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PRENATAL DETERMINATION OF FAT AND COLLAGEN CONTENT IN PORCINE MUSCLES

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Introduction

The early fetal environment plays a key role in development of skeletal muscle. Several studies have shown that intralitter variation, *in utero*, is reflected in the postnatal growth of skeletal muscle in the pig (Dwyer et al., 1994). It is well accepted that muscle growth is influenced by the number, size and type of the muscle fibres. Marked differences in fibre number have been demonstrated between the smallest and largest porcine littermates prenatally, with the smallest littermate having significantly fewer fibres than the largest littermate (Wigmore & Stickland, 1983).

Pilot studies carried out in our laboratory have indicated that the smallest littermate may also have a higher proportion of connective tissue in its muscle (Clelland, 2001). Muscle connective tissue provides structure to the muscle and is composed of ground substance, fibres and connective tissue cells (Purslow, 2002). A proportion of these elements of the connective tissue comprise collagen and fat deposits. These are important parameters to the meat industry as an increased amount of these components may impact on meat toughness and intramuscular fat respectively, and so influence the resultant meat quality.

Objectives

The primary objective of this study was to investigate the prenatal development of intralitter variation in content of fat, collagen and myosin (a major contractile protein found in skeletal muscle) of muscles. In the pig, it can be argued that differing levels of nutrition received, *in utero*, are a major cause of the observed intra-litter variation. The smallest and largest littermates were chosen and the content of fat, collagen I and myosin (embryonic) were analysed in the *M. semitendinosus* of both. This investigation will determine whether the differences in fat, collagen and myosin expression have a fetal origin.

Methodology

Fetal selection and preparation

A total of 23 pairs of porcine fetuses from a Large White-Landrace origin were used in this study, aged from 36-86 days gestation. This includes the timing of primary and secondary fibre formation and the ending of myogenesis (muscle formation) (Wigmore & Stickland, 1983). Runts were excluded from the study as outliers (Hegarty & Allen, 1978; Powell & Aberle 1980). The *M. semitendinosus* muscles were snap frozen in liquid nitrogen and stored at -80^{0}C. Complete transverse sections (10μm) were taken from the mid-belly region of the *M. semitendinosus* using a cryostat (Bright, U.K) at -25^{0}C. The sections were mounted on slides. Histochemistry and immunocytochemistry techniques were employed on the frozen transverse sections.

Histochemistry

The extent of fat deposition in the smallest and largest littermates was determined using Oil Red O stain and analysed using the Kontron Image Analysis software (Carl Zeiss, Germany). Analysis of fat deposition was made by measuring the area of stained fat within a unit area (mm^2) of muscle. A representative portion (at least 2%) (Clelland, 2001) across the muscle from the deep part to the superficial part was used to perform the analysis.

Immunocytochemistry

An antibody to Collagen I (Sigma Chemical Co., UK) (1:100 dilution) was used to identify collagen fibres within the connective tissue matrix and an antibody to myosin (embryonic) (Developmental Studies Hybridoma Bank) was used to identify this abundant motor protein found in individual fibres of skeletal muscle. A standard antibody protocol was followed (Clelland & Stickland, 2001), using a biotinylated rabbit anti-mouse IgG secondary antibody (1:200 dilution) (Vector Laboratories, CA) common to both antibodies. Slides were analysed using the Kontron Image Analysis software. Analysis was performed by comparing stained component against background within a unit area (mm^2) of muscle. Again, a representative portion (at least 2%) (Clelland, 2001) across the muscle from the deep part to the superficial part was used to perform the analysis.

Statistical Analysis

Paired t-Tests were performed on all results from the smallest and largest littermates analysed. Fetuses were grouped from day 36-60 (including the initiation of primary and secondary muscle fibre proliferation) and day 61-86 (including the period of differentiation of fibres and the ending of myogenesis). A P value of ≤ 0.05 was deemed to be of significance.

Results & Discussion

Histochemistry

Fig. 1 illustrates the transverse cryosections from the mid portion of the *M.semitendinosus* stained in Oil Red O solution for detection of lipid (fat) deposition. Qualitatively, more fat deposition is seen in the smallest fetus (Fig. 1a) compared to the largest littermate (Fig. 1b). Fat per mm^2 was determined for a representative portion of the section. A Paired t-Test showed the smallest littermate had significantly more fat per mm^2 present than the largest littermate (P=0.01) in the group of fetuses aged 36-60 days of gestation (Fig. 2). Also, in the group aged 61 – 86 days of gestation, the smallest littermate exhibited more fat per mm^2 than the largest littermate (P=0.04).

Immunocytochemistry I

An antibody against Collagen I was used (Fig. 3). Qualitatively it can be seen that in Fig. 3a the area of Collagen I in the smallest fetus is more densely packed than in the largest fetus (Fig. 3b). The endomysium is apparent around groups of fibres rather than individual fibres at this stage (60 days of gestation) (Fig. 3a & 3b).

Due to the acetone stage in the protocol only fetuses of 44-86 days of gestation could be analysed as this treatment proved to be too harsh for younger tissue sections. Fig. 4. illustrates Paired t-Test results demonstrating the smallest littermate had significantly more expression of Collagen I area per mm^2 than the largest littermate in the later stages of fetal life (61-86 days of gestation) (P=0.008).

Immunocytochemistry II

An antibody against myosin (embryonic) was used (Fig. 5a & b). Myosin per mm^2 was determined for a representative portion of the muscle section. A Paired t-Test showed the smallest littermate had significantly less myosin expression per mm^2 of muscle than the largest littermate (P=0.04) in the 36-60 gestational age group and the latter fetal age group (61-86 days of gestation) (P=0.004), Fig.6.

The results of the present study demonstrate that an increased proportion of fat and collagen I content was present in the muscles of the smallest littermate compared with the largest littermate, at least in the latter fetal stages. On the contrary, decreased expression of myosin was seen in the smallest littermate compared to the largest, indicating less muscle and more non-muscle may be present. This confirms the initial hypothesis.

The results presented here extend previous pilot studies carried out in our laboratory (Clelland, 2001). The increased non-muscle found in these pilot studies can now be confirmed as including increased fat and collagen I within the connective tissue composition of the smallest littermate. Decreased myosin expression confirmed the smallest littermate contains less muscle protein than the largest littermate.

In the present study it was demonstrated that significantly increased levels of fat per mm^2 were present in the smallest littermate compared with the largest littermate. The difference was seen at both the late embryonic to mid-fetal stages (36-60 days of gestation) and also the late fetal stages (61-86 days of gestation). A postnatal study by

Powell and Aberle (1981) showed that runts (pigs with severe intrauterine growth retardation) and small pigs have increased amount of intramuscular fat and perirenal fat compared to larger littermates. Fat deposition measured in the present study did not distinguish between intramuscular fat and fat found in adipocytes. However, it had been previously found that a higher number of small diameter adipocytes were present in runts (Powell & Aberle, 1981). Our results are further validated postnatally by Gondret et al, (2005). In their recent study the lightest and heaviest birthweight pigs were analysed at slaughter weight (mean weight of 111.8kg) and the lightest littermates contained higher levels of intramuscular lipid in *M. semitendinosus* compared with the heaviest littermate. Our result clearly demonstrates that the postnatal lipid differences have their origins prenatally.

Collagen is a major component of connective tissue. The fibrous material forms a continuous mesh within the extracellular matrix. Collagen is divided into seven different subtypes. The most important in fetal skeletal muscle is Collagen I (Listrat et al., 1999). In this present study elevated levels of Collagen I were found in the smallest littermate compared to the largest in later fetal life (61-86 days of gestation). Although debatable, collagen content has been linked with meat toughness (Fang et al, 1999). In the work discussed above (Gondret et al, 2005), low birth weight pigs at slaughter exhibited a low score for loin meat tenderness compared with the heaviest littermate (Gondret et al., 2005). These results may indicate a higher connective tissue in the less tender muscles. Our results indicate that differences in collagen content between littermates appear to have a prenatal origin.

Myosin is a major contractile protein found in muscle fibres. The results indicate that lower levels of myosin (expressed per mm^2) in the smallest littermate is associated with higher levels of fat and collagen. From previous studies (Wigmore & Stickland, 1983) it is also known that the smallest littermates have fewer muscle fibres. Together these studies demonstrate an influence on muscle at both cell and protein level when littermates are compared.

Conclusions

Arguably, intralitter variation provides us with a good naturally occurring model of differing levels of nutrients reaching the offspring. A variation in blood flow and hence nutrients, as a consequence of good and restricted access from the placenta results in a wide range of littermate sizes within the entire litter (McClaren & Michie, 1960; Warwick, 1928; Perry & Rowell, 1969). Previous studies have investigated the influence of prenatal nutrition, either directly or by studying intralitter variation on muscle development and consequences for postnatal growth (Dwyer et al, 1994). These studies have shown that low nutrition (by either method) impedes fibre number development and correlates with future postnatal growth (Dwyer & Stickland, 1992; Dwyer et al, 1994; Ward & Stickland, 1991; Wigmore & Stickland, 1983). In this study we have shown a prenatal nutritional influence also on the non-muscle components of muscle and, from other work, this appears to continue postnatally. In this way prenatal programming is influencing the longer term postnatal phenotype of muscle.

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Tables and Figures

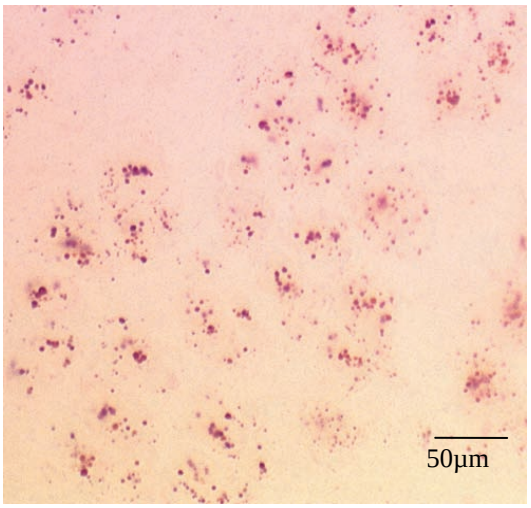


Fig. 1a

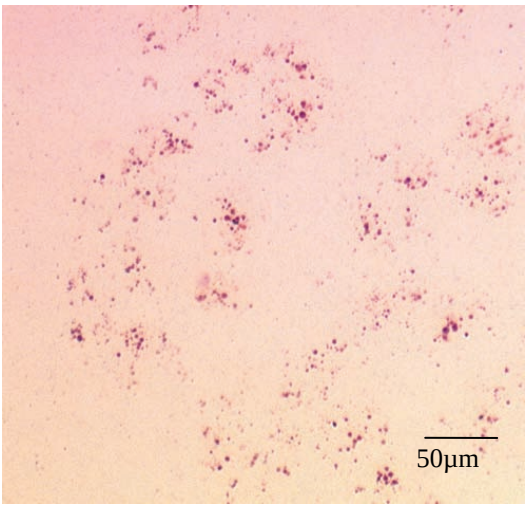


Fig. 1b

Fig. 1: Oil Red O stained sections from fetuses aged 60 days of gestation. a) Smallest littermate. b) Largest littermate.

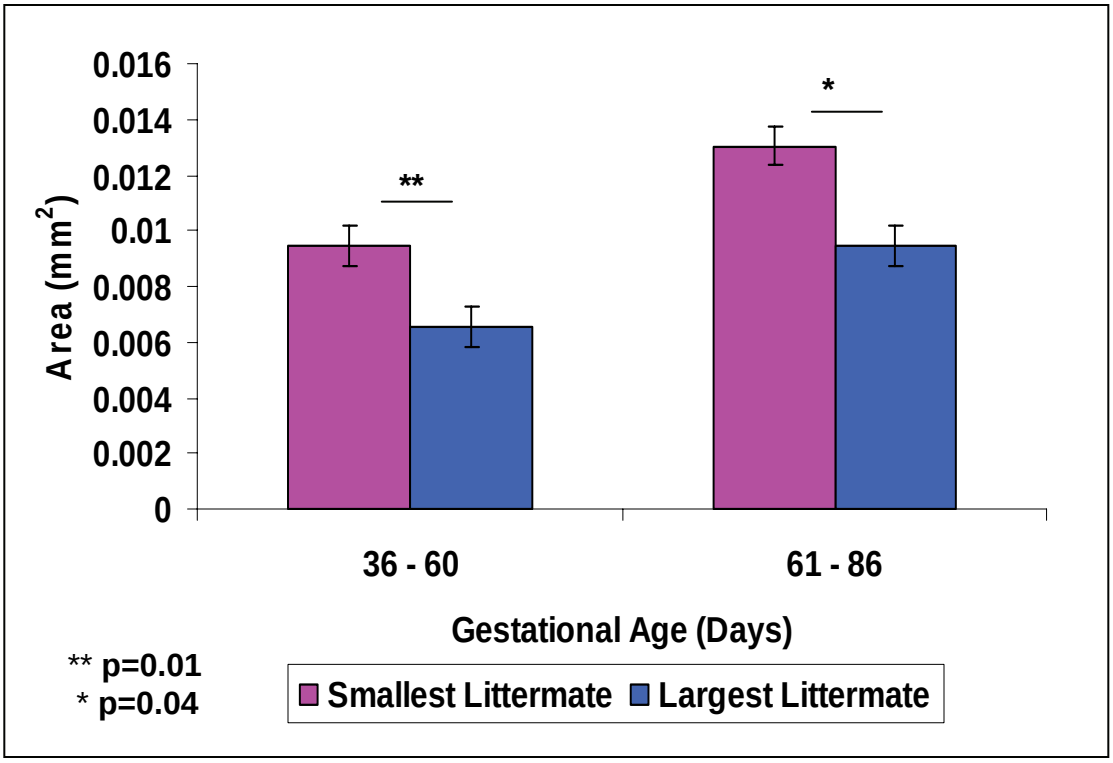
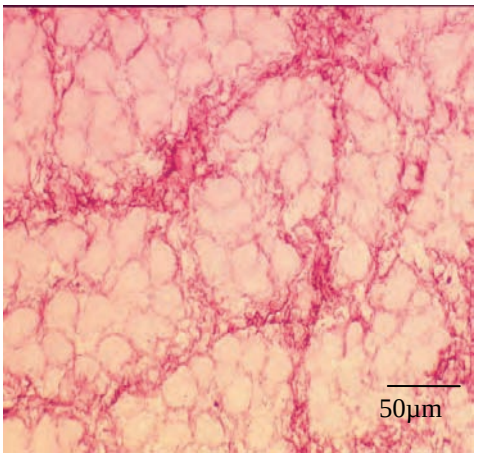


Fig. 2: Fat Area per mm^2 of Muscle for fetal samples aged 36-60 and 61-86 days of gestation. The smallest littermate has an increased amount of fat present compared to the largest littermate in the 36-60 days if gestation group (*P*=0.01) and the 61-86 days of gestation group (P=0.04).



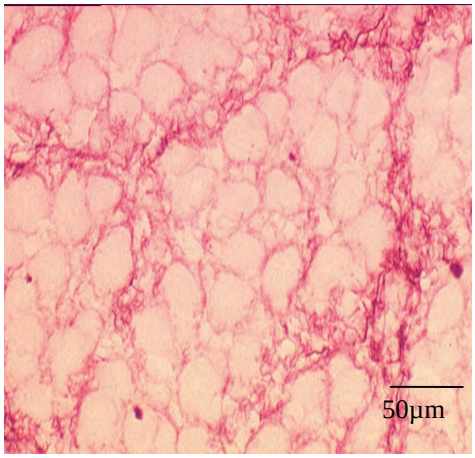


Fig. 3a Fig. 3b

Fig. 3: Sections stained with Collagen I antibody, fetuses aged 60 days of gestation. a) Smallest littermate. b) Largest littermate.

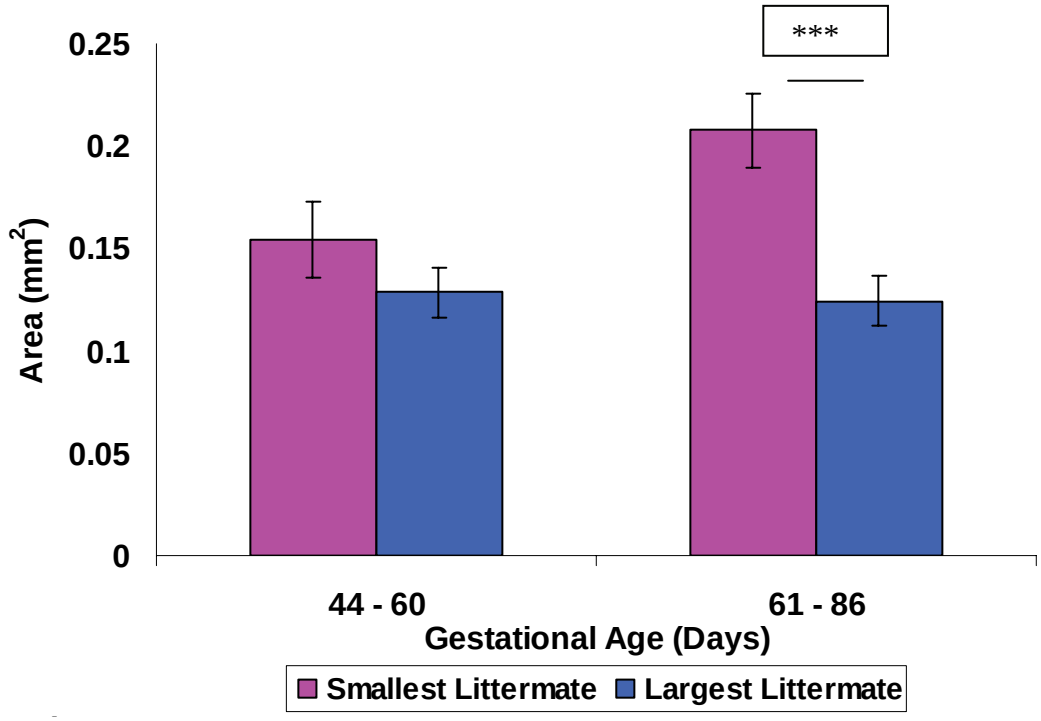
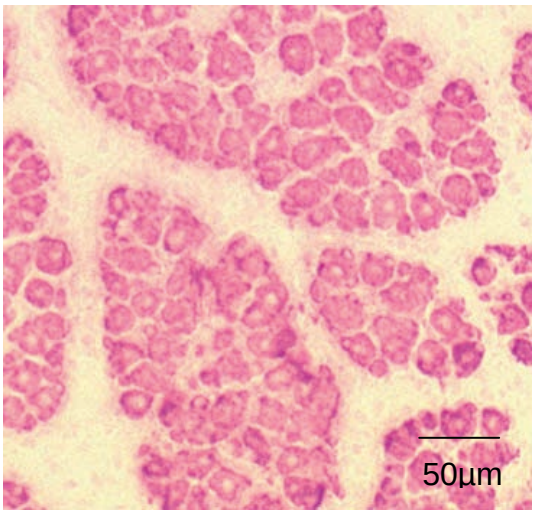


Fig. 4: Collagen I Area per mm^2 of Muscle for grouped fetal samples aged 36-60 and 61-86 days of gestation. The smallest littermate has a significantly increased amount of Collagen I present compared to the largest littermate (*P*=0.008) in the 61-86 days of gestation group.



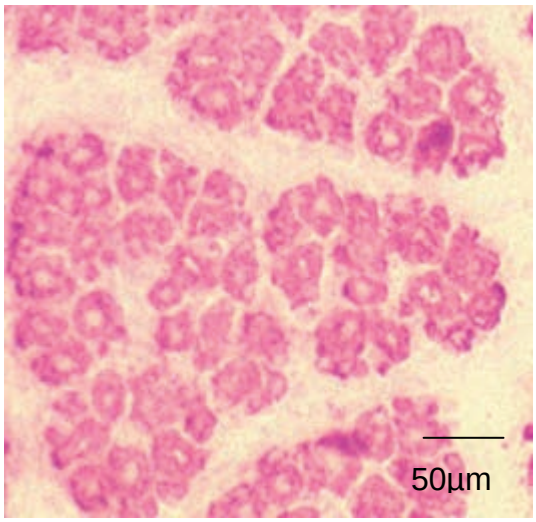
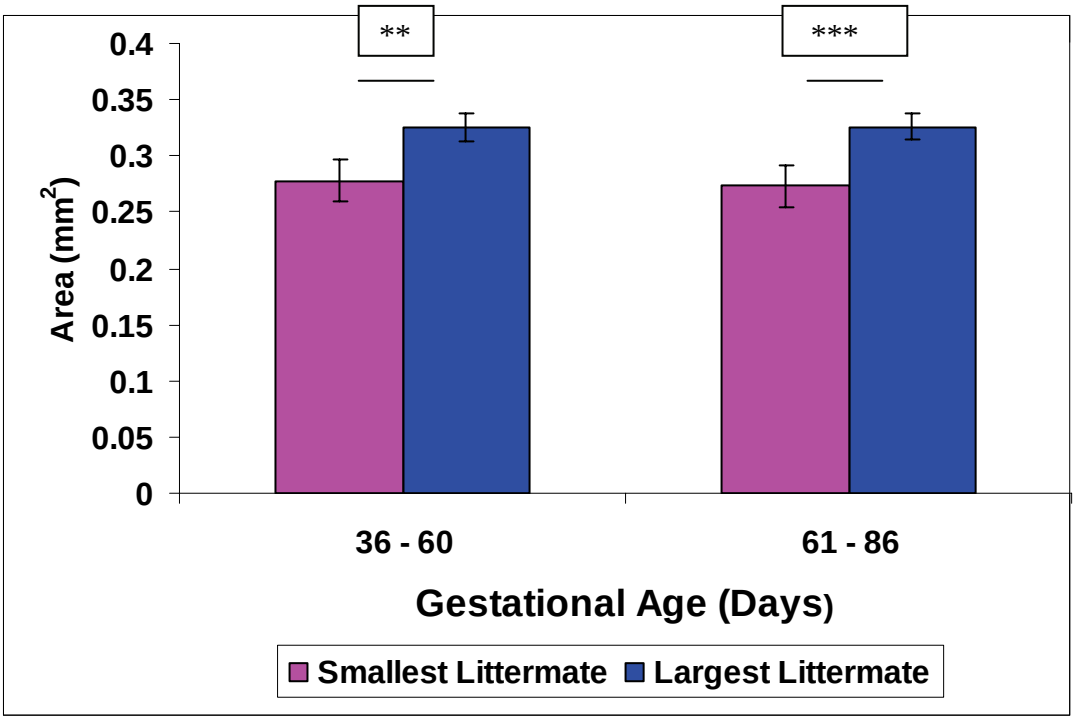


Fig. 5a Fig. 5b

Fig. 5: Sections stained with Myosin (embryonic) antibody, fetuses aged 60 days of gestation. A) Smallest littermate. B) Largest littermate.



**** p=0.04**
***** p=0.005**

Fig. 6: Myosin Area per mm^2 of Muscle for grouped fetal samples aged 36-60 and 61-86 days of gestation. The smallest littermate has an increased amount of myosin present compared to the largest littermate in the 36-60 days if gestation group (P=0.04) and the 61-86 days of gestation group (P=0.005).