Journal of Magnetic Resonance **157,** 267–276 (2002)
doi:10.1006/jmre.2002.2600

Changes in Porcine Muscle Water Characteristics during Growth—An *in Vitro* Low-Field NMR Relaxation Study

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Received February 8, 2002; revised June 28, 2002

This study investigates the effects of developmental stage and muscle type on the mobility and distribution of water within skeletal muscles, using low-field ^{1}H-NMR transverse relaxation measurements *in vitro* on four different porcine muscles (*M. longissimus dorsi, M. semitendinosus, M. biceps femoris, M. vastus intermedius*) from a total of 48 pigs slaughtered at various weight classes between 25 kg and 150 kg. Principal component analysis (PCA) revealed effects of both slaughter weight and muscle type on the transverse relaxation decay. Independent of developmental stage and muscle type, distributed exponential analysis of the NMR T_2 relaxation data imparted the existence of three distinct water populations, T_{2b}, T_{21}, and T_{22}, with relaxation times of approximately 1–10, 45–120, and 200–500 ms, respectively. The most profound change during muscle growth was a shift toward faster relaxation in the intermediate time constant, T_{21}. It decreased by approx. 24% in all four muscle types during the period from 25 to 150 kg live weight. Determination of dry matter, fat, and protein content in the muscles showed that the changes in relaxation time of the intermediate time constant, T_{21}, during growth should be ascribed mainly to a change in protein content, as the protein content explained 77% of the variation in the T_{21} time constant. Partial least squares (PLS) regression revealed validated correlations in the region of 0.58 to 0.77 between NMR transverse relaxation data and muscle development for all the four muscle types, which indicates that NMR relaxation measurements may be used in the prediction of muscle developmental stage.

Key Words: **T_2 relaxation; water mobility and distribution; age; protein; fat.**

INTRODUCTION

During development and growth, skeletal muscle characteristics change. Beside the longitudinal and radial growth of the muscle fiber, changes in the concentration of different ions, and noticeably, the amount of water and the protein content in the muscles have been reported (*1–5*). Accordingly, changes in water mobility and distribution as reflected in NMR relaxation can be expected, as observed in other systems, where the protein concentration has been found to affect proton T_2 relaxation (*6–9*). Nevertheless, apart from the study by Hazlewood *et al.* (*10*), hardly any investigations have been carried out with the aim of understanding if changes taking place during growth are accompanied by shifts in water mobility and distribution within the skeletal muscles. This is so even though water mobility and distribution within the skeletal muscles are factors known to be of vital importance for the final quality of pork (*11–15*). Moreover, from a clinical point of view, an understanding of how developmental stages are reflected in NMR relaxation characteristics of the muscles is of interest because of a potential use of NMR relaxation measurements in diagnosis of muscle tissue. Recently, Hatakeneka *et al.* (*16*) investigated how proton T_2 relaxation in human muscles was affected by age in a group of 22- to 76-year-olds. Interestingly they found that the relaxation time increased slightly with increasing age, in particular in elderly (60 to 76-year-olds), which was suggested to be caused by atrophic changes and an increase in extracellular space. However, in that study T_2 relaxation times were determined using only four different echo times, which makes advanced fitting of the T_2 relaxation decay impossible and hereby limits further understanding of which relaxation components are influenced by age. Consequently, further studies are needed to display age-related changes in water states as reflected in NMR T_2 relaxation characteristics and the principal causes for such changes. In order to contribute to a further understanding of age-related changes in NMR T_2 relaxation characteristics of muscle, we have examined how NMR transverse relaxation characteristics in different pig muscles are affected by age. *In vitro* low-field ^{1}H-NMR transverse relaxation measurements were carried out on four different muscles (*M. longissimus dorsi, M. semitendinosus, M. biceps femoris, M. vastus intermedius*) from pigs slaughtered at various weight classes between 25 and 150 kg.

RESULTS

Figure 1 shows the score and loading plots for PCA analysis on NMR relaxation data. The data reveal a tendency to grouping

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1090-7807/02 $35.00

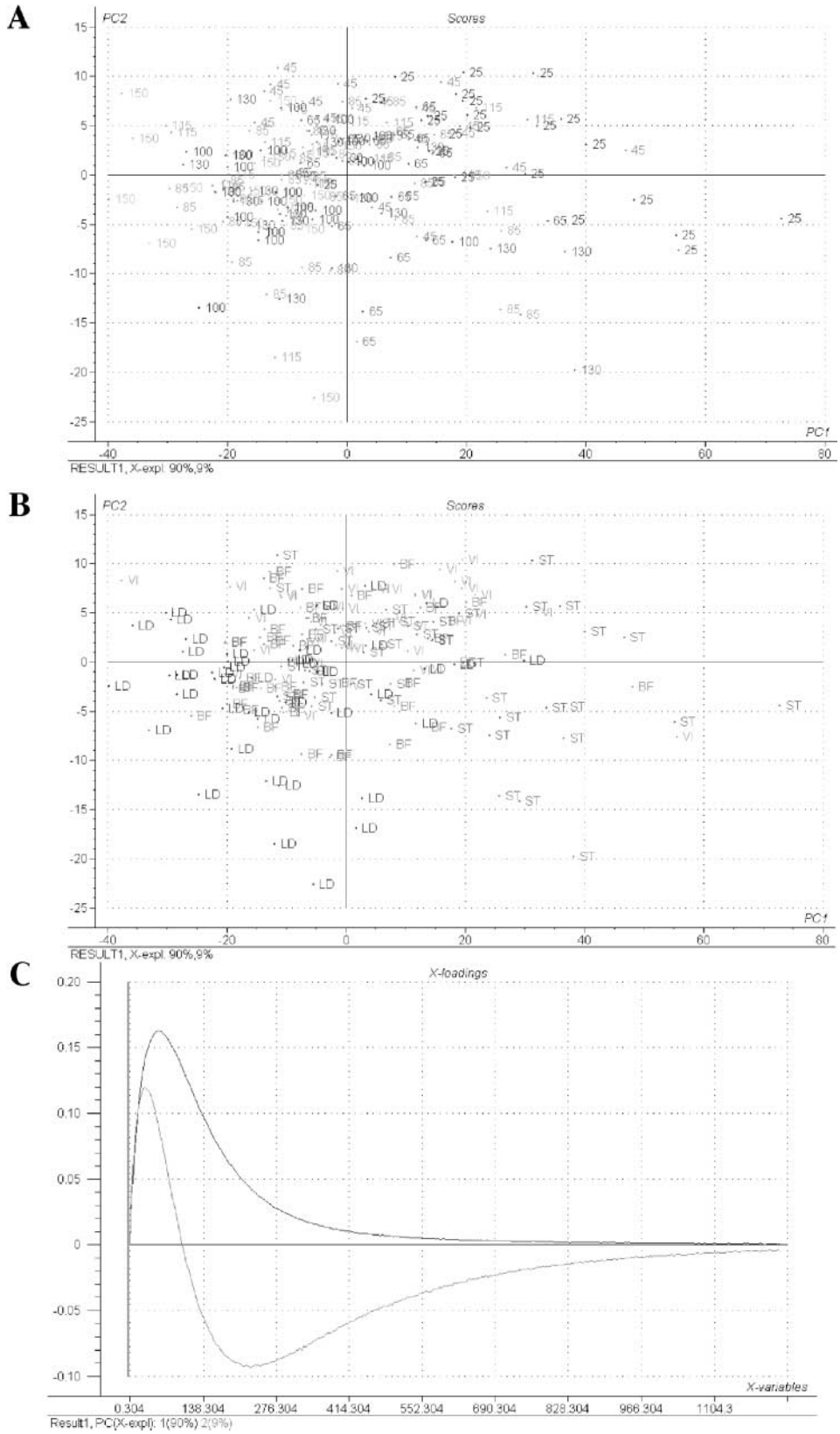

FIG. 1. PCA score plot (PC1 versus PC2) based on NMR transverse relaxation decay: (A) samples marked by weights (25, 45, 65, 85, 100, 115, 130, and 150 kg), (B) samples marked by muscle type (BF = *M. biceps femoris*, LD = *M. longissimus dorsi*, ST = *M. semitendinosus*, VI = *M. vastus intermedius*), and (C) corresponding loading plot.

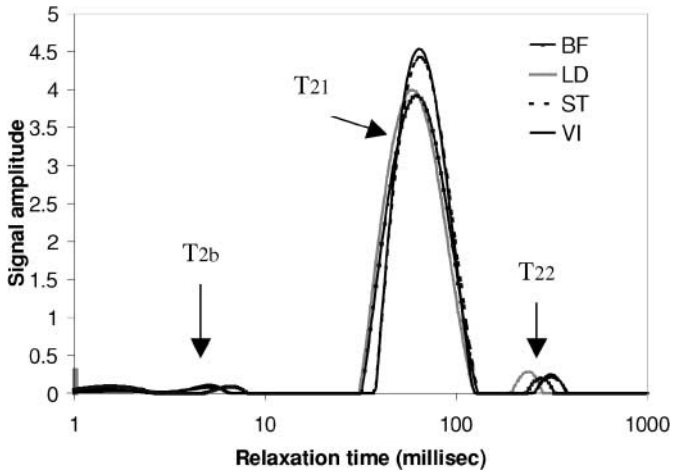

FIG. 2. Representative distribution of T_2 relaxation times for *M. biceps femoris*, *M. longissimus dorsi*, *M. semitendinosus*, and *M. vastus intermedius* muscle samples from a pig killed at a live weight of 25 kg. T_2 data were performed with a τ-value (time between 90° pulse and 180° pulse) of 150 μs. Measurements were performed at 25°C, and data were acquired from 4096 echoes as 16 scan repetitions with a repetition time of 2 s between two succeeding scans.

in both weight classes (Fig. 1a) and muscle types (Fig. 1b), which indicates differences in NMR relaxation characteristics between both weight classes and muscle types. Two principal components could explain 99% of the variance in the NMR relaxation data.

Figure 2 shows typical examples of results obtained upon distributed exponential fitting on T_2 relaxation data from the four different muscles from a representative animal weighing 25 kg. Three components, hereafter referred to as T_{2b}, T_{21}, and T_{22}, were detected in all muscle samples, a fast relaxing component between 1 and 10 ms (T_{2b}), a major component between 45 and 120 ms (T_{21}), and finally a slower relaxing component between 200 and 500 ms (T_{22}).

In Fig. 3 the mean time constants and corresponding proportions of the water population (area) for each relaxation component are given for the different muscle types for each weight class. Minor differences in the fastest relaxation time constant, T_{2b}, were observed between different muscle types, and independent of muscle type, a decrease in T_{2b}, was observed as the weight increased (Fig. 3a). The population of the T_{2b} was not significantly affected by muscle type and was almost independent of slaughter weight until 85 kg, whereupon a slight increase was observed (Fig. 3d). With increasing slaughter weight from 25 to 150 kg and independent of muscle type, a highly significant decrease corresponding to 24% was observed in the intermediate time constant, T_{21}. Furthermore, the T_{21} time constant was significantly affected by muscle type in the following order: *M. semitendinosus* > *M. vastus intermedius* > *M. biceps femoris* > *M. longissimus dorsi* (Fig. 3b). The population of T_{21} decreased slightly with increasing weight, and independent of slaughter weight minor differences in the T_{21} population were observed between muscle types with *M. vastus intermedius* > *M. biceps femoris* ≡ *M. semitendinosus* > *M. longissimus dorsi* (Fig. 3e). For all muscle types, a significant decrease in the slowest time constant, T_{22}, was observed with increasing slaughter weight. This decrease was most profound from 25 to 65 kg, after which T_{22} remained almost constant. The T_{22} time constant was significantly affected by muscle type with *M. vastus intermedius* > *M. semitendinosus* > *M. biceps femoris* > *M. longissimus dorsi*. However, the effect was most severe at slaughter weights of 25 and 45 kg, whereas higher slaughter weights produced less pronounced differences in the T_{22} time constant between muscle types (Fig. 3c). The population of T_{22} increased with increasing weight; again it was most pronounced at the low slaughter weights between 45 and 65 kg. Independent of slaughter weight, minor differences in the T_{22} populations were observed between muscle types with *M. longissimus dorsi* > *M. biceps femoris* ≡ *M. semitendinosus* > *M. vastus intermedius* (Fig. 3f).

In Fig. 4 mean fat, protein, and dry matter content are shown for the different muscle types at each slaughter weight. The fat content increased significantly with increasing slaughter weight from 25 to 85 kg, while becoming almost constant at slaughter weights beyond 85 kg. Independent of slaughter weight, *M. semitendinosus* had significantly higher fat content than the other three muscles (Fig. 4a). For all muscle types, the protein content, determined as total nitrogen content, increased significantly with increasing slaughter weight. *M. longissimus dorsi* and *M. biceps femoris* had the highest protein content, measured by total nitrogen. *M. semitendinosus* and *M. vastus intermedius* had identical protein content, which was significantly lower than that in the two other muscles (Fig. 4b). The dry matter content of muscles increased significantly with increasing slaughter weight (Fig. 4c).

In Table 1 the correlations between the NMR relaxation characteristics, i.e., time constants and corresponding populations, and fat, protein, and dry matter content are shown. Significant correlations were found between all NMR parameters and total nitrogen as well as dry matter content. The strongest correlation ($r = -0.88$) was obtained between the intermediate time constant, T_{21}, and the total nitrogen content in the muscles.

TABLE 1

Pearson Correlation Coefficients and Levels of Significance for the Correlations between the T_2 Time Constants and Corresponding Area and Muscle Content of Fat, total N, and Dry Matter ($n = 196$)

	Fat (%)	Total N (%)	Dry matter (%)
T_{2b} (ms)	−0.04 (N.S.)	−0.60***	−0.53***
T_{2b} area (%)	−0.03 (N.S.)	0.32***	0.26***
T_{21} (ms)	−0.10 (N.S.)	−0.88***	−0.79***
T_{21} area (%)	−0.38***	−0.47***	−0.58***
T_{22} (ms)	−0.29***	−0.69***	−0.74***
T_{22} area (%)	0.44***	0.42***	0.57***

Note. Levels of significance: * = $p \le 0.05$; ** = $p \le 0.01$; *** = $p \le 0.001$.

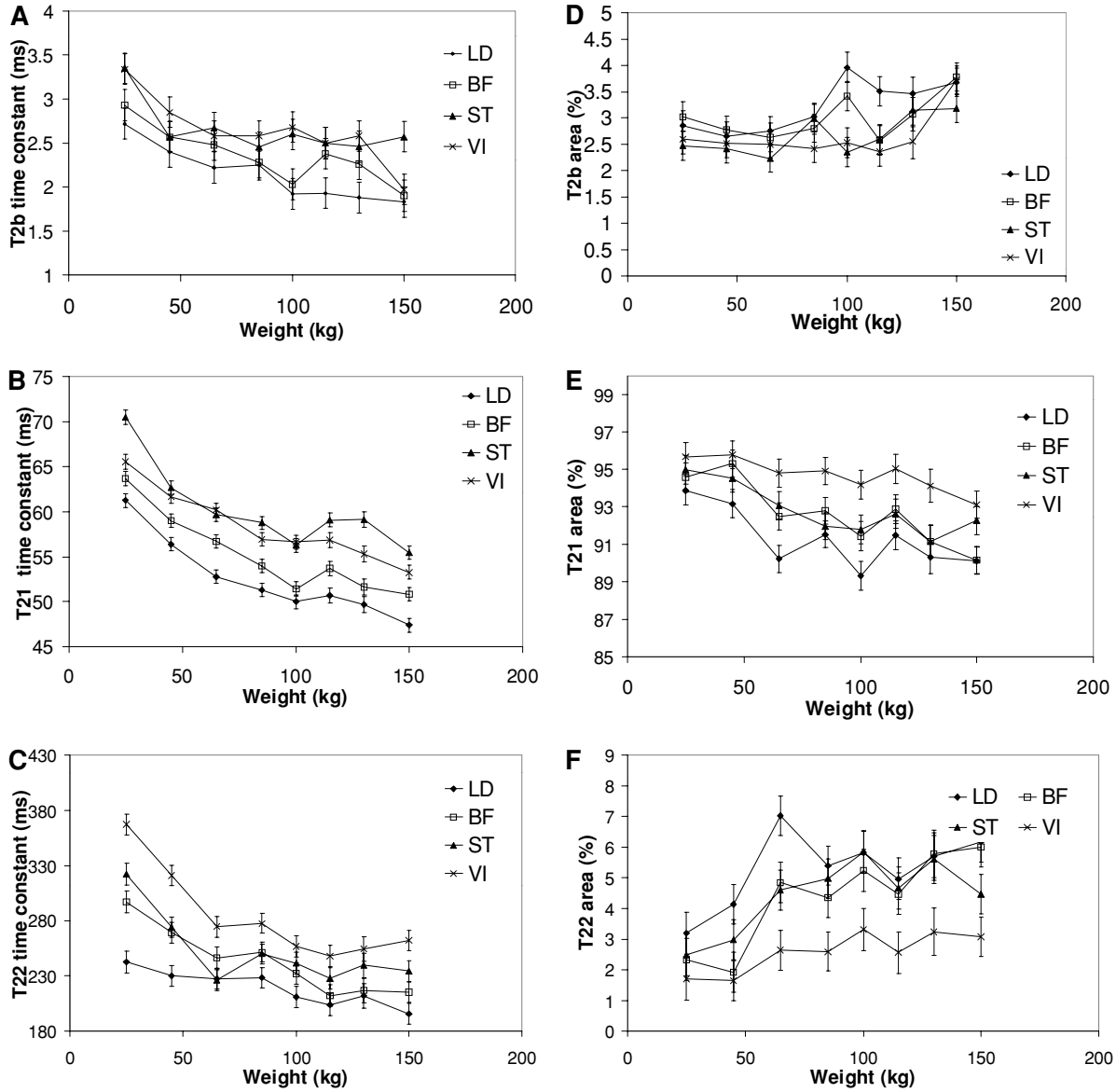

FIG. 3. The relaxation time constants and the population of relaxation described by these (area) for the different body weight classes (BF = *M. biceps femoris*, LD = *M. longissimus dorsi*, ST = *M. semitendinosus*, VI = *M. vastus intermedius*). (A) T_{2b}, (B) T_{21}, (C) T_{22} time constants and (D) T_{2b}, (E) T_{21}, (F) T_{22} populations. Least squares means are given. Bars show standard errors.

Table 2 presents the results from PLS calibration with NMR relaxation curves as *x*-variables, and body weights as *y*-variables for each of the four muscle types. Correlations between $r = 0.58$ and $r = 0.77$ were obtained.

The results of the microscopic analysis carried out on muscle samples from an animal with a slaughter weight of 100 kg are shown in Fig. 5. The *M. vastus intermedius* showed a distinct structure compared with the other three muscles, *M. longissimus dorsi, M. semitendinosus,* and *M. biceps femoris*, as the muscle fibers within *M. vastus intermedius* were smaller and considerably more densely packed, whereas especially *M. longissimus dorsi* showed a tendency to gaps between muscle fibers.

TABLE 2
Performances of PLS Regression Models with NMR Transverse Relaxation Data as *x*-Variables and Body Weight as *y*-Variable

	M. biceps femoris	*M. longissimus dorsi*	*M. semitendinosus*	*M. vastus intermedius*
#PC	2	2	2	2
RMSECV	25.0	24.8	31.2	25.5
r	0.75	0.77	0.58	0.75

Note. Numbers of principal components (PC), root mean squared error of cross validation (RMSECV) in %, and correlation coefficients are given ($n = 48$). The number of PC's resulting in the lowest RMSECV was chosen.

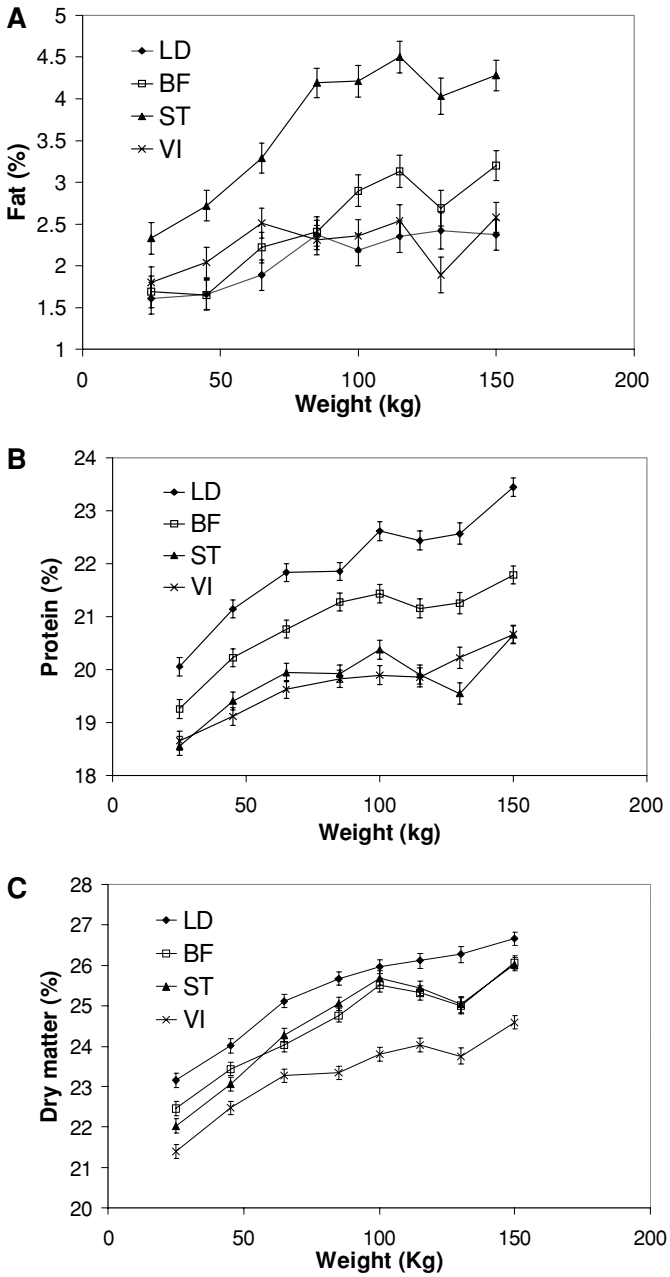

FIG. 4. (A) fat, (B) total nitrogen, and (C) dry matter contents in the muscles determined by chemical analysis. Values are reported in % or g/100 muscle (wet weight). Least squares means are given. Bars show standard errors.

DISCUSSION

It has been recognised that NMR relaxation in muscles is affected by several factors, e.g., species, tissue type, and age, as comprehensively reviewed by Bottomley *et al.* (*17*). However, most of these conclusions were drawn from comparisons of different studies, which included different species, tissue types, and ages and were carried out under various conditions. It is well known that differences in biological sample characteristics and in NMR instrumental and measurement parameters, as well as in how the data analysis is carried out, are critical factors, which often make it difficult to draw any quantitative conclusions in the comparison of different studies.

In the present study the significance of both skeletal muscle type and developmental stage, expressed as slaughter weight, for NMR transverse relaxation characteristics in pig muscles was investigated. Principal component analysis (PCA) on NMR transverse relaxation decays revealed a clear tendency to grouping according to both skeletal muscle type and slaughter weight, which shows that the water characteristics within skeletal muscles depend on both muscle type and developmental stage.

Partial least squares (PLS) regression revealed correlations from 0.58 to 0.77 between NMR transverse relaxation data and slaughter weights for the different muscle types and corresponding root mean squared errors of cross validation (RMSCV) of 31.2% and 24.8%, respectively. The result indicates that NMR relaxation is highly dependent on muscle developmental stage or age and suggests the possibility that techniques based on NMR relaxation measurements for characterization of muscle developmental stage can be developed. This may have implications for future diagnostic and growth performance evaluation within medicine and animal production, respectively.

Distributed exponential fitting analysis of T_2 relaxation data revealed three different water fractions, a minor fraction with very fast relaxation ($T_2 \sim 1$–10 ms), T_{2b}, an intermediate fraction ($T_2 \sim 45$–120 ms), T_{21}, and a slower fraction ($T_2 \sim$ 200–500 ms), T_{22}. The existence of three different water fractions in the present study is in agreement with earlier data obtained for muscle tissue (*15, 18–21*). It has been suggested that the T_{2b} component reflects water closely associated with macromolecules (*18*), that the T_{21} component reflects water trapped within the myofibrils, and that the T_{22} component corresponds to water outside the myofibrillar lattice, i.e., extramyofibrillar water (*21*).

Independent of muscle type, the three transverse relaxation components shifted toward lower time constants with increasing body weight, revealing muscle development during growth. In contrast, Hatakeneka *et al.* (*16*) observed that NMR T_2 relaxation time increased in human muscles with increasing age. The discrepancy is potentially due to the latter study having been carried out on fully developed muscles (ages 22–76), and therefore the increase in T_2 relaxation time is consistent with the present findings, as it probably reflects aging-related muscle atrophy.

Thus, both studies reveal dependence of the NMR T_2 relaxation and thereby water characteristics on the developmental stage of muscles. Consequently, this displays the potential for using NMR relaxation as a diagnostic tool in medicine, e.g., characterization of muscle atrophy. Moreover, the relationship between muscle growth and development in domestic animals is known to influence meat quality (*22–24*); why ongoing development of low-cost and flexible NMR relaxation equipment (*25*) may be implemented as a quality control tool in the near future.

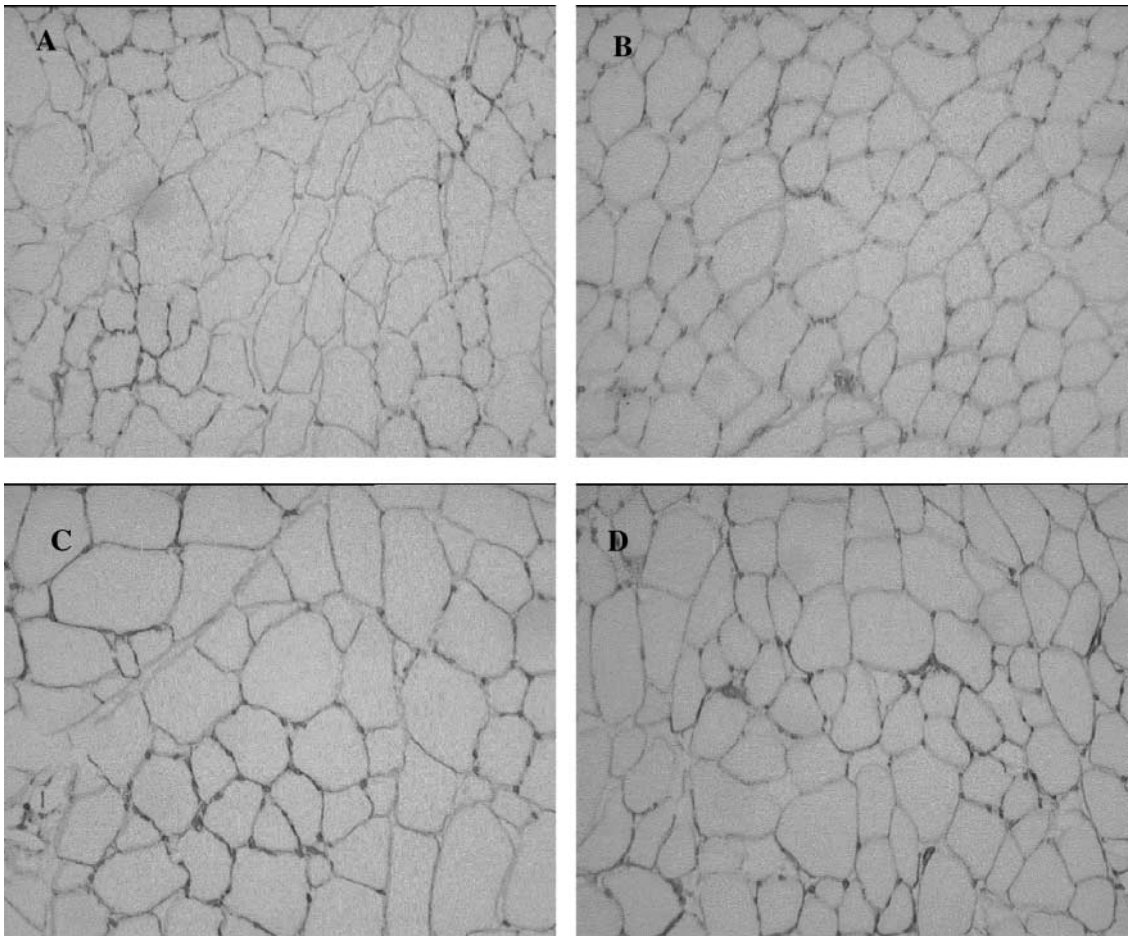

FIG. 5. Light microscopy of muscle samples from a representative pig with a weight of 100 kg: (A) *M. longissimus dorsi*, (B) *M. vastus intermedius*, (C) *M. semitendinosus*, and (D) *M. biceps femoris*. Bars indicate 100 μm.

Linear regression analysis showed a high correlation between all three relaxation time constants and dry matter content and protein content, respectively, with the latter being more pronounced. Consequently, the increases in relaxation rates with increasing weight should probably be ascribed to increased protein content within the muscle, which results in higher dry matter content. As the protein content will determine the local microstructure, the result verifies earlier proposed theories stating that the microenvironments are the determinants of NMR T_2 relaxation characteristics (*26–27*). Additionally, it confirms the results obtained by Damon *et al*. (*28*), who found that increases in the T_{21} time constant during exercise-induced increases in intracellular volume could be explained quantitatively by changes in protein concentrations. Kennan *et al*. (*29*) stated that the net protein or macromolecular concentration within each compartment determines the relaxation rate within each compartment. In the present study, the correlation to protein content was higher for the T_{21} component as compared with the T_{2b} and the T_{22} components (Table 1). During muscle growth the muscle protein mass rises due to increases in the myofibrillar protein proportion as well as the sarcoplasmatic protein proportion (*1–3*). An increase in the myofibrillar protein content would be expected to affect the T_{21} component, while a *post mortem* increase in the sarcoplasmatic protein content may be expected to affect both the T_{21} and the T_{22} component, as high amounts of sarcoplasmatic proteins have been discovered in the extrafibrillar fluid (*30*). Accordingly, the higher correlation between protein content and the T_{21} component as opposed to the T_{22} component seems to be explainable and also consistent with the suggestion that the T_{21} component reflects water trapped within the myofibrils, and that the T_{22} component corresponds to water outside the myofibrillar lattice, i.e., extramyofibrillar water (*21*).

For all the three relaxation components, variations in both time constants and corresponding populations were demonstrated between different muscle types. In previous studies investigating the effect of muscle type on NMR relaxation patterns, only muscles that were solely fast-twitch white and slow-twitch red have been included (*20, 31–33*). Various results have been reported from these studies, probably mainly due to use of different methods for analysis of the T_2 data, as also suggested by

English *et al*. (*20*). Using biexponential fitting, Le Rumeur *et al*. (*31*) found that the time constants were similar for fast-twitch and slow-twitch rat muscles, but the population of the slow relaxation component (T_{22}) was larger in slow-twitch red muscles compared with fast-twitch white muscles. Moreover, using continuous fitting English *et al*. (*20*) also found that the population of the slow relaxation component (T_{22}) was larger in slow-twitch red muscles compared with fast-twitch white muscles. In contrast, also using biexponential fitting, Adzamli *et al*. (*33*) found that both the fast (T_{21}) and the slow (T_{22}) relaxation component were significantly longer in slow-twitch red rabbit muscles compared with fast-twitch white muscles, whereas the populations were similar for the two muscle types. Using monoexponential fitting, Polak *et al*. (*32*) and Bonny *et al*. (*34*) reported higher T_2 values in slow-twitch muscles than in fast-twitch muscles. Despite the differences in effects on either time constant or population, all these studies in general come to the same conclusion, as they all find either a larger T_{22} time constant or a larger population in slow-twitch than in fast-twitch muscle. The following suggestions have been put forward to explain the reported differences in T_2 values between muscle fiber types:

(i) Differences in the fat content between muscle fiber types
(ii) Differences in the myoglobin content between muscle fiber types
(iii) Differences in the intra-/extra-cellular ratio between muscle fiber types
(iv) Differences in the hydrophobicity of the myosin isoforms in different muscle fiber types.

It has long been recognized that slow-twitch oxidative fibers contain more lipid than glycolytic fibers (*35–37*). Thus, due to the distinct relaxation characteristics of lipid compared with muscle tissue, the T_2 values may differ in the different fiber types. However, fat suppression using an inversion recovery sequence was recently demonstrated not to affect proton T_2 relaxation in muscles compared with the traditional use of a spin–echo sequence (*16*), which reveals insignificant contributions from fat to T_2 relaxation, as also suggested earlier by Bonny *et al*. (*34*). The present study supports this view, as the general tendencies to a correlation between the T_2 components and fat content (Table 1) are far from high enough to explain the observed variation in the T_2 values.

In the oxygenated state, myoglobin is diamagnetic, and therefore an increase in the myoglobin content is expected to cause an increase in the T_2 value. Accordingly, oxidative muscle fibers containing higher amounts of myoglobin (*35, 38*) should be expected to result in higher T_2 values in oxidative muscles. Of the muscle types included in the present study, the content of fiber type I (oxidative) has been characterized as follow: *M. vastus intermedius*: approx. 75%, *M. biceps femoris*: approx. 12%, *M. longissimus*: approx. 10%, and *M. semitendinosus*: approx. 5–20% type I fibers, depending on the part of the muscle (*39, 40*). Assuming that the content of myoglobin is the major decisive factor for the T_2 values, *M. vastus intermedius* should have the longest T_2 values, followed by *M. biceps femoris*, *M. longissimus*, and *M. semitendinosus*, respectively. However, the T_2 values were not found to be reflected according to the expected myoglobin content of the different muscle types in the present study (Fig. 3). Accordingly, it cannot be concluded that the observed differences between the different muscles in T_2 relaxation times should be ascribed solely to differences in the myoglobin content.

It has previously been suggested that the T_{21} population should mainly be ascribed to the intracellular space, and that the T_{22} population should mainly be ascribed to the extracellular space of muscle tissue (*19, 41, 42*). This theory is supported by results showing that the paramagnetic agent gadolinium-DTPA, known to be distributed only in the extracellular space, mainly affected the T_{22} values (*31, 33*). Consequently, in agreement with larger extracellular fluid spaces *in vivo* in red muscles compared with white muscles (*32*), it has been suggested that the differences in T_{22} values between red and white muscles should be ascribed to a larger extracellular space in the red muscles (*31, 32*). In contrast, in the present study it was found that the T_{22} population was lower in the oxidative *M. vastus intermedius* than in the three other muscles, which can be considered to be mainly glycolytic muscles. Moreover, the microscopic studies supported the conclusion that the extrafibrillar space was considerably smaller in meat of *M. vastus intermedius* than in meat of the three other muscles, which all were found to have more or less similar extrafibrillar spaces. The discrepancy in extrafibrillar fluid spaces between *in vivo* muscles and the corresponding 24 h *post mortem* muscles reveals differences in fluid distribution between different muscle types during the conversion of muscle to meat. Taking the microscopic results into consideration, the results in the present study are consistent with the theory that differences in the T_{22} values can be ascribed to differences in the extrafibrillar fluid spaces. Consequently, noticeable care should be taken if a direct comparison between *in vivo* and *post mortem* conditions is to be carried out.

Boyer *et al*. (*43*) found that the surface hydrophobicity of myosin from rabbit *M. semimembranosus proprius* (oxidative) was 1.5 times higher than that of myosin from rabbit *M. psoas major* (glycolytic). Accordingly, as the number of hydrophopic sites will determine the level of water associated with macromolecules this might also affect the T_2 relaxation. Bonny *et al*. (*34*) suggested that differences between muscle fiber types in T_2 relaxation times might be ascribed to differences in the hydrophobicity of the myosin isoforms. Assuming that the T_{21} component corresponds to water within the myofibrillar structures, the T_{21} component especially should be affected by differences in the hydrophobicity of myosin. However, as the hydrophobicity of myosin affects the amount of water entrapped within the myofibrillar structures, it will also affect the amount of water which is not closely associated with the myofibrillar stuctures, i.e., the extramyofibrillar water, represented by the T_{22} component. Accordingly, if myosin isoforms differ between pig muscles, the possibility cannot be excluded that such differences

contribute to the observed differences in T_2 characteristics among the different muscle types in the present study.

CONCLUSIONS

In conclusion, the present study has demonstrated a high dependence of NMR T_2 relaxation characteristics on both developmental stage and muscle type, which clearly reveals changes in the mobility and distribution of water during muscle growth. The dependence of NMR T_2 relaxation on developmental stage is highly consistent with an increased protein content within muscles during growth. Accordingly, the results reveal a close relationship between NMR T_2 characteristics and muscle protein content, and hereby also a relationship between NMR T_2 characteristics and muscle microstructure.

EXPERIMENTAL

Animals and Sampling

The experimental setup included a total of 48 pigs (crossbreed between Danish Duroc boars and Landrace/Yorkshire sows), which were divided into eight groups with a live weight of 25, 45, 65, 85, 100, 115, 130, or 150 kg, respectively (six animals in each weight class). The pigs were slaughtered in the experimental abattoir at Research Centre Foulum, and the same slaughter procedure was used for all animals. At 24 h *post mortem*, the muscles *M. longissimus dorsi, M. semitendinosus, M. biceps femoris,* and *M. vastus intermedius* were dissected from the carcasses. From each muscle, samples (approx. 3.5 cm long and 1×1 cm in sectional area, weight approx. 5 g) were cut along the fiber direction using a scalpel. Care was taken that the samples were always cut in the same position on the muscle. The samples were placed with vertical fiber direction in a cylindrical glass tube that fitted into the NMR probe and were immediately used for NMR measurements. The remaining part of the muscle was frozen and stored at -20°C prior to use for chemical analyses.

NMR Measurements

The relaxation measurements were performed on a Maran Benchtop pulsed NMR analyzer (Resonance Instruments, Witney, UK) with a magnetic field strength of 0.47 T and with a corresponding resonance frequency for protons of 23.2 MHz.

The NMR instrument was equipped with an 18-mm temperature variable probe. At 24 h *post mortem*, T_2 was measured using the Carr–Purcell–Meiboom–Gill sequence (CPMG; *44, 45*). The T_2 measurements were performed with a τ-value (time between 90° pulse and 180° pulse) of 150 μs, which according to Santyr *et al.* (*46*) should avoid spin-locking. The lengths of the pulses were 7.8 and 15.6 μs for the 90° and 180° pulse, respectively. Data from 4096 echoes were acquired as 16 scan repetitions. Signal-to-noise ratios (SNR), estimated by dividing intensity of the first point by the standard deviation of the last 400 points in the T_2 decay curve, were on acquired data found to be 3000–4000. The repetition time between two succeeding scans was 2 s, which corresponded to $2.5 \times T_1$ ($T_1 = 0.8$ s). All relaxation measurements were performed at 25°C.

Distributed exponential fitting analysis was performed on the T_2 relaxation data using the RI Win-DXP program (software release version 1.2.3) released from Resonance Instruments Ltd, UK. The RI Win-DXP program performs distributed exponential curve fitting. A continuous distribution of exponentials for a CPMG experiment may be defined by the equation

$$g_i = \int_0^\infty A(T) \times e^{-ti/T}\, dT, \qquad [1]$$

where g_i is the intensity of the decay at time ti, and $A(T)$ is the amplitude of the component with transverse relaxation time T. The RI Win-DXP program solves this equation by minimizing the function

$$\left(g_i - \int_{x=1}^{m} f_x e^{-\tau i/Tx}\right)^2 + \lambda \sum_{x=1}^{m} f_x^2, \qquad [2]$$

where $f_x = \int_{T_x}^{T_{x+1}} A(T)\, dT$. $\lambda \sum_{x=1}^{m} f_x^2$ is a linear combination of functions added to the equation in order to perform a zero-order regularization as described by Press *et al.* (*47*). λ is the weight, and on the basis of the quality of the fit, judged by χ^2, a value of 0.005 was used. The data were pruned from 4096 to 256 points using linear pruning, which on synthetic data was found to give stable solutions. This analysis resulted in a plot (see Fig. 2) of relaxation amplitude for individual relaxation processes versus relaxation time. From such analyses, average time constants for each water population were calculated from the peak position, and the area under each population curve (corresponding to the proportion of water molecules exhibiting that relaxation time) was determined by cumulative integration using an in-house program written in Matlab (The Mathworks Inc., Natick, MA).

Chemical Analysis

Fat, protein, and dry matter content were determined for all the muscles included in the study. Fat content was determined by Soxhlet extraction according to the Stoldt method (*49*) using petroleum ether (boiling point 40–60°C) for extraction. Protein content was determined as total nitrogen multiplied by 6.25 according to the Kjeldahl method (*49*). Dry matter content was determined by freeze-drying followed by drying at 100°C for 4 h (*50*). All values reported are in g/100 g muscle (wet weight).

Light Microscopy

Moreover, muscle samples for light microscopy were cut out at 24 h *post mortem*, mounted in an OCT embedding medium

(Sakura, Japan), frozen in isopentane, cooled in liquid nitrogen, and mounted for cryostat sectioning. The samples were stored at −80°C prior to use. Cryostat sections (10 μm) were immunohistochemically stained for collagen IV (diluted 1 : 500) (monoclonal mouse anti-human collagen IV, code M785, DAKO Co., CA) for 30 min at 37°C. Biotinylated rabbit anti-mouse IgG secondary antibody (diluted 1 : 300) (DAKO A/S, Glostrup, Denmark) was added for 15 min at 37°C. Horseradish peroxidase-labeled streptavidin (diluted 1 : 500) (DAKO A/S) was added for 15 min at 37°C followed by 3,3′-diaminobenzidine tetrahydrochloride (DAKO A/S) for 5 min at room temperature. Between the steps, the sections were rinsed with Tris-buffered saline (pH 7.6). Microscopy was carried out with an Olympus BH-2 microscope (Olympus Optical Co., Hamburg, Germany), allowing linear magnification of 8–20 times, fitted with a CCD black-and-white camera and a computer, based on a SB 1024-SA and SB-1024-RAM board provided with a high-resolution monitor. Microscopy was carried out on muscle samples from the four different muscles on three representative animals with weights of 40, 100, and 150 kg, respectively, and data from one representative animal are shown in the present paper.

Statistical Analysis

Principal component analysis (PCA) was performed on the transverse relaxation decay. PCA is a descriptive method, which allows the main variability aspects of a data set to be visualized without the constraint of an initial hypothesis concerning the relationship between samples and between samples and variables. The analysis aims at finding relationships between the different parameters (samples and variables) and to display possible clusters within samples or/and variables. To find the main sources of data variability and the relationship between or within samples and variables, data are arranged in a matrix, $X_{(n,m)}$, and decomposed into orthogonal components, linear combinations of which approximate the original data by a least squares method. The decomposition is formalized by

$$X_{(n,m)} = T_{(n,k)} P^T_{(k,m)} + E_{(n,m)},$$

where T is the score matrix, P is the loading matrix, E is the error matrix, n is the number of samples, m is the number of samples, and k is the number of principal components (*51*).

In order to obtain reasonable calculation times, only every 10th echo signal amplitude was included in the analysis (resulting in a total of 411 points per relaxation curve). Moreover, partial least squares regression (PLS) was performed on the obtained relaxation data with body weight as y-variable. PLS regression models were validated using full cross validation ("leave one out"), and all the stated results are validated (*51*). As an indicator of prediction ability, the root mean squared error of cross validation (RMSECV) is given. RMSECV is calculated according to the equation

$$RMSECV = \sqrt{\frac{\sum_{i=1}^{n} (\hat{y}_i - y_i)^2}{n}}, \qquad [3]$$

in which $\hat{y}_i$ is the predicted value of the y-variable for sample i, y_i is the actual value of the y-variable for test i, and n is the number of samples.

The multivariate calculations were performed using the program Unscrambler (Version 7.6, CAMO ASAS, Trondheim, Norway).

Analysis of variance was carried out using the GLM procedure from the Statistical Analysis System (*52*). The statistical models used included the fixed effects of muscle type (*M. longissimus dorsi, M. semitendinosus, M. biceps femoris*, and *M. vastus intermedius)*, weight (25, 45, 65, 85, 100, 115, 130, and 150 kg), and sex. Animal within weight group and sex were included as random effects if significant. Interactions between the main effects were only included when significant. In addition, for analysis of the relationship between fat, protein, and dry matter content and NMR populations, a linear correlation was carried out using the CORR procedure.

ACKNOWLEDGMENTS

The authors are grateful to the Ministry of Food, Agriculture, and Fisheries and to the Danish Bacon and Meat Council for financial support of this project. Moreover, Mr. Jimmi Jakobsen is greatly acknowledged for skilful technical assistance in sample preparations.

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