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Effect of Age on Structural Properties of Intramuscular Connective Tissue, Muscle Fibre, Collagen Content and Meat Tenderness in Pig *longissimus lumborum* muscle*

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Changes in the structure and properties of the intramuscular connective tissue, muscle fibre size, collagen content and meat tenderness of *m. longissimus lumborum* during growth was studied in 45 Polish Large White (PLW) pigs slaughtered at 90, 150 and 210 days of age. The results show that the endomysial sheath in *m. longissimus lumborum* consists of collagen fibrils of wavy appearance which run in all directions and form a loose network. The arrangement of collagen fibrils in the *endomysium* and *perimysium* becomes denser and more regular with increasing age of pigs. In addition, the increase in *endomysium* and *perimysium* thickness was paralleled by a significant increase in muscle fibre diameter, as well as an increase in shear force value with chronological aging. In contrast, the percentage of collagen area in muscle structure and the amount of total collagen and amount of soluble collagen decreased gradually with age of pigs. In conclusion, the structural changes in the arrangement of collagen fibres in the architecture of intramusclular connective tissue, as well as the decrease in soluble collagen content in *m. longissimus lumborum* during growth of pigs are important factors influencing shear force value, and thus raw meat tenderness.

Key words: Connective tissue, collagen, tenderness, *m. longissimus lumborum*, pig.

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One of the most important meat traits for consumer satisfaction is meat tenderness. Meat tenderness depends on several factors such as muscle fibre composition, sarcomere length, pH, intramuscular fat content and rate of tenderization (ESSEN-GUSTAVSSON 1993; KEMP *et al.* 2010). An important role in shaping meat tenderness is also played by connective tissue, which has been shown to be a critical factor in meat tenderness (BROOKS & SAVELL 2004; TORRESCANO *et al.* 2003; ŻOCHOWSKA *et al.* 2005; OSHIMA *et al.* 2009; CHRISTENSEN *et al.* 2011). In all muscles the connective tissue is divided into three hierarchal domains, namely the *endomysium*, *perimysium* and *epimysium*. These layers play significant roles in maintaining the structural integrity of muscle fibres and in completing muscle function as an active locomotor system. Because the *epimysium*, a dense connective tissue layer which is continuous with the tendons, is tough to eat, it is removed from meat. Therefore, the tenderness of meat originates mainly from the mechanical properties of the *endomysium* and *perimysium*. In general, the connective tissue is composed of collagen, elastin, proteoglycans and glycoproteins. In mammalian muscle, type I, III, IV, V and VI collagens have been detected, however the major types are type I and III (LISTRAT *et al.* 1999; NAKAMURA *et al.* 2003). Earlier studies indicated that the morphology, composition and amount of intramuscular connective tissue depends on animal genotype, nutrition, and muscle type (NAKAMURA *et al.* 2003; TORRESCANO *et al.* 2003; DAS *et al.* 2009; NISHIMURA *et al.* 2009; OSHIMA *et al.* 2009; CHRISTENSEN *et al.* 2011). Moreover, much evidence shows that chemical and structural collagen contents change with advanced age (NISHIMURA *et al.* 1996; FANG *et al.* 1999; VELLEMAN *et al.* 2003). However, no work has been reported on the structural changes in the architecture of the intra-

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muscular connective tissue during development of pig *longissimus* muscle, the most important muscle that determines carcass quality. Therefore, the aim of this study was to determine structural changes in the intramuscular connective tissue, muscle fibre, collagen content and meat tenderness of *m. longissimus lumborum* during growth of pigs.

Material and Methods

The study used 45 Polish Large White (PLW) pigs slaughtered at 90, 150 and 210 days of age (15 animals in each age group). Animals were kept from 60 to 210 days of age at the Agricultural Production Cooperative in Kędzierzyn-Koźle, Poland, and fed a complete diet according to Polish Feeding Standards (1993). All pigs were reared under the same environmental and production regime. Pigs were slaughtered in a commercial slaughterhouse according to routine procedure. Feed was withdrawn 12 h before slaughter but water was freely available in lairage. Immediately after slaughter muscle samples from the *longissimus lumborum* muscle were taken from the right carcass side at the level of the 3rd and 5th lumbar vertebra and used immediately for measurements and preparations.

For immunohistochemical analysis, muscle samples were cut into 1 cm^3 pieces, parallel to the muscle fibres, and frozen (45 min *postmortem*) in isopentane that was cooled using liquid nitrogen. Transverse sections (10-μm thick) were cut at -20^{o}C in a cryostat (Slee MEV, Germany). In order to determine the connective tissue (collagen fibres), frozen sections were immunohistochemically stained with rabbit polyclonal antibodies against type I-V collagens (ab27117, Abcam, 1:1000 diluted). The reaction was visualized using the NovoLink^{TM} Polymer Detection System (Leica, Germany), according to the manufacturer's instructions. Finally, all sections were dehydrated in a graded series of ethyl alcohol, cleared in xylene and mounted in DPX mounting medium (Fluka, Buchs, Switzerland). A minimum of 10 images (each 4 mm^2) were counted in each section using a Zeiss Axio Vision A.2 light microscope. The thickness of the *endomysium* and secondary *perimysium*, diameter of muscle fibre, and percentage of the collagen area (total *perimysium*+*endomysium*) in muscle structure were determined using an image analysis system (Axio Vision Rel. 4.8.3, Zeiss, Germany). A minimum of 100 fibres were examined from each cross-section to determine muscle fibre diameter.

To examine the collagen fibre architecture in the intramuscular connective tissue of *m. longissimus lumborum*, muscle samples collected 45 min *postmortem* were fixed in 2.5% glutaraldehyde solution in phosphate buffer 0.1 M (pH 7.2) for two days at 4°C. After this period, muscle samples were washed 3 times in 0.1 M PBS solution for 2 hours, and next muscle fibres were macerated in 2N NaOH solution for 6-8 days at room temperature according to the procedure described by IWAMOTO *et al.* (2001) with small modifications. After rinsing the preparations in distilled water for 5-8 days at room temperature (until the samples were pale and transparent), tissues were moved to 1% tannic acid (in PBS 0.1 M) for 2 h. Thereafter, they were washed in PBS 0.1 M for 6 hours and postfixed with 1% OsO_4 for 2 h. After washing in 0.1 M PBS for 1 hour samples were dehydrated in a graded series of ethyl alcohol, and were placed in t-butyl alcohol. Thus prepared histological material was mounted on holders, subjected to CO_2 critical point drying, and coated with gold in a sputtering device (Jeol JFC 1100E). The collagen architecture of the muscles was examined under a scanning electron microscope JSM-5410 (a gift from the Foundation for Polish Science SUBIN 94) at an accelerating voltage of 15 kV at the Scanning Microscopy Laboratory of the Faculty of Biology and Earth Sciences of the Jagiellonian University in Kraków.

The total amounts of collagen and soluble collagen content were determined in *m. longissimus lumborum* samples collected about 45 min *postmortem*. The total collagen content in muscle samples was determined after 24 h hydrolysis of 300 mg of meat with 25 cm^3 6 M HCl at 100°C using a modified method by REICH (1970). The hydrolysate was clarified with active carbon, neutralized with 10 M and 1 M NaOH, and diluted with distilled water to 250 cm^3. Hydrolysate (4 cm^3) and 2 cm^3 of chloramide T solution (1.41 g chloramines T, 10 cm^3 distilled water, 10 cm^3 *n*-propanol and 80 cm^3 citric buffer at pH 6.0) were mixed in a test tube and left for 20 min at room temperature. Next, 2 cm^3 of 4-dimethyl-aminobenzaldehyde (*p*-DABA) solution (10 g *p*-DABA, 35 cm^3 $HClO_4$-60% and 65 cm^3 isopropanol) was added. The solutions were shaken and heated at 60°C for 20 min. The samples were cooled for 5 min in tap water and the absorbance was measured at 558 nm. The amount of hydroxyproline was determined from a standard curve. The collagen content was calculated from hydroxyproline content using the coefficient 7.25. Soluble collagen was extracted according to the procedure described by LIU *et al.* (1994). Samples of 6 g of chopped meat were homogenized at 12,000 rpm with 24 cm^3 Ringer's solution diluted with distilled water at 1:3. Homogenates were heated for 70 min at 77°C, and centrifuged (2300×g, for 30 min). The sediments were mixed

with 24 cm^3 of diluted Ringer's solution and centrifuged again. Next the sediments were dried at 105°C and 100 mg of the dried mass was hydrolyzed with 25 cm^3 6 M HCl. The collagen content of the sediment was determined as described for total collagen. Soluble collagen was calculated as the difference between the total and insoluble collagen contents and expressed as percentage of total collagen.

For measurements of tenderness, and to avoid cold-shortening, raw samples of meat (80 g) were taken parallel to muscle fibre orientation at the level of 3rd and 4th lumbar vertebra and kept at room temperature until measurements of tenderness (about 2 h *postmortem*) were performed. Shear force value, in 5 raw samples from each animal, was determined as the maximum force (kg/cm^2) perpendicular to the fibres using INSTRON 5542 equipped with a Warner-Bratzler blade.

Differences among the age groups of pigs were assessed using analysis of variance (General Linear Model procedure), and tested for differences by the Tukey test. The probability of $P<0.05$ was considered statistically significant. The data were expressed as least squares means (LSM) ± standard error of the mean (SEM).

Results and Discussion

The results of measurements of the connective tissue, muscle fibres, the percentage of collagen area and of the total and soluble collagen content in *m. longissimus lumborum* of pigs, depending on age, are shown in Table 1, while the structure of intramuscular connective tissue is presented in Fig. 1.

Analysis of intramuscular connective tissue under light and scanning electron microscopes demonstrated that each muscle fibre is surrounded by a thin, honeycomb-like layer of connective tissue (*endomysium*). In the youngest group of 90-day-old pigs, the *endomysial* sheath in *m. longissimus lumborum* consists of collagen fibrils of wavy appearance which run in all directions and form a loose network structure. The advancing age of the pigs was paralleled by a gradual increase in *endomysium* thickness and an increase in collagen fibre density, to the effect that in the oldest group of pigs slaughtered at 210 days of age, the orientation of collagen fibrils in the *endomysium* became more perpendicular to the axis of muscle fibres, and fibrils bind ever more closely with each other. Moreover, in agreement with previous reports (FANG *et al.* 1999) the current study also showed that in *m. longissimus lumborum* of 210-day-old pigs, muscle fibres (of lower diameter) are located in the muscle bundle centre surrounded by thicker *endomysium* compared to the *endomysium* surrounding muscle fibres located in the bundle perimeter. It is well established that muscle fibre orientation in *m. longissimus lumborum* of pigs is highly specific, with red muscle fibres (I) located mainly in the muscle bundle centre and surrounded by intermediate fibres (IIA) and the outermost white fibres (IIB), which suggests that type I fibres have thicker *endomysium*. These suppositions may be confirmed by KOVANEN *et al.* (1984), who in isolated muscle fibres of rat *m. gastrocnemius* showed higher collagen concentrations in type I (slow twitch) compared to type II (fast twitch) muscle fibres. Similarly, in a recent review of intramuscular connective tissue LEPETIT (2009) reported that white muscles contain collagen at a lower concentration than red muscles. In the skeletal muscle, muscle fibres are grouped into bundles surrounded

Table 1

Least squares means (LSM) and standard error of the mean (SEM) of the physical, chemical and structural characteristics of *m. longissimus lumborum* depending on the age of pigs

Traits	Age (days)			SEM
	90	150	210	
Thickness of endomysium (μm)	2.654^{a}	3.62^{b}	5.25^{c}	0.24
Thickness of perimysium (μm)	22.16^{A}	31.67^{B}	41.53^{C}	0.82
Muscle fibre diameter (μm)	43.97^{A}	56.2^{B}	67.8^{C}	0.46
Collagen area (%)	18.44^{a}	17.32^{b}	16.25^{c}	0.23
Collagen content (mg/g)	5.48^{a}	4.69^{b}	3.85^{c}	0.19
Soluble collagen (%)	22.74^{a}	18.23^{b}	13.65^{c}	0.63
Shear force (kg/cm^2)	3.42^{A}	5.18^{B}	6.87^{C}	0.31

LSMs marked with different capital letters (A, B) differ significantly at $P<0.01$; LSMs marked with different small letters (a, b) differ significantly at $P<0.05$.

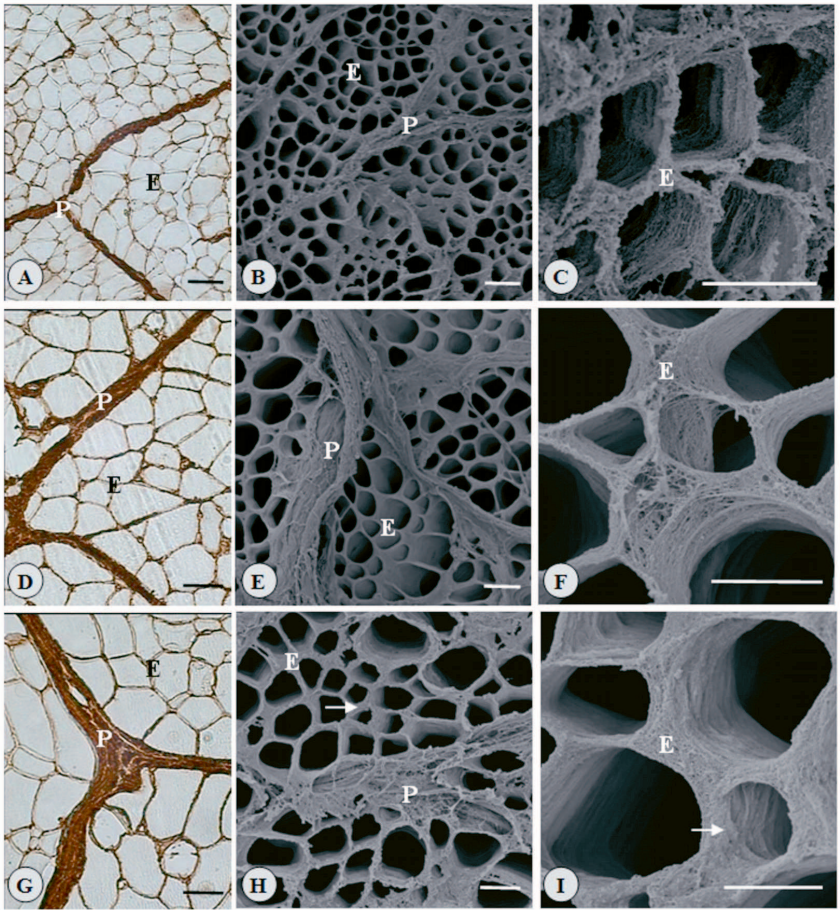

Fig. 1. Exemplary cross section of *longissimus lumborum* muscle of Polish Large White pigs slaughtered at 90 (A, B, C), 150 (D, E, F) and 210 (G, H, I) days of age: immunohistochemical detection of type I-V collagen (A, D, G); *SEM* elektronograms (B, C, E, F, H, I); E – *endomysium*; P – *perimysium* (secondary); arrows – thicker *endomysium* surrounded smaller muscle fibre located in the muscle bundle centre. Scale bar = 50 Fm.

by connective tissue – the *perimysium*. The *perimysium* is classified into two domains, namely the primary (thin) *perimysium* which surrounds a group of muscle fibres to form a primary muscle bundle, and the secondary *perimysium* (thick) which surrounds a group of primary muscle bundles. In the present study, secondary *perimysium* is composed of several layers of tightly bundled collagen fibres that generally run in waves parallel to one another and concentrically around muscle bundles. These structural arrangements are similar to those noted earlier in skeletal muscle of various animal species (ROWE 1981; NISHIMURA *et al.* 1996; FANG *et al.* 1999; IWAMOTO *et al.* 2001; DAS *et al.* 2009). In addition, as in the case of the *endomysium*, a gradual increase in *perimysium* thickness was also found as pigs grew older. Many earlier studies indicate that both collagen structure and content are related to meat tenderness. NISHIMURA *et al.* (1996) and FANG *et al.* (1999) found that meat tenderness decreases with animal age. The present results also show that shear force value increased significantly during the growth of pigs. On the other hand, the percentage of collagen area in the structure of *m. longissimus lumborum* decreased significantly from 90 to 210 days of age. Similarly, total collagen content and soluble collagen content decreased gradually with age of pigs. The decrease in collagen percentage and content in *m. longissimus lumborum* structure with the advancing age of pigs is associated mainly with a significant increase in muscle fibres. Muscle fibre size is determined by several factors such as genotype (BOCIAN *et al.* 2012; GIL *et al.* 2008), age, body weight, sex and diet (BROCKS *et al.* 1998; DEPREUX *et al.* 2002; MIGDAŁ *et al.* 2004). The increase in muscle fibre diameter, observed in the present study with age, concurs with the results of previous studies (STICKLAND *et al.* 1975; ČANDEK-POTOKAR *et al.* 1999; REHFELDT *et al.* 2000), which showed that in the postnatal period, muscle growth is mainly based on an increase in length and on the diameter of muscle fibres, and not on the increase in their amount. NISHIMURA *et al.* (2009) showed that the total collagen content of skeletal muscle incompletely reflects mechanical properties of the intramuscular connective tissue and that there are some additional factors deter-

mining meat tenderness. ROWE (1981) noted that meat tenderness does not depend on the amount of collagen, but significantly depends on the size and arrangement of collagen fibrils in the intramuscular connective tissue. VELLEMAN *et al.* (2003) demonstrated that *endomysium* and *perimysium* had different functions in muscles. *Endomysium* is responsible for nutrition supply, while *perimysium* functions more for posture maintenance and power transmission (KOVANEN *et al.* 1987). MCCORMICK (1999) noted that *endomysium* accounts for less than 10% of total connective tissue in muscles. Thus, a major proportion in total connective tissue in muscles is represented by *perimysium*, the amount of which largely determines meat tenderness. These conjectures are in agreement with LIU *et al.* (1996), who demonstrated, in various chicken skeletal muscles, that the thickness of the secondary *perimysium* correlates significantly with the shear force value of raw chicken, and they suggest that the structure of the secondary *perimysium* is a major factor determining tenderness of raw chicken. Similarly, AN *et al.* (2010) found that shear force values correlated negatively with the thickness of *endomysium* and positively with the thickness of *perimysium*. FANG *et al.* (1999) revealed that thickening of the *perimysium* in *semitendinosus* muscle is closely related to an increase in the toughness of pork during growth of pigs. BROOKS & SAVELL (2004) noted that *perimysium* thickness would be a poor indicator of Warner-Bratzler shear force in several bovine muscles. Meanwhile, NISHIMURA *et al.* (1996), who analysed structural changes in the intramuscular connective tissue of bovine *semitendinosus* muscle, showed that collagen fibres of the *perimysium* increased in thickness with chronological age of animals and the wavy pattern became more regular. In turn, BAILEY & NICHOLAS (1989) report that the diameter of collagen fibrils is related to the type of collagen molecule which makes up the fibrils, and, significantly, during growth of animals these collagen fibrils become more stable and rigid. This is determined by the formation of covalent intermolecular cross-links of collagen (TANZER 1973) which hinder the natural breakdown and renewal of collagen fibres, and reduce their flexibility and elasticity. As demonstrated by AVERY *et al.* (1996), in young animals the *perimysium* contains a mixture of thermally labile and thermally stable cross-links, whereas the *endomysium* contains thermally stable cross-links. Moreover, these authors showed that as the animal increases in age, the thermally labile, intermediate cross-links are increasingly converted into thermally stable, mature cross-links, which may explain the gradual decrease in soluble collagen level with the age of pigs and the associated increase in shear force, found in the present study. Likewise, GERRARD & GRANT (2003) noted that the extent and type of cross-linking between collagen fibres plays a significant role in shaping meat tenderness. These authors show that with age, the increase in the amount of the cross-linking is paralleled by an increase in insoluble collagen content. This is why younger animals will produce more tender meats than will older animals.

In conclusion, the results obtained indicate that structural changes in the architecture of the intramusclular connective tissue (increase in collagen fibre density and increase in the thickness of both *endomysium* and *perimysium* with age) as well as the decrease in the content of soluble collagen in *m. longissimus lumborum* during growth of pigs are important factors influencing shear force value and thus raw meat tenderness.

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