to be explored.

A high rigor temperature of 35 °C generated muscle fibre fragments with more debris apparent within the suspension, as demonstrated in Figure 4. The increase in debris could be explained by an in increase in myofibril fragments due to accelerated proteolytic activities typical of high rigor temperatures (see Kim et al. (2014) for a review), or due to aggregated sarcoplasmic proteins and may also had a role in the development of light scattering.

ACCEPTED MANUSCRIPT

Interestingly, both the redness and yellowness values were similar (P>0.05) between treatments, as shown in Table *1* indicating that a non-pigment mechanism was responsible for the changes in lightness. High rigor temperatures have previously been associated with higher redness and yellowness values, indicative of more myoglobin denaturation and elevated autoxidation, as reviewed by Kim et al. (2014). No change in either redness or yellowness was observed in this study and we would assume myoglobin autoxidation was minimal at the time of measurement (although no measurement of myoglobin oxidation was performed).

Table 1 shows that the ultimate pH values, although significant, were only slightly higher for the 5 °C treatment compared to the higher rigor temperatures (5.57, 5.52, 5.47 for 5,15, 35 °C, respectively; LSD =0.064; P<0.05). This slightly lower pH has previously been found for pork muscles going through high rigor temperature similar to our conditions (Warner et al., 1997). It is likely that the slower pH decline and slower glycolytic rate of the lower temperature muscles were involved, but there would be other metabolic reasons why this occurred, as discussed previously (Honikel et al., 1983; Jeacocke, 1977).

Figure 6 shows the effect of pH cycling on the global brightness and width of individual fibre fragments of the stretched 15 °C muscle and Figure 7 shows reflection confocal micrographs of fragments at the various stages marked with the letters A-E in Figure 6. A corresponding movie (pH manipulation of one fibre_15C stretched.avi) is included in the supplemental information. These figures show that the width and global brightness are partially reversible but that some semi-permanent changes occurred to the muscle fibre fragment during the pH cycling process. Increasing the buffer pH (stages A to B) induced a swelling of the muscle fibre fragment which occurred simultaneously with a reduction in global brightness and a gradual increase in fibre width. Subsequently lowering the pH (stages B to C) generated the opposite scenario, with an increase in the global brightness and a decline in fibre width- although there appeared to be a time lag effect, see Figure 6 (c) (shown as red arrow). The lag is the time taken for the washing solution to penetrate into the muscle fibre fragment and cause the necessary structural changes required for swelling and shrinkage. For the first cycle, this lag period occurred between 7 min 45 sec and 11 min 12 sec, and

ACCEPTED MANUSCRIPT

therefore had a duration of 3 min 27 sec. The second and third lag times occurred for a duration of 4 min 38 sec and 2 min respectively, but may have continued for longer, and suggest that the events occurred quite rapidly within the cell, possibly due to cell membrane rupture. While it would be interesting to explore the time course of these events further, these preliminary results indicate a direct relationship exists between the muscle fibre fragment dimensions and the quantity of scattered light, with the pH of the surrounding medium a key determinant, and supports previous work on chicken *pectoralis* muscles (H. Swatland, 2008) and isolated beef *longissimus* muscle fibres (Hughes et al., 2017).

After the first cycle shown in Figure 6, when the pH was lowered for a second time, the global brightness did not reach the same intensity as in the previous cycle, indicating some permanent modification in the muscle structure had occurred. There may have been a loss of scattering material from the structure during the first washing procedure and we suggest this scattering material may have been sarcoplasmic proteins, which could have been bound onto the stretched I-band of the sarcomere or the myofibril surface and was relocated elsewhere (possibly extracellularly) when the pH was increased. This pH cycling investigation was successfully completed on the 15 °C stretched muscle, and it is still uncertain if the same effects would have occurred consistently in the 35 °C muscle, where we believe some permanent denaturation of myosin is likely to have occurred. From the results shown in Figure 6 and 7 and the movie in the supplementary material, we can confirm our hypothesis (c), that the increased global brightness caused by lowering the pH is not able to be fully reversed after the muscle fibre fragment has been washed with a high pH solution, most likely as a result of the loss of some washed material from either the I-band of the sarcomere or the surface of the myofibrils.

4. Conclusion

Manipulation of sarcomere length, rigor temperature and pH has provided tools to dissect the various sources of light scattering by muscle fibres. Sarcomere length alone did not impact on the light scattering properties of the muscle. A high rigor temperature increased lightness and global brightness

ACCEPTED MANUSCRIPT

of the muscle and was associated with alterations in the structural configuration of the muscle, creating increased light scattering. When stretched, the high rigor temperature promoted muscle fibre shrinkage in the transverse orientation, which generated more light scattering. When in the unstretched configuration, the high rigor temperature muscles retained a longer sarcomere, which was associated with more light scattering compared to lower rigor temperatures. It appears the fibre width, was not solely responsible for this light scattering development and indicates other mechanisms are involved. Together, these findings indicate there is no single mechanism responsible for the development of light scattering, and more than one structural attribute of the muscle is involved.

The pH cycling part of the experiment confirmed the interaction that occurs between muscle fibre width, pH and light scattering, with reductions in the environmental pH causing shrinkage of the muscle fibre fragment, whilst increasing light scattering. However, once the muscle fibre was exposed to the low pH conditions, some permanent, irreversible structural modifications occurred, indicating there may have been a loss of scattering material from the structure or some permanent modification to the components of the muscle fibre. The exact mechanisms responsible for this irreversibility have yet to be determined.

In terms of application in the meat industry, any optimisation of the temperature during rigor would be opportune for improving the structural attributes of the muscle and avoiding extremes of either excessively pale or dark meat.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

5. Acknowledgments

The authors and CSIRO acknowledges funding provided by Australian Meat Processor Corporation (AMPC) and matching funds provided from the Australian Government, via Meat and Livestock Australia (MLA), to support the research and development detailed in this publication. The support of Griffith University, and in particular the Imaging and Image Analysis Facility, is also gratefully acknowledged. We also acknowledge the support of FONCyT (PRH-PICT 2013-3292). The authors have no competing interests to declare.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 1: Effect of rigor temperature (5, 15 or 35 °C) and muscle configuration (stretched or unstretched) on beef *M.sternomandibularis* pH, colour (L*, a*, b*) at the meat surface and the global brightness and peak intensities (transverse and longitudinal) measured using reflectance confocal laser scanning microscopy (rCLSM) on images of muscle fibre fragments isolated from each muscle. Values are least squares means and the least significant differences (LSD) and *P*-values are shown, n = 4 or [1]n = 3 for each treatment.

	Stretched			Unstretched			LSD			*P*-value		
Temperature (°C)	5	15	35	5	15	35	Temp	configuration	Temp x configuration	Temp	configuration	Temp x configuration
Lightness (L*)	30.4	28.6	32.8	29.2	28.2	31.2	1.35	1.10	1.91	<0.001	0.060	0.664
Redness (a*)	13.5	12.4	13.9	14.3	15.0	13.8	1.63	1.33	2.30	0.958	0.095	0.244
Yellowness (b*)	1.4	1.2	1.5	1.0	1.5	2.0	0.75	0.61	1.06	0.331	0.634	0.472
pH[2]	5.56	5.53	5.47	5.57	5.50	5.47	0.064	0.052	0.090	0.028	0.921	0.769
Global brightness[1]	119	108	145	110	132	133	16.9	13.3	24.7	0.011	0.734	0.072
Transverse peak intensity[1]	182	169	208	177	186	189	10.4	8.5	14.7	0.002	0.555	0.008
Longitudinal peak intensity[1]	186	178	211	178	184	193	16.6	13.5	23.4	0.027	0.313	0.332

[2] The pH was measured at 18-19 h PM for the 15 and 35 °C treatments and at 30 h PM for the 5 °C treatment.

ACCEPTED MANUSCRIPT

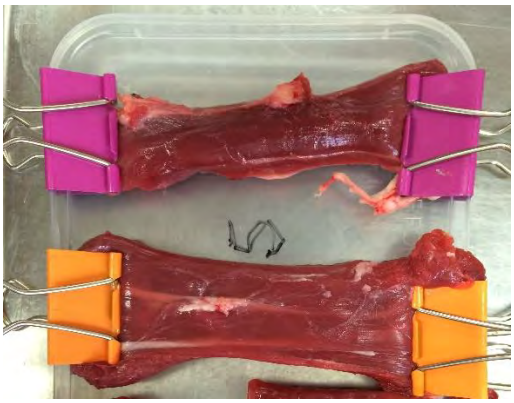
ACCEPTED MANUSCRIPT

Table 2: Effect of rigor temperature (5, 15 or 35 °C) and muscle configuration (stretched or unstretched) on beef *M. sternomandibularis* muscle fibre fragments isolated from each muscle. The sarcomere length (SL) was measured using light microscopy and the fibre width (FW), distance between intensity global brightness peaks or periodicities either in the transverse (transverse peak distance, TPD) or longitudinal (longitudinal peak distance, LPD) was measured using reflectance confocal laser scanning microscopy (rCLSM) images. Values are least squares means and the least significant differences (LSD) and *P*-values are shown, n = 4 or [1]n = 3.

	Stretched			Unstretched			LSD			*P*-Value		
Temperature or Temp (°C)	5	15	35	5	15	35	Temp	configuration	Temp x configuration	Temp	configuration	Temp x configuration
Sarcomere length (µm)	2.53	2.68	2.63	2.03	1.94	2.22	0.112	0.091	0.158	0.038	<0.001	0.019
Fibre width (µm)	33.7	35.4	25.3	31.6	33.6	35.0	3.93	3.21	5.55	0.096	0.222	0.008
Transverse peak distance[1] (µm)	2.17	2.25	1.97	2.16	2.00	1.87	0.34	0.278	0.481	0.286	0.358	0.750
Longitudinal peak distance[1] (µm)	2.35	2.37	2.49	2.18	2.20	2.00	0.315	0.257	0.446	0.962	0.038	0.440
Volume (µm ^{2})	3084	3558	1860	1897	2142	2563	826.2	674.6	1168.4	0.279	0.063	0.031
LPD / SL	0.94	0.87	0.95	1.08	1.16	0.90	0.154	0.126	0.218	0.387	0.05	0.109
TPD / FW	0.062	0.064	0.074	0.071	0.060	0.056	0.011	0.0093	0.0161	0.656	0.316	0.056
TPD / SL	0.86	0.83	0.75	1.06	1.05	0.84	0.154	0.126	0.218	0.072	0.012	0.642

ACCEPTED MANUSCRIPT

(a)



(b)

Figure 1: Images depicting beef *M. sternomandibularis* from (a) stretched (clamped with 2 clips) or (b) unstretched (clamped with only one clip) configuration. Muscles were wrapped in plastic film after fixing to the plastic tray.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

(a) (b)

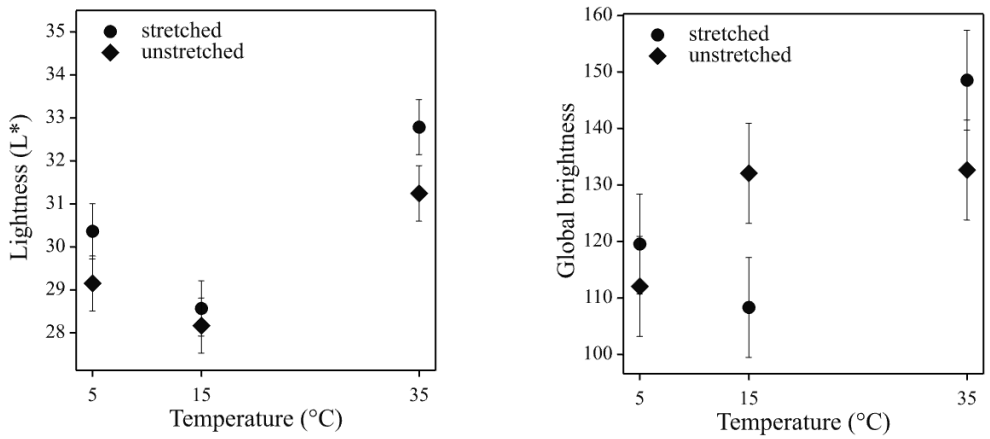


Figure 2: Effect of rigor temperature (5, 15 or 35 °C) and muscle configuration (stretched or unstretched) on (a) Minolta lightness or L* (n = 4 per treatment) and (b) rCLSM global brightness (n = 3 per treatment) of beef *M. sternomandibularis* muscle fibres. Each point is a least squares means and the vertical bar represents ± standard error of differences (SED) values. Effect of temperature, $P<0.05$, muscle configuration, $P>0.05$; interactions $P>0.05$ for both global brightness and lightness.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

(a) (b)

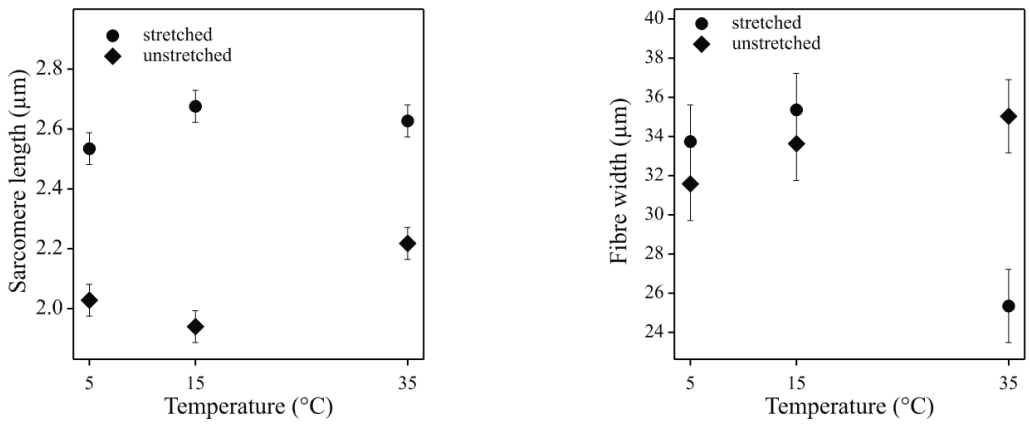


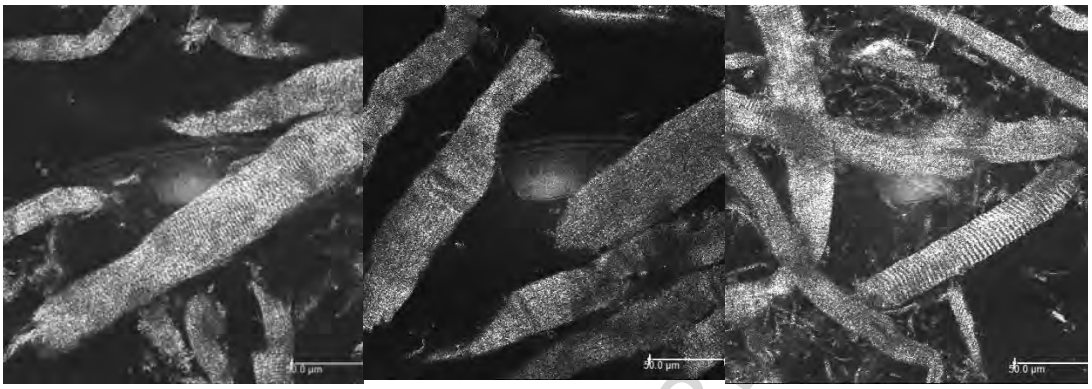
Figure 3: Effect of rigor temperature (5, 15 or 35 °C) and muscle configuration (stretched or unstretched) on beef *M. sternomandibularis* muscle fibres. Each point is a least squares means and the vertical bar represents ± standard error of differences (SED) values. (a) Sarcomere length showed an effect of temperature ($P<0.05$), but no effect ($P>0.05$) was observed of configuration or the interaction. (b) Fibre width showed no effect of main treatments ($P>0.05$), but there was an interaction ($P<0.05$), (n = 4 per treatment).

ACCEPTED MANUSC

ACCEPTED MANUSCRIPT

(a) 5 °C (b) 15 °C (c) 35 °C

stretched



(d) 5 °C (e) 15 °C (f) 35 °C

unstretch
ed

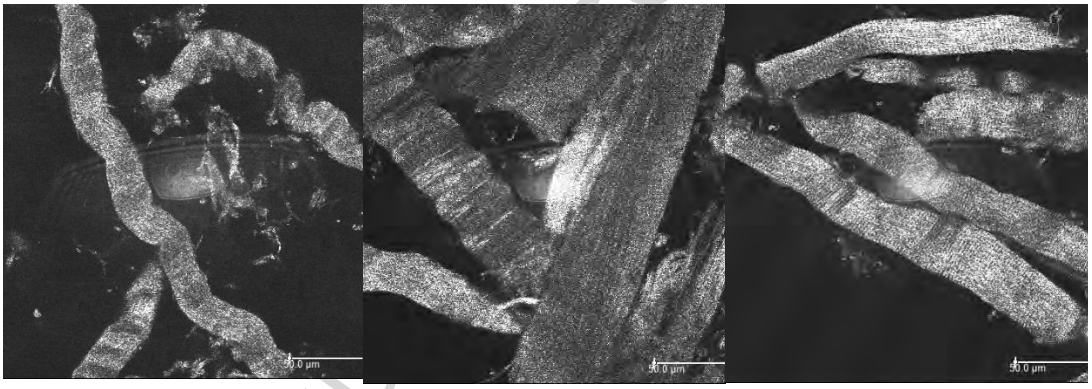


Figure 4: Example of effect of rigor temperature (5 °C, a, d; 15 °C b, e; or 35 °C, c, f) and muscle configuration (stretched, a, b, c; or unstretched, d, e, f) on reflection confocal laser scanning microscopy (rCLSM) images of beef *M. sternomandibularis* muscle fibre fragments. Scale bar 50 µm.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

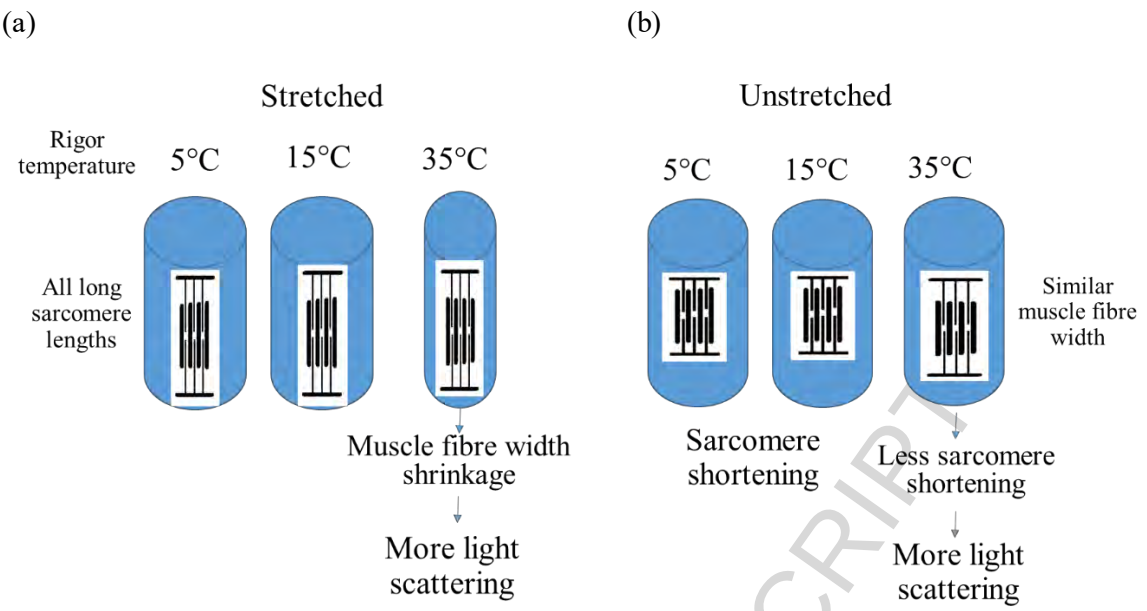
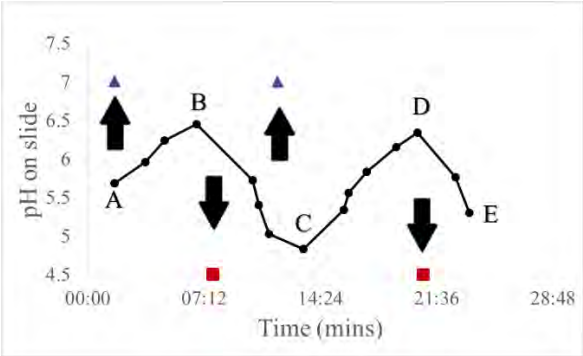


Figure 5: Diagram illustrating the effect of configuration; either (a) stretched or (b) unstretched and rigor temperature; 5, 15 or 35 °C on the structural attributes of beef *M. sternomandibularis* muscle fibres and sarcomere lengths.

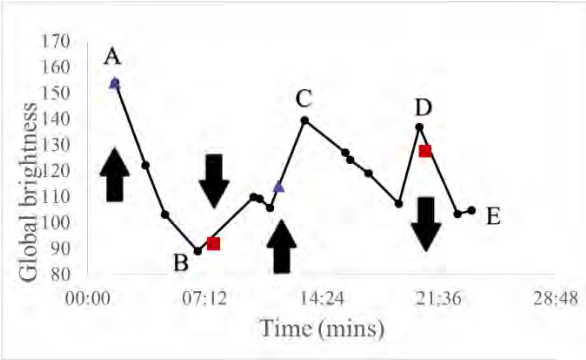
ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

(a)



(b)



(c)

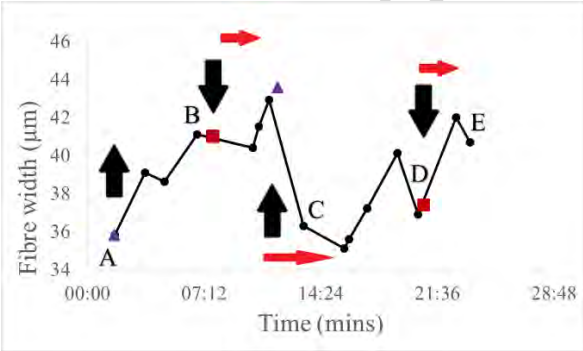


Figure 6: Effect of pH cycling on beef *M. sternomandibularis* muscle fibre fragment (15 °C, stretched), as visualised using reflection confocal laser scanning microscopy (rCLSM), showing (a) pH measured on the slide (b) global brightness and (c) fibre fragment width. The pH cycling was completed by exposing the muscle fibre fragment to the same homogenising buffer (380 mM mannitol + 50 mM potassium acetate buffer) at either pH 4.5 (red square) or 7.0 (purple triangle) to decrease or increase the pH of the surrounding medium (as shown with large black arrows). Each stage of pH cycling is represented, as described in Figure 7 with events marked A to E: A- start of

ACCEPTED MANUSCRIPT

initial pH increase; B-1st high pH peak; C- 1st low pH trough; D- 2nd high pH peak; E- 2nd low pH trough. The red arrow in (c) depicts the lag effects.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

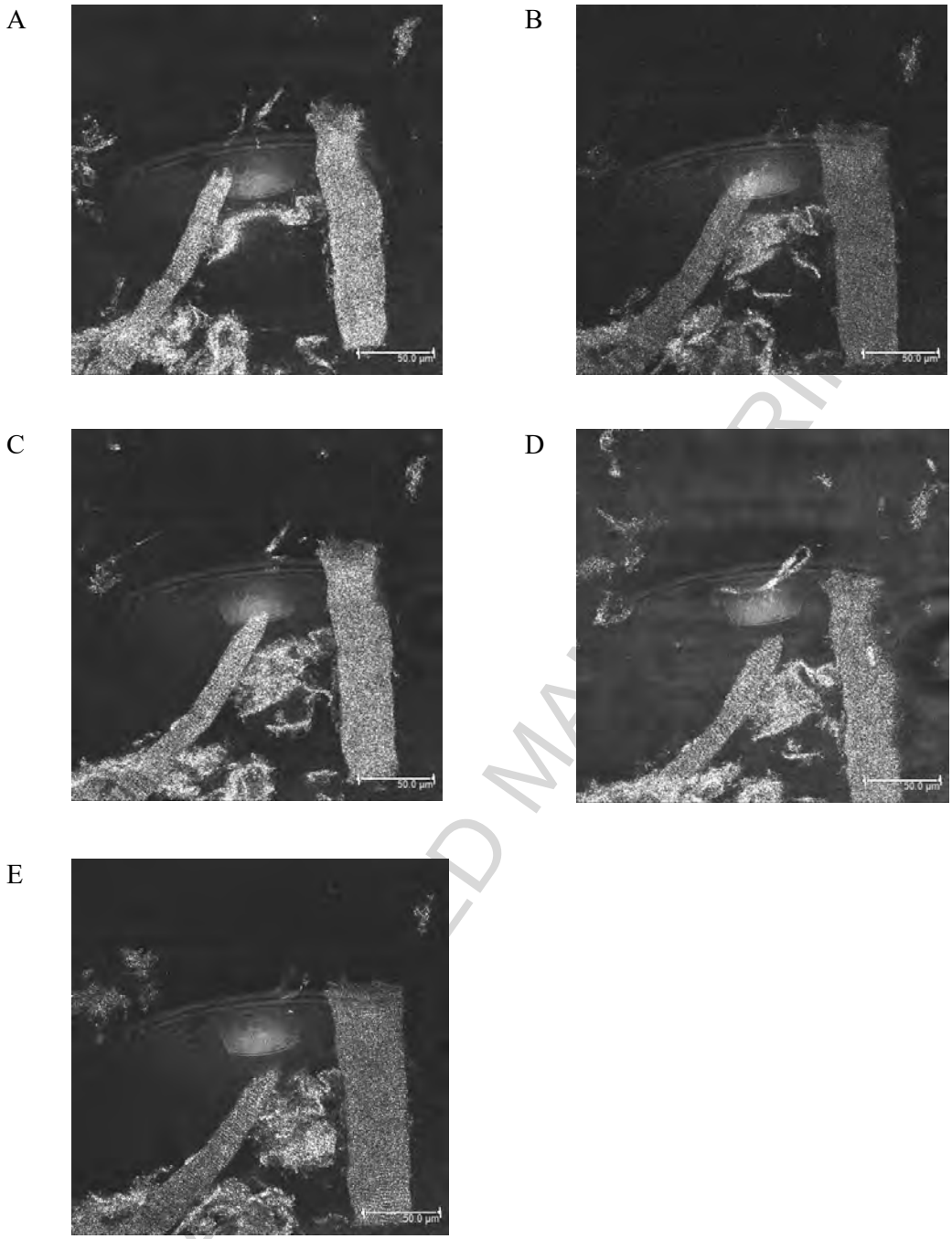


Figure 7: Effect of pH cycling on beef *M. sternomandibularis* muscle fibre fragments (15 °C, stretched) visualised using reflection confocal laser scanning microscopy (rCLSM). The pH cycling was completed by exposing the muscle fibre fragment to the same homogenising buffer (380 mM mannitol + 50 mM potassium acetate buffer) at either pH 4.5 or 7.0 to decrease or increase the pH of the surrounding medium. Each stage of pH cycling is represented, as described in Figure 6 with

ACCEPTED MANUSCRIPT

events marked A to E: A- start of initial pH increase; B-1st high pH peak; C- 1st low pH trough; D- 2nd high pH peak; E- 2nd low pH trough. Scale bar 50 µm.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

References

Bozler, E. (1958). Light scattering and water content of extracted muscle fibers as related to contraction and relaxation. *Archives of Biochemistry and Biophysics, 73*(1), 144-152. doi: http://dx.doi.org/10.1016/0003-9861(58)90249-2

Brown, L., Gonzalez-Serratos, & Huxley, A. F. (1984). Sarcomere and filament lengths in passive muscle fibres with wavy myofibrils. *Journal of Muscle Research & Cell Motility, 5*, 293-314.

Clarke, F. M., Shaw, F. D., & Morton, D. J. (1980). Effect of electrical stimulation post mortem of bovine muscle on the binding of glycolytic enzymes. Functional and structural implications. *Biochem. J., 186*(1), 105-109.

Honikel, K. O., Roncalés, P., & Hamm, R. (1983). The influence of temperature on shortening and rigor onset in beef muscle. *Meat Science, 8*(3), 221-241.

Hughes, J., Clarke, F., Purslow, P., & Warner, R. (2017). High pH in beef *longissimus thoracis* reduces muscle fibre transverse shrinkage and light scattering which contributes to the dark colour. *Food Research International, 101*, 228-238. doi: https://doi.org/10.1016/j.foodres.2017.09.003

Hughes, J., Oiseth, S., Purslow, P., & Warner, R. (2014). A structural approach to understanding the interactions between colour, water-holding capacity and tenderness. *Meat Science, 98*, 520-532.

Irving, T. C., Swatland, H. J., & Millman, B. M. (1989). X-Ray Diffraction Measurements of Myofilament Lattice Spacing and Optical Measurements of Reflectance & Sarcomere Length in Commercial Pork Loins. *Journal of Animal Science, 67*(1), 152-156. doi: 10.2134/jas1989.671152x

Jeacocke. (1977). The temperature dependence of anaerobic glycolysis in beef muscle held in a linear temperature gradient. . *Journal of the Science of Food and Agriculture, 28*(6), 551-556. doi: 10.1002/jsfa.2740280613

Jeacocke, R. (1984). Light scattering from muscle during the onset of rigor mortis. *European Meeting of Meat Research Workers, 30th, Bristol, UK*, 104-105.

Kim, Y. H. B., Warner, R. D., & Rosenvold, K. (2014). Influence of high pre-rigor temperature and fast pH fall on muscle proteins and meat quality: A review. *Animal Production Science, 54*(4), 375-395. doi: http://dx.doi.org/10.1071/AN13329

Liu, J., Arner, A., Puolanne, E., & Ertbjerg, P. (2016). On the water-holding of myofibrils: Effect of sarcoplasmic protein denaturation. *Meat Science, 119*, 32-40. doi: http://dx.doi.org/10.1016/j.meatsci.2016.04.020

MacDougall, D. B. (1982). Changes in the colour and opacity of meat. *Food Chemistry, 9*(1-2), 75-88.

MediaCybernetics. (2012). Image Pro Premier. Retrieved from http://www.mediacy.com

Millman, B. M. (1998). The Filament Lattice of Striated Muscle. [Journal Article]. *Physiological reviews, 78*(2), 359-391.

Offer, G., Knight, P., Jeacocke, R., Almond, R., Cousins, T., Elsey, J., . . . Purslow, P. P. (1989). The structural basis of the water-holding, appearance and toughness of meat and meat products. *Food Microstructure, 8*, 151-170.

Periasamy, A., Burns, D., Holdren, D., Pollack, G., & Trombitas, K. (1990). A-band shortening in single fibers of frog skeletal muscle. *Biophysical Journal, 57*, 815-828.

Purslow, P. O., S, Hughes, J., & Warner, R. (2016). The structural basis of cooking loss in beef: variations with temperature and ageing. *Food Research International, 89*(1), 739-748.

Rasband, W. (2014). ImageJ.Ink Retrieved 6/2/14, 2014, from http://imagej.nih.gov/ij/index.html

Stabursvik, E., Fretheim, K., & Frøystein, T. (1984). Myosin denaturation in pale, soft, and exudative (PSE) porcine muscle tissue as studied by differential scanning calorimetry. *Journal of the Science of Food and Agriculture, 35*(2), 240-244. doi: 10.1002/jsfa.2740350218

ACCEPTED MANUSCRIPT

Swatland. (2002). Relationship between pork muscle fiber diameter and optical transmittance measured by scanning microphotometry. *Canadian Journal of Animal Science, 82*(3), 321-325. doi: 10.4141/a01-096

Swatland, H. (2008). How pH causes paleness or darkness in chicken breast meat. *Meat Science, 80*(2), 396-400.

Totland, G. K., & Kryvi, H. (1991). Distribution patterns of muscle fibre types in major muscles of the bull (*Bos taurus*). *Anatomy and Embryology, 184*(5), 441-450. doi: 10.1007/bf01236050

Warner, R. D., Kauffman, R. G., & Greaser, M. L. (1997). Muscle protein changes post mortem in relation to pork quality traits. *Meat Science, 45*(3), 339-352.

Wilding, P., Hedges, N., & Lillford, P. J. (1986). Salt-induced swelling of meat: The effect of storage time, pH, ion-type and concentration. *Meat Science, 18*(1), 55-75. doi: http://dx.doi.org/10.1016/0309-1740(86)90066-5

Winger, R. J., & Pope, C. G. (1981). Osmotic properties of post-rigor beef muscle. *Meat Science, 5*(5), 355-369. doi: http://dx.doi.org/10.1016/0309-1740(81)90034-6

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

6. Highlights

- Beef meat colour is impacted by light scattering
- Sarcomere length alone is not directly causative of light scattering
- A high rigor temperature promotes light scattering
- Increasing rigor temperature commercially could reduce the occurrence of dark meat

ACCEPTED MANUSCRIPT

Accepted Manuscript

A high rigor temperature, not sarcomere length, determines light scattering properties and muscle colour in beef M. sternomandibularis meat and muscle fibres

J. Hughes, F. Clarke, P. Purslow, R. Warner

PII: S0309-1740(18)30283-3
DOI: doi:10.1016/j.meatsci.2018.05.011
Reference: MESC 7561

To appear in: *Meat Science*

Received date: 10 March 2018
Revised date: 17 May 2018
Accepted date: 18 May 2018

Please cite this article as: J. Hughes, F. Clarke, P. Purslow, R. Warner , A high rigor temperature, not sarcomere length, determines light scattering properties and muscle colour in beef M. sternomandibularis meat and muscle fibres. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Mesc(2017), doi:10.1016/j.meatsci.2018.05.011

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.