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Perimysial collagen crosslinking in Belgian Blue double-muscled cattle

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Abstract

Relative quantities of five collagen crosslinks in meat from Belgian Blue normal, heterozygous and homozygous double-muscled (DM) bulls ($n=31$, mean age = 20 months) were investigated using *m. gluteobiceps, m. semitendinosus* and *m. pectoralis profundus*. The crosslinks pyridinoline, dihydroxylysinorleucine (DHLNL), hydroxylysinorleucine (HLNL), histidinohydroxymerodesmosine (HHMD) and Erlich chromogen (EC) were measured. Concentrations (per mol collagen) of pyridinoline and DHLNL were greater in the DM animals than normal and heterozygous animals for the muscles combined; HHMD was lower. The collagen content in the DM animals was about 65% of the normal animals; that of the heterozygous animals was about 87%. Consequently, the HHMD, HLNL and EC crosslink concentrations (per g wet meat) were significantly lower in the DM animals than in normal and heterozygous animals. All other measured crosslink concentrations and the thermal solubility of the collagen were not significantly affected by the animal type. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Both hyperplasia (an increase in the number of muscle fibres) and, to a lesser extent, hypertrophy (an enlargement of individual muscle fibres) are consequences of an inherited condition commonly known as "double muscling" (Ansay, 1976; Hanset, Michaux, Dessy-Doize, & Burtonboy, 1982; Swatland & Kieffer, 1974). Cattle exhibiting this condition are often referred to as "double-muscled" or "hypertrophied" and characteristically have some enlarged muscles, in particular, in the regions of the proximal fore and hind quarters (Ménissier, 1982). Double-muscled (DM) cattle also have less bone, less fat, more muscle and a higher proportion of "expensive" cuts of meat compared to normal cattle (Ménissier, 1982; Shahin & Berg, 1985a, 1985b; Shahin, Berg, & Price, 1991). These characteristics make DM cattle attractive as potential meat animals and, in Europe, a premium price is paid for their carcasses (Arthur, 1995; Boccard, 1982). However, their potential is associated with reduced fertility, dystocia, low calf viability and increased stress susceptibility (Arthur, 1995).

The DM condition in cattle was first documented by Culley in 1807 and described in detail by Kaiser in 1888 (Arthur, 1995). However, it is only in the last decade that the similar map positions of the myostatin gene and the *mh* locus, and the identification of relatively severe mutations of the myostatin gene of several cattle breeds, has led McPherron and Lee (1997), Kambadur, Sharma, Smith, and Bass (1997) and Grobet et al. (1998) to conclude that the mutations were responsible for the double muscling phenotype. Prior to the identification of the *mh* locus, classification of DM cattle was usually based on external physical assessment of the animals and, where available, pedigree records (Arthur, 1995). Some semi-objective scoring systems were also developed, based on physical characteristics and/or biochemical measurements (Hanset & Michaux, 1985; Rollins, 1967; Rollins, Tanaka, Nott, & Thiessen, 1972; Vissac, Ménissier, & Perreau, 1973). Generally, these methods were effective in classification of "extreme" animals, both normal and DM.

The use of these "extreme" animals in research may have resulted in findings not typical of either DM or

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normal animals. Furthermore, due to a lack of classification of animals heterozygous for the *mh* allele, some heterozygous animals would have been grouped with DM or normal animals, perhaps even in both groups in the same experiment. Inclusion of these animals could therefore have resulted in erroneous and confusing data, or at least "diluted" some of the characteristics associated with the DM condition.

Regardless of the faults of these earlier methods of classification, a lower collagen content has been consistently reported in meat from DM animals than in normal animals (for example, Hanset et al., 1982; Lawrie, Pomeroy, & Williams, 1964; Uytterhaegen et al., 1994). Furthermore, Bailey, Enser, Dransfield, Restall, and Avery (1982) observed that there was little difference in the nature of collagen crosslinks in meat from DM and normal cattle. The collagen obtained from DM meat did contain a slightly higher proportion of reducible crosslinks per mg of collagen and a slightly lower relative proportion of stable crosslinks. Collagen is one of the major intramuscular connective tissue proteins and it is generally accepted that it plays an important, but not clearly understood, role in meat texture. Quantification of collagen and determination of the nature of collagen are therefore important for an understanding of this role and its effect on meat quality. Use of meat from DM animals can provide an interesting and extreme contrast to that from normal animals in terms of some meat characteristics, in particular its collagen content.

The aim of this study was to investigate the relative quantities of intramuscular collagen and some collagen crosslinks in three muscles obtained from Belgian Blue DM (*mh*/*mh*), heterozygous (*mh*/+) and normal (+/+) bulls.

2. Materials and methods

2.1. Materials

These included animals and chemicals.

2.1.1. Animals

Thirty-one Belgian Blue bulls of ages ranging from 16 to 25 months (mean = 20 months), from different farms and fattened on high-concentrate diets, were slaughtered at the abattoir of Ghent University. The dressed carcasses were hung by Achilles tendon suspension in a chiller at 2 °C. At 24 h post mortem, *m. gluteobiceps* (Gb), *m. semitendinosus* (St) and *m. pectoralis profundus* (PP) were removed and standardised sections (approximately 500 g) taken from the centre of the muscles were vacuum-packaged and transported, chilled, arriving at INRA Theix within 24 h. The muscle sections were aged for 4 weeks at 2 °C. These sections of muscles were used fresh for crosslink analyses and after freezing and frozen storage (−18 °C) for total and soluble collagen measurements and moisture contents. The remaining pieces of the muscles were stored at 2 °C for 11 days, prior to freezing at −18 °C. Lipid content measurements were undertaken on these latter pieces. The animals sampled in this study were part of a larger trial, of which some meat quality data have been published (De Smet, Claeys, Balcaen, van den Brink, Seynaeve, & Demeyer, 2000; Ngapo, Berge, Culioli, Dransfield, De Smet, & Claeys, in press).

2.1.2. Chemicals

All chemicals and reagents used were at least of analytical grade. Water was deionised.

2.2. Methods

These included muscle classification, preparation and reactions.

2.2.1. Double muscling classification

Genotyping for the mutation nt821 (del 11) in the mysotatin gene responsible for the DM phenotype (DM, *mh*/*mh*; heterozygous, *mh*/+; normal, +/+) in the Belgian Blue breed was done according to the method of Grobet et al. (1998).

2.2.2. Preparation of intramuscular connective tissue (IMCT)

The method used was based on that of Light and Champion (1984) for extraction of perimysial collagen. Muscle samples (about 100 g) were cut into 2 cm^3 pieces and homogenised in saline solution (0.15 M NaCl, 0.05 M Na_2HPO_4, pH 7.5, 4 °C; 600 ml) for 10 s at low speed and 10 s at high speed in a Waring blender. The homogenate was filtered through PVC mesh (1 mm^2 pore size). The combined retained material from 400 g of muscle was rehomogenised in saline solution (500 ml) and filtered. This step was repeated twice. Visible blood vessels and extraneous matter were removed from the retained material prior to blotting with filter paper (thereafter referred to as intramuscular connective tissue or IMCT). A portion of IMCT (about 3 g) was removed for reduction. The remainder was freeze-dried, vacuum-packaged and stored at 4 °C, protected from light.

2.2.3. Reduction

The method of reduction of IMCT at pH 7.5 with sodium borohydride was adapted from the method of Robins, Shimokomaki, and Bailey (1973) and undertaken with minimal exposure to light. Wet IMCT (3.0 g) was suspended overnight in saline solution (0.15 M NaCl, 0.05 M Na_2HPO_4, pH 7; 100 ml). Immediately prior to its addition, a solution of 100 mg/ml $NaBH_4$ was prepared in 1 mM NaOH. A dry weight ratio of

borohydride:collagen of between 1:30 and 1:15 was calculated, assuming that the collagen content was 15% of the wet weight of the IMCT. The $NaBH_4$ solution (150 μl) was added to the IMCT suspension which was vigorously stirred throughout the reduction process. The pH was maintained at 7.5 or slightly less for 30 min, by the addition of 9 M acetic acid. A second addition of a freshly prepared $NaBH_4$ solution was then added and the pH was maintained at 7.5 or slightly less for a further 30 min. The pH was then reduced to 4 and the IMCT sample was thoroughly washed with water, filtered through plastic mesh (1 mm^2 pore size) and blotted with filter paper. The reduced IMCT was freeze-dried, vacuum-packaged and stored at 4 °C, protected from light.

2.2.4. Hydrolysis

The following procedures were based on the methods of Black, Duncan, and Robins (1988) and were undertaken with minimal exposure to light. Dry IMCT (150 mg) was cut into pieces (4 mm^3) and accurately weighed into a glass vial with a teflon joint. Six samples were repeated (three repeats for each of three DM muscles and four repeats for each of three normal muscles). A solution of 6 M HCl (3 ml) was added, the samples were purged with N_2 gas and the vials securely closed. The samples were heated at 115 °C for 16 h. When the samples had cooled to room temperature, glacial acetic acid (3 ml) was added and the samples were vortexed. Samples were transferred to 5 ml syringes fitted with 13 mm 0.45 μm Durapore (PVDF) syringe driven filter units (Millex-HV, Millipore Corporation) and filtered into 10 ml volumetric flasks. The hydrolysis vials were rinsed with three volumes of butanol:acetic acid:water in the ratio 4:1:1 (1 ml per rinse), filtering after each rinse and transferring the rinse solutions to the volumetric flasks. The flasks were made up to 10 ml, using the butanol:acetic acid:water solution. Sample solution (100 μl) was removed to an Eppendorf flask and stored at −18 °C for hydroxyproline analyses.

2.2.5. Cellulose chromatography

The following method was based on the procedure described by Black et al. (1988). A suspension of 5% (w/v) CF1 cellulose was prepared in an organic phase of butanol:acetic acid:water in the ratio 4:1:1. Aliquots (3 ml) of this slurry were removed to large test tubes. The sample solutions in the 10 ml volumetric flasks were added to the cellulose slurry in test tubes. The flasks were rinsed with three volumes of butanol (2 ml per rinse) and the rinse solutions added to the test tubes. A further volume of butanol (6 ml) was added to each test tube and vortexed. The sample-slurry mixtures were left for 30 min in the dark.

Columns were prepared by applying 6 aliquots of CF1 cellulose slurry (4 ml per aliquot) to plastic polypropylene columns (1.5×12 cm), each fitted with a porous polymer bed support (Bio-Rad Laboratories). When the columns had settled, the sample-slurry mixtures were added. The tubes were rinsed with three volumes of butanol:acetic acid:water (4:1:1) solvent (5 ml per rinse) and each rinse added to the column. A further three aliquots of the solvent were added (15 ml total). The crosslinks were eluted with five aliquots of water (30 ml total). Samples were freeze-dried overnight in a vacuum centrifuge, 600***g*** at about 200 mbar (Heto Maxi Dry Plus, type VR Maxi, Heto-Holten A/S, Denmark) at 50 °C. Using a buffer of 0.2 M sodium citrate and 0.1% (w/v) phenol (pH 4.61; Solvent A; 450 μl) the residues were transferred to 1 ml plastic syringes, fitted with 13 mm 0.45 μm Durapore (PVDF) syringe driven filter units (Millex-HV, Millipore Corporation), filtered into Eppendorfs and stored at −18 °C.

2.2.6. High-performance liquid chromatography (HPLC)

This included selected instrumentation and methods.

2.2.6.1. Instrumentation. The HPLC analyses were performed, using a system consisting of a LabPRO Solvent Selector (PR100-106, Cotati, CA), a Kontron HPLC High Pressure Pump System 522 operating at 0.50 ml/min, a Kontron HPLC 560 Autosampler, fitted with a 100 μl loop and a Kontron Column Thermostat 482, operated at 52 °C (Kontron Instruments, Milan, Italy), a pre-injection nitrogen absorbing column (for the solvent only, resine desamoniacante, DC 3A Li+, 300×4.6 mm, CIL Cluzeau Info Labo, Sainte-Foy-La-Grande, France) and an AMINOSep AA-911 ion-exchange column (AAA-99-8553, Transgenomic, Omaha, NE), fitted with a pre-column filter (A.316, Upchurch Scientific, Oak Harbour, WA). Ninhydrin peak development was achieved by addition of a ninhydrin solution to the line immediately after the ion-exchange column, using a Kontron Pump 422 (Kontron Instruments, Milan, Italy) operating at 0.25 ml/min. The ninhydrin solution was mixed with the mobile phase prior to entering a Reacticil oven (total volume 500 μl, 0.5 mm i.d., CIL Cluzeau Info Labo, Sainte-Foy-La-Grande, France), operating at 110 °C. The ninhydrin-developed peaks were measured on a Kontron HPLC 540 Diode Array Detector operated at 546 nm, and the chromatograms analysed using the KromaSystem 2000 software package (Kontron Instruments, Milan, Italy).

2.2.6.2. Methods. The procedure for elution of the crosslinks was adapted from the methods described by Horgan, Jones, King, Kurth, and Kuypers (1991). The HPLC system was equilibrated for 15 min with Solvent A [0.2 M sodium citrate and 0.1% (w/v) phenol, pH 4.61] at 0.50 ml/min, prior to sample injection. After 165 min, a step change to Solvent B [0.35 M sodium citrate

and 0.1% (w/v) phenol, pH 5.28] was made and the system run for a further 80 min, after which the column was rinsed for 30 min with Solvent C (0.05 M H_3BO_3, 0.15 M NaCl, 0.05 M NaOH, pH 10.1). Repeats of randomly selected samples were made throughout the study.

A 0.1 M ninhydrin solution was prepared using an adaptation of the method of Moore (1968). A solution of 4 M lithium acetate was prepared, adjusted to pH 5.2 with glacial acetic acid and filtered (0.45 μm HVLP Durapore Membrane filters, Millipore Corporation). Minimising exposure to light, the lithium acetate solution (125 ml) was added to dimethyl sulfoxide (DMSO; 375 ml) while purging with nitrogen and vigorously stirring. The nitrogen purging and vigorous stirring continued throughout the preparation. After 20 min, ninhydrin (9 g) was added, followed by hydrindantin (0.5 g). After a further 20 min, the solution was filtered (0.45 μm HVLP Durapore Membrane filters, Millipore Corporation). The ninhydrin was transferred immediately to the HPLC system and maintained under helium and protected from light.

Pyridinoline peaks were well separated and identified by comparison with (1) profiles obtained using a highly purified sample of pyridinoline (a kind gift from the laboratory of Dr. Ronald Kuypers, Brisbane, Australia), (2) profiles of sample solutions spiked with this purified pyridinoline solution, and (3) to pyridinoline concentrations obtained by other workers (Bosselmann, Möller, Steinhart, Kirchgessner, & Schwarz, 1995; Horgan et al., 1991; Smith & Judge, 1991; Steinhart, Bosselmann, & Möller, 1994). Quantification was achieved using a colour yield relative to leucine (2.99), obtained by comparison of leucine to the purified pyridinoline sample. The reducible crosslink amino acids, dihydroxylysinorleucine (DHLNL), hydroxylysinorleucine (HLNL), and histidinohydroxymerodesmosine (HHMD), were identified by comparison of reduced and non-reduced IMCT samples and with published results of analyses using tissues known to contain these amino acids (Robins, 1982). These crosslink amino acids were quantified using colour yields relative to leucine of 1.84, 1.75 and 3.5, respectively (Horgan et al., 1991). Note that some deterioration of ninhydrin was observed and so all values of peak areas were adjusted accordingly. The crosslink concentrations were expressed as both mol/mol collagen and nmol/g wet meat.

2.2.7. Erlich chromogen (EC)

The method of analysis of EC was based on that of Horgan et al. (1991). Finely-diced, reduced IMCT (100 mg) was suspended in Tris–HCl buffer (50 mM Tris–HCl, 1 mM $CaCl_2$, pH 7.5; 5 ml) and stored at 2 °C for 24 h. The suspension was then denatured at 65 °C for 30 min, vortexing at 0, 15 and 30 min, cooled to 37 °C and vortexed again. A solution of 20 mg/ml trypsin in the Tris–HCl buffer (125 μl) was added to the suspension and it was maintained at 37 °C for 4 h with vortexing every 15 min. This was followed by 20 min at 65 °C with vortexing at 0 and 20 min. The suspension was then centrifuged for 30 min at 28 000***g*** (25 °C). The supernatant was filtered [13 mm 0.45 μm Durapore (PVDF) syringe driven filter units; Millex-HV, Millipore Corporation]. Filtrate (1 ml) was removed to an Eppendorf and a solution of 5% (w/v) *p*-dimethylaminobenzaldehyde and 0.01% (w/v) mercuric chloride in 4 N perchloric acid (200 μl) was added. The mixture was filtered (13 mm 0.45 μm Durapore (PVDF) syringe driven filter units; Millex-HV, Millipore Corporation). Five minutes after the addition of the *p*-dimethylaminobenzaldehyde solution, the absorbance of the solution was measured at 572 and 640 nm (the latter reading was considered a baseline measure and therefore subtracted from the former reading). The Erlich Chromogen concentration was expressed as mol/mol collagen and nmol/g wet meat using a molar extinction coefficient of 25 000 (Kemp & Scott, 1988). A second aliquot of the filtrate (500 μl) was removed and collagen concentration of the trypsin digest was obtained (see hydroxyproline analyses). Fifteen samples were repeated.

2.2.8. Hydroxyproline

This was determined in meat and digests.

2.2.8.1. Meat. Hydroxyproline contents of total and insoluble collagen in whole meat samples were determined using the colorimetric method of Bergman and Loxley (1963), adapted by Bonnet and Kopp (1984) and using a Technicon Autoanalyzer AAII (Bran and Luebbe, Norderstedt, Germany). The collagen content was expressed as mg/g wet meat and % of fat free dry matter, assuming that collagen weighed 7.14 times the measured hydroxyproline weight (Etherington & Sims, 1981) and had a molecular weight of 300 000. Five repetitions of each sample were made.

Insoluble collagen was measured according to the method of Bonnet and Kopp (1992). Five minced samples (3 g) were added to boiling tubes containing 0.02 M Tris (pH 7.4; 15 ml) and let stand for 2 h. The tubes were then heated at 90 °C for 2 h in a waterbath, filtered (pure cotton cellulose filters, Durieux 00B) and washed with water (100 ml) which had been preheated to 50 °C. The solids with filter paper were then added to boiling tubes and the hydroxyproline content measured as above (the solids replacing the minced sample in the above method). Soluble collagen content was calculated by difference.

2.2.8.2. IMCT and trypsin digests. The filtrate (100 μl) removed just prior to cellulose chromatography was diluted with water (900 μl). Three repeats were then taken from this filtrate (100 μl each) and further diluted

with water (900 μl), these being the solutions to be assayed. The hydroxyproline concentration of the IMCT samples was determined by the method of Neuman and Logan (1950). Absorbance was read at 558 nm. Collagen content was estimated, assuming that collagen weighed 7.14 times the measured hydroxyproline weight (Etherington & Sims, 1981) and had a molecular weight of 300 000.

The hydroxyproline content of the trypsin digests was obtained by diluting the digest (20 μl) in water (980 μl) directly into the 25 ml test tube. The method for IMCT samples was then used beginning with the addition of 0.01 M $CuSO_4$ (1 ml).

2.2.9. *Intramuscular fat content*

The analysis of intramuscular fat content was undertaken according to the ISO 1444-1973 method on the meat samples from Gb and St.

2.2.10. *Statistical treatment*

Soluble collagen data, expressed as a percentage, was transformed to the arcsine of the square root prior to statistical analyses. The effects of animal type (DM, heterozygous and normal) and muscle (Gb, St and PP) were analysed by analysis of variance (ANOVA) and detected by least square means test for multiple means comparison using the General Linear Models (GLM) procedure of SAS (1996). Age was used as a covariant.

3. Results

3.1. *HPLC analyses—repeatability*

The means and coefficients of variation (CV) of crosslink concentrations from 3–4 repeats of HPLC analyses are presented in Table 1. The repeats are from samples of the same piece of IMCT for each muscle, each repeat being taken independently through all the stages of extraction, purification, chromatography and peak integration. The average CVs for the crosslinks ranged from 10 to 21% and, in general, the CVs were less than 25%. The CVs of the pyridinoline in muscles from normal animals appeared lower than those in the DM animals. Analysis by muscle showed that DM St CVs appeared greater than those for the other muscles, ranging from 17 to 41%, and with an average CV of 29% (compared to averages from 10 to 17% for the other muscles).

It is not within the scope of this work to determine the statistical probability of detection of differences or even the number of samples required to have a significant difference between samples at a given probability level. The repeats here are limited in number and do not account for animal variation for a given muscle type. These repeats were undertaken merely to illustrate the variability within a sample of connective tissue, arising as a consequence of both intra-sample variability and, more particularly, in view of the process undertaken to obtain the concentration values. There are many steps in the process from the relatively large scale IMCT extraction using half a kilogram of meat to crosslink concentration estimation on a milligram scale, each step introducing experimental error. Furthermore, some of the chromatographic peaks obtained are relatively small and not clearly defined, in particular the DHLNL and HLNL peaks. While the method is carefully undertaken, ensuring minimal introduction of error, an element of subjectivity remains in terms of defining these peaks for crosslink quantification as is perhaps reflected in the greater CVs for HLNL compared to the more clearly defined peaks of HHMD and pyridinoline.

The repeatability of the chromatographic procedure was also tested, running repeats of the same final solution

Table 1
Means and coefficients of variation (CV) of crosslink concentrations measured by HPLC for IMCT repeats

	DM Gb	DM St	DM PP	Normal Gb	Normal St	Normal PP	Mean	Standard deviation
Number of repeats	3	3	3	4	4	4		
Mean concentration (mol/mol collagen)								
HLNL	0.036	0.068	0.029	0.045	0.065	0.045		
DHLNL	0.104	0.086	0.073	0.055	0.072	0.090		
HHMD	0.205	0.254	0.106	0.259	0.249	0.271		
Pyridinoline	0.238	0.190	0.197	0.199	0.137	0.205		
CV (%)								
HLNL	19	41	17	22	17	11	21	10
DHLNL	17	28	10	24	11	10	17	8
HHMD	12	28	12	13	15	8	15	7
Pyridinoline	12	17	11	7	4	9	10	4
Mean CV (%)	15	29	13	17	12	10		
Standard deviation CV (%)	4	10	3	8	6	1		

through the HPLC. These repeats were made randomly throughout the study and gave chromatographic profiles which were virtually identical for the same solutions.

3.2. Crosslink concentrations on a collagen basis—muscles combined

Means and standard deviations of the crosslink (per mol collagen), and total and soluble collagen concentrations are presented in Tables 2 and 3 for the three muscles combined. The means and standard deviations of the ages for each group of animals are also given in Table 2. The ANOVA, using age as a covariate, showed that there was no significant effect of age (for the range used here) on any of the crosslink or collagen concentrations.

The ANOVA did show that the animal type significantly affected total collagen, pyridinoline, DHLNL and HHMD concentrations (Tables 2 and 3). The muscle type had significant effects on pyridinoline, HHMD and HLNL concentrations only ($P<0.001$) and so the muscles were further analysed on an individual basis. Neither animal nor muscle type affected EC or soluble collagen concentrations. No animal type/muscle interactions were observed.

Examination of the least square means for animal type shows that both measures of total collagen content decreased significantly from normal to heterozygous to DM animals. The mean collagen content was 31–36% lower in the DM animals than the normal and 21–27% lower than in the heterozygous animals. The DHLNL, HHMD and pyridinoline concentrations in the DM animals were significantly different from those in the normal and heterozygous animals. These crosslink concentrations in the heterozygous animals were not significantly different from those in the normal animals. More specifically, the mean pyridinoline concentration in the DM animals was 22% greater than in the normal and 16% greater than in the heterozygous animals. The mean DHLNL concentration was greater by 14 and 27% in the DM than the normal and the heterozygous animals, respectively. The mean HHMD concentration was 25% lower in the DM animals than either the normal or heterozygous animals.

3.3. Crosslink concentrations on a collagen basis—individual muscles

Means and standard deviations of the crosslink (per mol collagen), and total and soluble collagen concentrations are presented in Tables 2 and 3 for the individual muscles. The animal type had a significant effect on the total collagen content for all three muscles and for HHMD concentrations of the St muscle (Tables 2 and 3). The animal type did not significantly affect the concentrations of the other crosslinks or soluble collagen.

Table 2
Means and standard deviations (S.D.) of total and soluble collagen contents[a]

	Normal ($n=13$)		Heterozygous ($n=8$)		DM ($n=10$)		Animal type significance[b]
	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Muscles combined							
Soluble collagen (% total collagen)	19	8	22	7	25	8	
Total collagen (mg/g wet meat)	11.7a	2.1	10.3b	1.3	8.1c	1.7	***
Total collagen (% fat free dry matter)	4.7a	0.9	4.1b	0.6	3.0c	0.5	***
Gb							
Soluble collagen (% total collagen)	20	9	23	8	29	9	
Total collagen (mg/g wet meat)	11.3a	2.5	10.3a	1.7	8.0b	1.9	**
Total collagen (% fat free dry matter)	4.7a	1.0	4.1b	0.7	3.1c	0.8	***
St							
Soluble collagen (% total collagen)	18	9	23	7	23	7	
Total collagen (mg/g wet meat)	11.3a	1.8	10.1a	1.3	7.3b	0.6	***
Total collagen (% fat free dry matter)	4.7a	0.7	4.1b	0.6	2.9c	0.2	***
PP							
Soluble collagen (% total collagen)	19	8	20	6	23	7	
Total collagen (mg/g wet meat)	12.3a	2.0	10.6a	1.1	8.9b	2.1	**
Animal age (months)	19	3	20	2	21	3	

[a] Within rows, means with differing letters are significantly different at $P<0.05$.
[b] Where * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

The least square means analyses showed that total collagen (% fat free dry matter) was lowest in the DM animals, greatest in the normal animals and intermediate in the heterozygous animals. On a gramme of wet meat basis, the total collagen content was significantly lower in the DM animals than the in normal animals. However, the values for the heterozygous animals were not significantly different from those of the normal animals. In the St muscle, the mean HHMD concentration was significantly lower in the DM animals than the heterozygous or normal animals, by 32%.

3.4. Crosslink concentrations on a meat basis

Means and standard deviations of the crosslink concentrations (per g wet meat) are presented in Table 4. The ANOVA showed that age had no significant effect on the crosslink concentrations. For all muscles combined, the ANOVA did show that animal type significantly affected HLNL, HHMD and EC concentrations. Furthermore, concentrations of HLNL, HHMD and pyridinoline were affected by muscle type ($P<0.035$, 0.001 and 0.0001, respectively) and DHLNL, by animal type/muscle type interactions. The data were therefore further analysed by individual muscle.

Analysis by individual muscle showed that the animal type was a significant factor for HHMD and EC concentrations for all three muscles. The concentrations of HLNL and DHLNL in the St were also significantly affected by the animal type. No animal type/muscle interactions were observed.

Examination of the least square means showed that the crosslink concentrations, which were significantly different in the DM animals, were lower than those in the normal animals. Crosslink concentrations in the heterozygous animals were intermediate, but not always significantly different from those in the normal or DM animals.

4. Discussion

The muscles used in this study were selected because they have relatively high quantities of intramuscular

Table 3
Means and standard deviations (S.D.) of crosslink concentrations (per mol collagen)[a]

Crosslink (mol/mol collagen)	Normal ($n=13$)		Heterozygous ($n=8$)		DM ($n=10$)		Animal type significance[b]
	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Muscles combined							
HLNL	0.047	0.015	0.048	0.014	0.043	0.012	
DHLNL	0.069a	0.022	0.062a	0.017	0.079b	0.020	*
HHMD	0.25a	0.09	0.25a	0.10	0.19b	0.04	**
Pyridinoline	0.18a	0.04	0.19a	0.04	0.22b	0.05	**
EC	0.39	0.05	0.37	0.03	0.36	0.05	
Gb							
HLNL	0.039	0.011	0.042	0.017	0.038	0.010	
DHLNL	0.062	0.021	0.070	0.020	0.084	0.021	
HHMD	0.24	0.08	0.28	0.09	0.20	0.03	
Pyridinoline	0.19	0.04	0.20	0.03	0.23	0.05	
EC	0.39	0.04	0.37	0.03	0.34	0.03	
St							
HLNL	0.060	0.013	0.058	0.010	0.050	0.006	
DHLNL	0.079	0.023	0.061	0.014	0.071	0.018	
HHMD	0.31a	0.09	0.31a	0.10	0.21b	0.04	*
Pyridinoline	0.15	0.03	0.16	0.03	0.18	0.04	
EC	0.38	0.06	0.37	0.03	0.38	0.05	
PP							
HLNL	0.041	0.011	0.045	0.010	0.042	0.015	
DHLNL	0.066	0.021	0.055	0.015	0.080	0.022	
HHMD	0.20	0.07	0.17	0.03	0.16	0.04	
Pyridinoline	0.20	0.05	0.21	0.05	0.24	0.06	
EC	0.39	0.07	0.37	0.03	0.34	0.05	

[a] Within rows, means with differing letters are significantly different at $P<0.05$.
[b] Where * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

collagen and they exhibit increased muscle mass in double-muscled (DM) animals compared to the same muscles in normal animals (Boccard, 1981). The collagen crosslinking in these muscles was measured in extracts of IMCT, comprised largely of perimysium using the methods of extraction employed here (Light & Champion, 1984), and being the collagen fraction of greatest interest in terms of meat quality characteristics (Light, Champion, Voyle, & Bailey, 1985).

The average total collagen content in the muscles from the DM animals was about 65% of that in the normal animals and is consistent with other studies (for example, Bailey et al., 1982; De Smet, Claeys, Buysse, Lenaerts, & Demeyer, 1998; Hanset et al., 1982; Lawrie et al., 1964; Uytterhaegen et al., 1994). It was also observed that heterozygous animals had intermediate levels of collagen content (about 87% of the content in the normal animals). Using a larger population of animals, of which the animals in this study are a subset, De Smet et al. (2000) observed that carcass compositional data of heterozygous animals were generally intermediate between DM and normal animals, and often closer to the normal animals. The trends in levels of crosslinks and collagen concentrations in heterozygous animals observed are consistent with the reported conformational data.

No significant effect of animal type on collagen heat-solubility was observed. This finding is similar to that of Kopp and Bonnet (1981) who studied muscles from 2-year-old DM and normal Charolais bulls. In contrast, however, is the finding of Boccard, Dumont, and Schmitt (1967) of higher collagen heat-solubility in muscles from 3-year-old DM steers compared to normal animals. It was suggested that less polymerisation of the collagen (due to differences in mechanical activity or physiological maturity) was one of the factors resulting in its higher heat-solubility in DM animals.

In the present work, combining all three muscles showed that the quantities of DHLNL and pyridinoline (per mol collagen basis) were greater in the DM animals than in the normal animals; the HHMD concentration was smaller. Bailey et al. (1982) observed similar differences for DHLNL, as well as a higher proportion of HLNL in the collagen from the DM cattle. The higher

Table 4
Means and standard deviations (S.D.) of crosslink concentrations (per g wet meat)[a]

Crosslink (nmol/g wet meat)	Normal ($n=13$)		Heterozygous ($n=8$)		DM ($n=10$)		Animal type significance[b]
	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Muscles combined							
HLNL	1.8a	0.6	1.6a	0.5	1.2b	0.4	***
DHLNL	2.6	0.9	2.1	0.7	2.1	0.8	
HHMD	9.4a	3.5	8.6a	3.2	5.1b	1.2	***
Pyridinoline	6.9	2.0	6.6	1.5	5.9	2.2	
EC	15a	4	13b	2	10c	2	***
Gb							
HLNL	1.4	0.4	1.4	0.6	1.0	0.4	
DHLNL	2.3	0.6	2.4	0.8	2.3	0.7	
HHMD	8.9a	3.2	9.4a	3.0	5.4b	1.4	*
Pyridinoline	7.0	2.1	7.0	1.6	6.4	2.9	
EC	15a	4	13a	3	9b	3	***
St							
HLNL	2.3a	0.6	1.9a	0.5	1.2b	0.2	***
DHLNL	3.0a	1.0	2.1b	0.6	1.7b	0.4	**
HHMD	11.4a	3.4	10.4a	3.5	4.9a	0.9	***
Pyridinoline	5.6	1.0	5.5	1.4	4.4	1.1	
EC	14a	3	12a	2	9b	2	***
PP							
HLNL	1.7	0.4	1.6	0.3	1.2	0.5	
DHLNL	2.7	0.8	1.9	0.5	2.4	1.0	
HHMD	8.0a	3.3	6.0b	0.9	4.8b	1.4	**
Pyridinoline	8.2	1.7	7.4	0.9	6.8	1.4	
EC	16a	4	13b	2	10b	3	**

[a] Within rows, means with differing letters are significantly different at $P<0.05$.
[b] Where * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

concentrations of reducible crosslinks (DHLNL and HLNL) led these workers to conclude that the collagen from the DM animals was less mature than that from normal animals. The immaturity of the DM animals was also apparent by the higher proportions of oleic acid in fat depots compared to normal animals.

In an earlier study, Shimokomaki, Elsden, and Bailey (1972) observed that DHLNL and HLNL concentrations from bovine intramuscular collagen increased from fœtus to about 18 months in normal animals, after which an exponential decrease was observed until the crosslinks were virtually absent at maturity. Another reducible compound, probably HHMD (compare with Avery, Sims, Warkup, & Bailey, 1996), followed a similar profile. Using goat perimysial collagen, Horgan et al. (1991) observed that concentrations of Erlich chromogen (EC) showed similar changes with age, increasing to a maximum concentration at about 1 year, and thereafter decreasing exponentially. The ages of the animals in the present study ranged from 16 to 25 months. Because crosslink concentrations show age-related changes, in particular, maximal levels at the approximate ages of these animals, it is not possible to draw conclusions about the relative maturities of the collagen using these crosslinks as it is not known on which side of the peak maxima the values lie.

In contrast to the other crosslinks, pyridinoline concentration only increases with animal age (Horgan et al., 1991). The age range in this study was sufficiently small that no significant increase in pyridinoline concentration with age was observed. However, the greater concentrations of pyridinoline observed in muscles from DM animals could indicate a more mature collagen than that in normal animals of the same age. This finding is in opposition to that of Bailey et al. (1982), as mentioned above. Furthermore, in terms of animal maturity, frequent occurrences of delays in puberty in DM heifers (Vissac, Perreau, Mauleon, & Ménissier, 1974) and DM males [Bizard (1964) referenced in Ménissier, 1982; Dimitropoulos, 1972; Sanchez Garcia, 1976] have been reported. It is therefore suggested that physiological maturity of the animal is not directly related to, or solely responsible for, collagen maturation in these animals.

Not surprisingly, the trends in crosslink concentrations, on a gramme of wet meat basis, were not the same as those observed on a mole of collagen basis. The greater concentrations of pyridinoline and DHLNL observed on a per mole of collagen basis in DM animals, compared to normal, were likely negated by the lesser amounts of collagen in the muscles of the DM animals.

The relative contributions of the individual crosslinks to the collagen characteristics, such as its strength and thermal stability, are not known and there is debate as to the existence, or the nature, of the relationship between collagen crosslinks and meat toughness. Dransfield (1977) demonstrated that muscles from the same animal with relatively high collagen contents are tougher than those with less collagen. Horgan et al. (1991) also concluded that the major determinant of the collagen toughness, of different meat cuts, appears to be the quantity, not the quality, of the intramuscular collagen. However, Light et al. (1985) found that a combination of collagen crosslinking and differences in collagen content and perimysial fibre diameter accounted for the variations in toughness. Other workers have observed that, in general, muscles with high collagen content (for example, *m. biceps femoris* or *m. soleus*) possess higher crosslink concentrations (per unit of collagen) than muscles with low collagen content (such as, *m. longissimus dorsi*) in normal animals (Horgan et al.; Light et al.; Shimokomaki et al., 1972). As a consequence of the lower levels of collagen in DM animals, lower concentrations of all crosslinks in the collagen might therefore be expected from DM animals when comparing the same muscles to those in normal animals. However, this was not observed for all crosslinks, either in the present study or the work of Bailey et al. (1982).

In age-related toughening of meat it has been suggested that it is the quality of collagen which is responsible for progressive toughening of meat as animals grow older (Bailey, 1990). The progressive toughening that occurs in meat from animals of increasing age and maturity has been related to the progressive maturation of muscle collagen (Bailey, 1972; Bailey & Light, 1989; Bailey, Restall, Sims, & Duance, 1979; Bailey & Shimokomaki, 1971; Bailey & Sims, 1977; Shimokomaki et al., 1972) and the relative proportion of non-reducible crosslinks (often referred to as mature crosslinks and including pyridinoline, histidino-hydroxylysinorleucine (HHL) and perhaps also Erlich chromogen) is thought to be important in determining the texture of cooked meat (Bailey, 1990; Bailey & Light). However, Horgan et al. (1991) observed that the increased thermal stability of goat intramuscular collagen with age, from birth to 14 years, could not be explained simply in terms of the crosslinks measured. More recently, Avery et al. (1998) were unable to show a significant correlation between pyridinoline concentration in bovine longissimus muscle and variations in meat tenderness from animals aged from 400 to 800 days. In the present work, a significantly greater concentration of pyridinoline was observed in the collagen of DM animals compared to normal animals, yet no significant difference in the heat solubility of the collagen was found.

The implication of the lower intramuscular collagen content, and therefore reduced levels of crosslinks, in meat from DM cattle, has been a lower toughness. Indeed, compared to normal animals, more tender meat from DM cattle has been reported by several workers

(Bailey et al., 1982; Boccard, 1981, 1982; Bouton, Harris, & Shorthose, 1982). However, contradictory to these studies, Uytterhaegen et al. (1994) reported that cooked meat from DM cattle had a significantly higher shear force than meat from normal animals and De Smet et al. (1998) observed no significant differences in shear force of cooked meat between the two groups of animals. Uytterhaegen et al. (1994) suggested that the lower toughness from reduced collagen content in meat from DM animals was overridden by a decreased myofibrillar tenderisation, resulting in overall tougher meat. In a complementary study, using the Gb and St from the present experiment, significant differences in cooked shear force were observed for Gb only (Ngapo et al., in press), but no relationship between collagen or crosslinks concentrations and shear force was established.

It is evident from this discussion that relatively little is known about factors which affect crosslinking patterns in muscle collagen. Furthermore, there is a large area of unexplored territory, in terms of collagen crosslinking in meat, in particular from DM animals and effects on the tenderness of the meat in these animals. While one can speculate, it is not possible to draw conclusions as to the reasons for differences in crosslinks from the available data, or the effects on meat texture. This study has shown that differences do exist in crosslink concentrations between collagen from DM and normal animals, but that the differences in the total content of collagen appear so great as to negate these differences in meat. The differences in crosslinks warrant further investigation in terms of implications for the texture of the meat from these animals.

5. Conclusions

Total collagen content significantly decreased as the number of mh alleles increased. Collagen solubility was not affected by animal type. The concentrations of three crosslinks, on a collagen basis, were found to be significantly different between the DM and normal animals. Greater quantities of pyridinoline per mol of collagen in meat from DM animals suggest a more mature collagen in the muscles of these animals than in normal animals. Concentrations of crosslinks in heterozygous animals were intermediate with respect to DM and normal animals, but not consistently significantly different from either. Per gramme of meat, the greater quantities of collagen in the normal animals resulted in significantly fewer of crosslinks in the DM animals than normal animals, except for pyridinoline and DHLNL, which were not significantly affected by animal type. The differences in crosslinks warrant further investigation in terms of implications for the texture of the meat from these animals.

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