Developmental programming of skeletal muscle phenotype/metabolism

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Skeletal muscle is a highly dynamic and malleable tissue that is able to adapt to different stimuli placed upon it, both during gestation and after birth, ultimately resulting in anatomical changes to muscle fibre composition. Variation in nutrient supply throughout gestation is common, whether in livestock or in the human. The specific effects of maternal nutrition on foetal development are at the forefront of scientific research. However, results describing how different maternal feeding strategies affect skeletal muscle fibre development in the offspring are not fully consistent, even where the same time windows during gestation have been examined. The aim of this study is to determine the effects of increased maternal nutrition (above the recommended levels) on the Musculus semitendinosus phenotype of progeny. In all, 24 pregnant sows were assigned to one of four feeding regimes during gestation; T1 (control group): 30 MJ digestible energy per day (MJ DE/day) throughout gestation, T2: same as that for T1 but increased to 60 MJ DE/day from 25 to 50 days of gestation (dg), T3: same as that for T1 but increased to 60 MJ DE/day from 50 to 80 dg, T4: same as that for T1 but increased nutrition to 60 MJ DE/day from 25 to 80 dg. Light- and heavy-weight littermate pairs of the same sex were selected at birth and individually fed to slaughter (c. 158 days). Histochemical and immunohistochemical staining were used to identify the predominantly oxidative (deep) and less oxidative (superficial) regions of the M. semitendinosus, and to determine total fibre number and proportions of fibre types. The results demonstrate that increased maternal nutrition alters skeletal muscle phenotype in the offspring by changing fibre-type proportions, leading to an increased oxidative capacity due to an increase in Type IIA fibres. No change in total muscle area, total muscle fibre number, or fibre cross-sectional area is observed. The precise molecular mechanism(s) by which these findings occur is being investigated.

Keywords: muscle, fibre types, nutrition, gestation, pig

Implications

The influence of maternal nutrition on skeletal muscle development has been at the forefront of scientific research for many years. The main implication of this study is meat quality, which is highly variable. Muscle phenotype characteristics have a direct effect on many meat quality parameters. Maximising meat quality would be of major benefit for the consumer and farmer, and may ultimately lead to an increased production economy. Furthermore, this study provides a model for the developmental programming of diseases such as obesity in the human, and is of relevance to the programming of muscle for athletes.

Introduction

The development of skeletal muscle is crucial to its function. Mammalian myogenesis is a biphasic process involving the formation of an early population of primary (P) fibres and a later population of secondary (S) fibres. In the pig, myogenesis encompasses an intense period of proliferation and differentiation from 35 days of gestation (dg) until approximately 90 dg. An initial rapid wave of primary embryonic myoblast fusion occurs from 35 to 55 dg, resulting in the formation of primary myotubes, which...
subsequently mature into primary muscle fibres. A longer second wave of differentiation occurs between 55 and 90 dg to produce the larger population of later-forming smaller $S$ fibres. As a direct result of the primary and secondary myogenic waves, central clusters of slow-contracting Type I fibres are surrounded by a ring of fast Type II fibres (Lefaucheur et al., 1995; Quiroz-Rothe and Rivero, 2004); a type grouping formation described as a rosette pattern or metabolic bundle. Generally, the central fibre of each cluster of Type I fibres originates from a primary myotube and develops as a slow primary muscle fibre. All other fibres originate from secondary muscle fibres (Wigmore and Stickland, 1983), which mature to either Type I or Type II fibres, depending on muscle function. The total number of fibres is determined in utero, and hence is fixed by the time of birth (Wigmore and Stickland, 1983). In the adult pig, four distinct muscle fibre types have been characterised; slow Type I and fast Type IIA, IIX and IIB (Lefaucheur, 2001). Fibre type classification is based upon the dominant myosin heavy chain (MyHC) isoform expressed by the individual fibre; MyHC$I$, MyHC$Ia$, MyHC$Ix$ or MyHC$Ib$. These largely define the biochemical and functional phenotype not only of the individual muscle fibre, but also of the whole muscle. Properties such as contractile performance, oxidative and glycolytic capacity, fibre size and lipid content co-vary between fibre types. Type I fibres are slow contracting, fatigue resistant and highly oxidative. These properties decrease through Type IIA to IIX to Type IIB fibres, which are highly glycolytic and fatigue rapidly.

Developmental programming is becoming an increasingly researched topic with respect to its influence on the variation in growth and developmental performance of an adult in later life. During the critical embryonic period, a foetus is highly responsive to abnormal environmental conditions such as a nutritional stress or stimulus. Altered foetal nutritional and endocrine status may result in developmental adaptations that permanently alter the anatomical structure, physiology and metabolism of the offspring, predisposing them to metabolic, cardiovascular and endocrine diseases in adulthood (for a review, see Wu et al., 2006). Postnatal variation in body composition and growth performance may be pre-programmed during foetal development, in utero, by both internal (e.g. genetic) and external factors (e.g. maternal nutrition).

Body composition measurements provide insight into the regulation of normal development as well as the interactions among nutritional, physiological and biochemical functions. The pig is considered to be an ideal candidate for such studies that require an animal model in which body composition at birth, and postnatal changes are comparable to those in human infants (Shulman, 1993). Furthermore, the physiological and anatomical similarity between the pig and the human species is above any other laboratory species (Hughes, 1986; Miller and Ullrey, 1987). The semitendinosus muscle (Musculus semitendinosus) is used in this study as it is well characterised with a simple fusiform structure. Anatomically, it contains two portions that exhibit striking differences in muscle fibre composition; a deep, more oxidative area and a superficial, less oxidative area. A distinct transition between the deep and superficial areas exists. The $M$. semitendinosus is commonly used to assess fibre typing and is also an important meat muscle.

Developmental intra-litter variation in utero affects the postnatal growth of skeletal muscle in the pig (Dwyer et al., 1994; Gatford et al., 2003). According to requirements, pregnant sows are restrictively fed. Ad libitum feeding of sows during early gestation shows that the voluntary feed intake is two to three times as high as the requirement (Nissen et al., 2003). It is widely accepted that maternal nutrition affects secondary muscle fibre development, hence having an impact on ultimate muscle fibre composition. The lightest littermate is shown to have a reduced muscle fibre number as a result of a decreased secondary to primary $(S:P)$ fibre ratio, compared to its heaviest littermate pair (Wigmore and Stickland, 1983). Pilot studies in our laboratory have shown that the differential expression of MyHC$Ia$ is significantly greater $(P \leq 0.05)$ in the $M$. semitendinosus of the heavyweight littermate, compared with its lightweight littermate pair between 80 and 100 dg (Ashton, 2002); the mechanism(s) by which this occurs is unknown. Many studies report the effects of nutrition on birth weight, total fibre number $(TNF)$ and $S:P$ fibre ratio, but few have outlined differences in the exact fibre type composition or the oxidative capacity of the muscle, both of which are of fundamental importance. Within an agricultural production system, the composition of fibres within a muscle can ultimately affect many meat quality parameters, e.g. tenderness (Maltin et al., 2003). Furthermore, there is evidence to suggest that restricted nutrition during gestation could have harmful consequences later in life, including reduced glucose tolerance, Type 2 diabetes and a reduced lifespan (Hales and Ozanne, 2003; Ozanne et al, 2003). Studies have linked muscle fibre type with obesity (Tanner et al., 2002), Type 2 diabetes and insulin resistance (Oberbach et al., 2006). In obese women, skeletal muscle possesses fewer Type I fibres and more Type IIB fibres than lean women, exhibiting a reduced oxidative and an increased glycolytic capacity (Hickey et al., 1995; Tanner et al., 2002). Similar observations have been made in Type 2 diabetic patients (Hickey et al., 1995; Oberbach et al., 2006).

The aim of this study was to investigate the effects of increased nutrition, throughout three different time periods of gestation, on the muscle fibre phenotype of the $M$. semitendinosus in the offspring at slaughter weight. We hypothesised that increased maternal nutrition has an influence on skeletal muscle phenotype in the offspring at slaughter weight $(c. 158$ days old), with a specific effect on the $S$ fibre population.

Material and methods

**Nutritional treatments**

A total of 24 multiparous Landrace × Large White sows were fed *ad libitum* until service, when they were randomly assigned to one of four gestational nutritional treatments;
Maternal nutrition and skeletal muscle phenotype

Table 1 Ingredient composition and nutrient content of experimental diets (g/kg)

<table>
<thead>
<tr>
<th>Diet type</th>
<th>Gestation</th>
<th>Lactation</th>
<th>Starter¹</th>
<th>Link¹</th>
<th>Weaner</th>
<th>Finisher</th>
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<tr>
<td>Steam flaked wheat</td>
<td>892.9</td>
<td>423.9</td>
<td>455.4</td>
<td>404</td>
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<td></td>
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<tr>
<td>Wheat</td>
<td>75</td>
<td>350</td>
<td>225</td>
<td>180</td>
<td>200</td>
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<tr>
<td>Soya</td>
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<td>40</td>
<td>10.0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Full fat soya</td>
<td>1.5</td>
<td>1.5</td>
<td>3.0</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Soya oil</td>
<td>0.5</td>
<td>2.0</td>
<td>4.0</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Minerals and vitamins²</td>
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<td>0.7</td>
<td>2.0</td>
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<tr>
<td>Lysine HCl³</td>
<td>4.0</td>
<td>0.8</td>
<td>1.5</td>
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<tr>
<td>α-Methionine³</td>
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<td>5.0</td>
<td>5.0</td>
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</tr>
<tr>
<td>L-Threonine³</td>
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<td>12.0</td>
<td>11.0</td>
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</tr>
<tr>
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<td>3.0</td>
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</tr>
<tr>
<td>Pulmotil4</td>
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<td>0.1</td>
<td>+</td>
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<td></td>
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</tr>
<tr>
<td>Phytase 5000 IU/⁵</td>
<td>0.1</td>
<td>0.1</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysed chemical composition (g/kg)

| Dry matter        | 871 | 873 | 870 | 870 | 872 | 870 |
| Crude protein     | 132 | 158 | 200 | 200 | 196 | 178 |
| Fat               | 31  | 56  | 90  | 75  | 43  | 27  |
| Crude fibre       | 45  | 35  | 25  | 30  | 36  | 37  |
| Ash               | 44  | 46  | 60  | 60  | 50  | 44  |
| Lysine            | 6.2 | 9.1 | 16.0| 15.0| 13.1| 11.1|
| Digestible energy (MJ/kg) | 13.0 | 14.2 | 16.3 | 15.4 | 14.1 | 13.7 |

¹Commercial diets for which the ingredient composition was not disclosed.
²Provided per kilogram of complete diet:
³Synthetic amino acids.
⁴Link diet contained 200 mg Tilmicosin per kg of feed provided from Pulmotil G100 (Eli Lilly and Company Limited, Basingstoke, Hampshire, England).
⁵Sow, weaner and finisher diets contained 500 FTU phytase per kg finished feed from Natuphos 5000 (BASF, Ludwigshafen, Germany).

(T1-control) 30 MJ digestible energy per day (MJ DE/day) throughout gestation (gestation length = 113 days), (T2) same as that for T1 but increased to 60 MJ DE/day from 25 to 50 dq, (T3) same as that for T1 but increased to 60 MJ DE/day from 50 to 80 dq and (T4) same as that for T1 but increased to 60 MJ DE/day from 25 to 80 dq (n = 6 sows/treatment; see Table 1 for details of experimental diets).

Between 110 dq and farrowing, all sows were fed 25 MJ DE/day of lactation diet (see Table 1 for diet composition). Once farrowed, sows were liquid-fed the lactation diet by a computerised feeding system (Big Dutchman, Vechta, Germany). A lactation feed curve increasing from 25 MJ DE/kg at farrowing to 95 MJ DE/kg at weaning (28 ± 0.9 days) was used, and sows fed twice daily. Sow feed intake was monitored and recorded during gestation, lactation, and between weaning and oestrus (5 ± 2.1 days after weaning). In the very rare case of a sow refusing to consume her feed, immediate removal from the experiment followed (e.g., this occurred in two cases when sows were sick). However, it must be stressed that it was rare for a sow not to consume the full daily feed allocation.

Following weaning at days 26 to 28, the lightest and heaviest littermate pairs of the same sex were selected from each litter based on birth weight (g). Within each treatment, an equal number of males (n = 6) and females (n = 6) were selected. Pigs were housed as individuals at Teagasc, Moorepark Research Centre. Each pig was fed 2 kg Easi Start (Devenish Nutrition, Belfast, Northern Ireland), 5 kg Vital (Devenish Nutrition) and weaner diet to 49 days post-weaning. Finisher diet was then fed as a liquid mix by a computerised wet feed system (Big Dutchman) from 49 days post-weaning up to slaughter at 130 days post-weaning (see Table 1 for diet compositions). Sow feed intake and growth rate (average daily gain) was recorded to slaughter at c.158 days old. Treatment had no effect on sow weight, daily gain or feed conversion efficiency (for details, see Lawlor et al., 2007).
Muscle samples
On the occasion a pig was not slaughtered with the rest of the treatment, e.g. due to sickness or being underweight, it was removed from the experiment. Consequently, a standardised n = 10 muscles from five littermate pairs were used for analyses in each treatment. The M. semitendinosus was collected from the left hind-limb of heavy- and light-weight littermate pairs (n = 10/treatment), and immediate weight, length and girth measurements were taken. A standardised complete transverse section (3 cm thick) from the midst-belly region of each muscle, encompassing both the superficial and deep areas, was excised for the purpose of histochemical and immunohistochemical analyses. Samples were quartered, mounted on to a corkboard and snap-frozen in liquid nitrogen. Ten serial transverse sections (12 μm) were obtained from each block with a Bright cryostat (Bright Instruments, Huntingdon, UK) and thaw mounted onto Superfrost slides (Fisher Scientific, Loughborough, UK). Toluidine blue basic general stain (Sigma UK Ltd, Poole, UK) was used as a visual check on the plane of the section and its quality. Sections were air-dried and slides were stored at −80°C until required for histo- and immunohisto-chemical staining.

Histochemical staining
Oxidative differentiation and muscle fibre typing: succinate dehydrogenase staining. Succinate dehydrogenase (SDH) is an enzyme located on the inner mitochondrial membrane. Its activity correlates with the total number of mitochondria, which ultimately determines muscle oxygen demand. SDH staining is stronger for Type I and IIA fibres, and it was thus used as a marker for oxidative capacity. Serial sections were stained for SDH activity according to Nachlas et al. (1957). Sections were air-dried for 30 min before being incubated in pre-heated succinate substrate solution (15 ml 0.2 M phosphate buffer solution; 15 ml 0.2 M sodium succinate solution and 30 ml tetrazolium salt) at 37°C for 20 min. Following two washes in distilled water, sections were allowed to air-dry for 30 min. They were then placed in a fixative solution (4 ml 10% formalin in 96 ml distilled water) for 10 min before being washed in tap water and mounted in glycerol jelly.

Muscle fibre typing: alkaline myosin ATPase staining. Additional sections were stained for myosin ATPase (mATPase) activity according to Guth and Samaha (1970). Alkaline pre-incubation was used at pH 10.5 for 15 min at room temperature (RT), and sections were incubated for 1 h at 37°C. Fibre types were classified using this staining method as Type I, Type IIA and Type IIB. However, fibres with 'IIb' mATPase profile may contain MyHCIIx, and so could, in fact, be classified as IIX (Lefaucheux et al., 2002; Toniolo et al., 2004).

Lipid detection: Oil Red O staining. The number of fibres containing fat was determined using the Oil Red O staining technique (Bancroft and Gamble, 2001). Stock Oil Red O solution was prepared by adding 0.5 g Oil Red O powder to 100 ml 98% isopropanol. Prior to staining, 60 ml of this solution was diluted in 40 ml distilled H₂O and left to stand for 24 h. The working solution was then filtered the following day with grade number 42 Whatman filter paper (Whatman, Maidstone, UK) to remove any Oil Red O crystals. Slides were air-dried for 30 min and stained in Oil Red O solution for 10 min. Sections were then de-differentiated in 60% isopropanol (Sigma UK Ltd), washed in distilled water and mounted in glycerol jelly.

Capillary detection: alkaline phosphatase staining. Sections were stained for alkaline phosphatase to reveal the endogenous enzyme in the capillary endothelium (Bancroft and Gamble, 2001). Frozen transverse sections were air-dried at RT for 30 min and then re-hydrated in NTMT buffer (For 50 ml buffer: 1 ml 5 M NaCl; 1.25 ml 1 M MgCl₂; 5 ml Tris-HCl pH 9.5; 500 μ1% Tween 20; 42.25 ml distilled H₂O) for 1 min. Muscle samples were stained with 18.8 mg/ml nitro blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolylphosphate toluidine salt (NBT–BCIP) in 67% DMSO (dimethyl sulphoxide) (Sigma UK Ltd, Poole, UK) stain, diluted 20 μl:: 1 ml in NTMT buffer, for 8 min at RT. NBT–BCIP is a chromogenic phosphatase substrate, producing a blue-coloured precipitate at the site of enzymatic activity. Sections were washed in NTMT buffer for 1 min and in 1× phosphate buffered saline (PBS) for a further minute, before being mounted in glycerol jelly.

Muscle fibre typing: immunohistochemical labelling of MyHCⅡa
Serial sections were stained for Type IIA fibres using SC71 monoclonal antibody (Schiaffino et al., 1989), which has been previously characterised in pig muscle to specifically stain for MyHCⅡa (Lefaucheux et al., 2002). Slides were air-dried for 30 min. Sections were fixed in 4% paraformaldehyde for 10 min, and then washed three times in 1× PBS (100 mM NaCl, 20 mM phosphate buffer, pH 7.4). Goat serum, at 5%, was used to block the sections. Monoclonal antibody SC71 was used at a concentration of 1 : 200 (previously optimised) and left to incubate overnight at RT. PBS substituting for the primary antibody served as a negative control. The following day, sections were washed three times in PBS containing 0.1% Tween (Sigma UK Ltd). A biotinylated goat anti-mouse IgG secondary antibody, diluted 1 : 500 (Sigma UK Ltd) in 5% goat serum was incubated on sections for 1 h at RT, followed by three further washes with PBS–0.1% Tween. Vectorstain ABC (avidin/biotin) kit (Vector Laboratories Ltd, Peterborough, UK) was applied to the sections for 30 min followed by three PBS washes. The colour reaction was developed using diaminobenzidine tetrahydrochloride, made up as per manufacturers’ instructions (3,3′-Diaminobenzidine (DAB) Peroxidase Substrate tablet kit; Sigma UK Ltd), on the sections for a maximum of 3 min, or until colour reaction developed, and was visualised under a microscope. The reaction was stopped in PBS and dehydrated in a progression of 30% to 100% ethanol. Slides were mounted in Di-n-butylphthalate in Xylene (DPX; Sigma UK Ltd).
Muscle areas

Whole muscle areas were established by drawing around the perimeter of the cut surface onto acetate sheet, and calculating them with a custom-made macro using Kontron Analysis Software (RVC Imaging Suite, London, UK). The transitional line between the superficial (close to the surface of the muscle) and deep (close to the bone) region of each muscle was determined upon analysis of SDH stained sections, allowing the more oxidative deep area to be distinguished from the less oxidative superficial area.

Image analysis

Morphometric analysis of histochemical and immunohistochemical staining was carried out using an interactive image analysis system (Leica Imaging Software, Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Fibre type percentage was calculated by counting 500 to 1000 fibres from random selected frames in both deep and superficial areas, from which estimations of the TNF were calculated using total muscle area values. Mean fibre cross-sectional area (CSA) was also determined from the same frames for both superficial and deep areas. Fibre shape was determined on the basis of Feret’s ratio (minimum/maximum Feret’s diameter). This is a measure of ellipticity generated by the analysis software, and serves to provide a check that sections were taken on a transverse plane. All fibres were within 10% of being a perfect circle (maximum Feret ratio of 1.0) and therefore measurements generated from them are true transverse representations.

Statistical analysis

Statistical analysis was performed on each treatment (n = 10 littermates), and where applicable on lightweight (n = 5) and heavyweight (n = 5) littermate pairs using GraphPad PRISM® version 5.0. Following initial muscle analysis (see Results for more detail), light- and heavy-weight littermate pairs were not considered as separate groups for all subsequent analyses. Furthermore, no significant gender effects were found; hence males and females were also combined within each treatment.

Data were analysed by one- or two-way ANOVA and, provided a significant F Ratio (P = 0.05) was obtained, post-hoc Bonferroni tests were performed. Comparisons of deep, superficial or whole muscle phenotype × treatment interaction generated numerical values which were presented as means ± standard error of the mean (s.e.) for 10 replicate pigs (combined light and heavy littermate pairs)/treatment.

Results

Growth analysis

Earlier pig growth and performance analysis to slaughter showed no significant effect of treatment on the mean birth weight or weaning weight of pigs (P > 0.05) (Lawlor et al., 2007). However, the separation of light- and heavy-weight littermates within each treatment demonstrates a large intra-litter variation, with a significant difference in weight seen at birth and at weaning (light v. heavy littermates) (data not shown). Interestingly, lightweight littermates were heavier at birth in the control treatment than respective littermates in all other treatments; however, by weaning no differences are seen, suggesting these littermates have undergone postnatal catch-up growth. A very similar situation is seen in the heavyweight littermates.

Muscle weight, length and girth

Maternal nutrition had no effect on the intra-litter variation of M. semitendinosus weight, length and girth (data not shown). Similarly, there was no effect of maternal nutrition on muscles from light- or heavy-weight littermates between treatments (data not shown).

Fibre typing

M. semitendinosus samples were stained for SDH activity; MyHCIIa, using SC71 monoclonal antibody; mATPase, following alkaline (pH 10.5) pre-incubation; and lipid, using Oil Red O. SDH and alkaline mATPase methods both produced three levels of staining intensity. Type I fibres are stained a green-blue colour with SDH and negative with mATPase. Type IIA fibres are purple with SDH and mid-brown with mATPase, and Type IIB fibres show very little SDH staining but are black with mATPase (Figures 1a and 2a). Staining with SC71 monoclonal antibody confirmed the identity of IIA fibres (Figures 1b and 2b).

Primary and secondary fibres were also quantified from mATPase stained sections. P fibres were characterised as those that showed inhibited ATPase activity and were also the largest fibres in the centre of the metabolic cluster. The total number of fibres minus the number of P fibres is the estimate for the number of S fibres, allowing the S:P ratio to be determined.

Comparison between stains allowed the determination of three different fibre types (Figure 1). Serial sections stained for SDH, MyHCIIa and lipids indicate that the more oxidative the muscle fibre type is, the greater is its lipid content (Figure 2). Fibres that stain positively for SDH activity correlate with those that stain positively with Oil Red O. Unfortunately, staining was not consistent enough to analyse more in-depth results on intramuscular lipid content.

Deep, superficial and total muscle area

The mean total muscle area from each treatment is outlined in Table 2; increased maternal nutrition had no significant affect on total muscle area. This agrees with the results from gross measurements of the muscle, where no significant differences in muscle weight, length or girth were found (data not shown). Staining for SDH activity was used to identify the transitional line between the deep (oxidative rich) and superficial (less oxidative) regions of the muscle, allowing their percentage areas to be established (Table 2). All treatments significantly increased the deep area of the muscle compared with control, with the greatest increase seen in T2 (P ≤ 0.001).

Total number of fibres

Increased maternal nutrition had no significant effect on the TNF in the deep, superficial and whole M. semitendinosus

Maternal nutrition and skeletal muscle phenotype
area, although an increase was evident in all treatments v. control (Figure 3a).

**Muscle fibre cross-sectional area**
The mean CSA of a representative sample of all fibre types present, and specifically of type IIA muscle fibres, were not influenced by increased maternal nutrition in the deep, superficial or whole *M. semitendinosus* area (Table 3).

**Oxidative capacity**
Staining for SDH activity allowed confirmation of oxidative and non-oxidative fibre type proportions (Figure 3b) and numbers (Figure 3c), as well as the identification of the areas of the deep and superficial regions of the muscle (Table 2).

A significant increase in oxidative fibre type proportion was seen in T2 in the whole muscle area \( (P < 0.05) \) and in the deep portion \( (P < 0.01) \). A trend increase is also seen in the whole muscle in T4 \( (P = 0.08) \) (Figure 3b). Similarly,
muscles from all treatments showed an increase in the number of oxidative fibres in the deep and whole muscle area compared with control, with significance in both T2 (P < 0.01) and T4 (P < 0.05). No significant effects were seen within the superficial portion (Figure 3c).

**MyHCIIa antibody staining**

Immunohistochemical staining with monoclonal antibody SC71 confirmed the histochemical identification of IIA fibres (Figures 1b and 2b). All treatments increased IIA fibre proportion compared with control in the deep, superficial and whole muscle areas. T2 had the greatest effect, increasing IIA fibre proportion by 7% (P < 0.001) in the deep area and 6% (P < 0.01) in the whole muscle (Figure 3d). A significant increase was also evident in the deep portion in T3, with an increase of 4% from control, and in the superficial portion and whole muscle in T4, with an increase of 5% and 4.5% IIA fibres respectively, compared to control (P = 0.01).

Furthermore, T2 had the greatest significant effect on the mean absolute number of IIA fibres. An increase above all other treatments was seen in the deep portion (P = 0.001 v. control) and in the whole muscle area (P = 0.01 v. control) (Figure 3e).

**S : P ratio and primary fibre number (mATPase staining)**

No significant effects on the S : P ratio were seen (Figure 3f); however, an increase is evident in the deep and whole M. semitendinosus area in all treatments, compared with control. Similarly, increased maternal nutrition had no significant effect on the P fibre number (Figure 3g). As a trend increase is seen in the TNF but no change in P fibre numbers, a trend increase in S : P ratio is in accordance.

**Type I fibres (mATPase staining)**

Type I fibre type proportions are described in Figure 3h, and are consistent with earlier findings that they range from approximately 4% in the superficial portion to 45% in the deep portion (Beermann et al., 1990). Both the proportion and absolute number of Type I fibres were not affected by increased maternal nutrition in the whole muscle. However, a significant decrease was seen in Type I fibre proportion and absolute number in the superficial portion in all treatments (Figure 3h and i).

**Capillary detection (alkaline phosphatase staining)**

Staining for alkaline phosphatase activity allowed for the detection of capillaries supplying the muscle. Oxidative fibres (Type I and IIA) have a greater capillary density than less oxidative fibres (Type IIX and IIB) (Hudlicka et al., 1992). The increased capillary density seen in the deep, more oxidative area compared with the superficial, less oxidative area can clearly be seen in Figure 4a and b, respectively. Increased maternal nutrition had no effect on capillary number or capillary to fibre (C : F) ratio compared with control in any part of the M. semitendinosus (Figure 4c). However, a greater C : F ratio is seen in the deep portion of muscles from T2 compared with T3 and T4 (Figure 4d).

**Discussion**

The aim of these studies was to determine the effects of increased maternal nutrition during three different gestational time windows on M. semitendinosus phenotype at slaughter weight. The control treatment delivers a constant level of nutrition (30 MJ DE/day) throughout the entirety of gestation; T2 delivers increased nutrition from 25 to 50 dg, which is immediately before the onset of secondary muscle fibre formation; T3 increases maternal nutrition during secondary muscle fibre formation (50 to 80 dg); and T4 encompasses both developmental time periods (25 to 80 dg). Previously reported data have shown that increased feeding throughout gestation did not affect the birth weight, weaning weight, or intra-litter variation in this study (Lawlor et al., 2007). Furthermore, post-weaning weight up until slaughter, actual slaughter weight and the carcass weight were not affected.

A number of studies have investigated the specific effects of maternal nutrition on foetal muscle development (Dwyer et al., 1994; Gatford et al., 2003; Nissen et al., 2003; Bee, 2004). However, results describing how different maternal feeding strategies affect muscle fibre development in the offspring are not consistent, even where the same time windows during gestation have been examined. This study administers increased maternal feed intake to double that of control (30 MJ DE/day), a level that is approximately 50% of ad libitum. The sows’ requirement for energy during gestation is 15–25 MJ net energy per day (MJ NE/day) (Gatford et al., 2003; Nissen et al., 2003). The feeding regime in our study delivers 21.25 MJ NE/day in the control treatment (compliant with requirements), which is increased to 42.5 MJ NE/day in T2, T3 and T4, representing a value that can be argued to correspond to ad libitum levels.
The findings of these studies outline the effect of increased maternal nutrition on skeletal muscle phenotype in the offspring at slaughter weight, with a particular focus on muscle fibre type and oxidative capacity. Increasing the level of maternal intake at all three time periods significantly increased the area comprising the more oxidative deep portion of the *M. semitendinosus*, with no change in total muscle area. This latter finding is supported by analyses of the gross measurements of the *M. semitendinosus*, which find that increased maternal nutrition did not influence muscle weight, length or girth. The most significant increase in deep area is seen in the progeny of sows fed increased nutrition from 25 to 50 dg (T2). No significant effects are seen on the TNF. Earlier findings report that the TNF is decreased in the smallest littermate (excluding runts) as a result of *in utero* or ‘natural’ under-nutrition (Wigmore

**Figure 3** The effect of increased gestational nutrition on the (a) mean total fibre number, (b) proportion of oxidative fibres, (c) absolute number of oxidative fibres, (d) proportion of Type IIA fibres, (e) absolute number of Type IIA fibres, (f) S: P (secondary to primary fibre) ratio, (g) absolute number of primary fibres, (h) proportion of Type I fibres and (i) absolute number of Type I fibres in the deep, superficial, and whole *M. semitendinosus* area. Serial sections were stained for succinate dehydrogenase (SDH) activity, mATPase activity with alkaline (pH 10.5) pre-incubation, and myosin heavy chain (MyHC)-IIa with SC71 monoclonal antibody. □ = T1 (Control); ■ = T2; □ = T3; ■ = T4. Numerical values are expressed as arithmetic means ± s.e.; n = 10/ treatment. Within each muscle area, means without a common letter are significantly different (*P* < 0.05).
This often results in a reduced TNF with a larger cross-sectional area. From a meat production perspective, previous studies have shown that pigs which exhibit a greater number of muscle fibres, with a small CSA, correlate with faster and more efficient growth, a leaner carcass and a better meat quality (Dwyer and Stickland, 1991; Oksbjerg et al., 2000). Our findings show no significant difference in TNF, \( S : P \) ratio or fibre CSA due to an increased level of maternal nutrition, and are consistent with earlier studies (Nissen et al., 2003; Bee, 2004) that found that increased maternal feed intake had no effect on \( M. \) semitendinosus fibre number or area in offspring at slaughter weight.

An increase in the proportion of IIA fibres is seen in all treatments in all areas of the muscle. This is seen to be significant in TNF, \( S : P \) ratio or fibre CSA due to an increased level of maternal nutrition, and are consistent with earlier studies (Nissen et al., 2003; Bee, 2004) that found that increased maternal feed intake had no effect on \( M. \) semitendinosus fibre number or area in offspring at slaughter weight.

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>1 (control)</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole muscle</td>
<td>6754.22 (±188.2)(^a)</td>
<td>6516.16 (±648.1)(^a)</td>
<td>6646.53 (±194.2)(^a)</td>
<td>6432.50 (±184.1)(^a)</td>
</tr>
<tr>
<td>Deep</td>
<td>6688.11 (±269.8)(^a)</td>
<td>6652.23 (±1152.9)(^a)</td>
<td>6460.09 (±257.5)(^a)</td>
<td>6402.35 (±228.3)(^a)</td>
</tr>
<tr>
<td>Superficial</td>
<td>6843.67 (±252.4)(^a)</td>
<td>6412.88 (±353.7)(^a)</td>
<td>6389.97 (±291.8)(^a)</td>
<td>6443.39 (±289.7)(^a)</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole muscle</td>
<td>5693.38 (±171.9)(^a)</td>
<td>5525.95 (±173.4)(^a)</td>
<td>5323.20 (±162.1)(^a)</td>
<td>5385.65 (±145.9)(^a)</td>
</tr>
<tr>
<td>Deep</td>
<td>5385.87 (±193.8)(^a)</td>
<td>5313.79 (±248.7)(^a)</td>
<td>5135.71 (±180.6)(^a)</td>
<td>5285.80 (±190.2)(^a)</td>
</tr>
<tr>
<td>Superficial</td>
<td>6271.28 (±324.1)(^a)</td>
<td>5822.31 (±225.4)(^a)</td>
<td>5659.01 (±314.2)(^a)</td>
<td>5737.52 (±227.7)(^a)</td>
</tr>
</tbody>
</table>

Numerical values are expressed as arithmetic means \( ± \) s.e.; \( n = 10/treatment \). Within each muscle area, means without a common letter are significantly different (\( P < 0.05 \)).

### Figure 4

Postnatal \( M. \) semitendinosus capillary staining analysis. The deep area (a) is dense in capillaries, compared to the superficial, less oxidative area (b), which has much fewer capillaries. Capillaries stain blue with alkaline phosphatase staining. Magnification is \( × 100 \) and scale bar is shown in (a). The effect of increased maternal nutrition on the capillary number (c) and C : F (capillary to fibre) ratio (d) in the deep, superficial and whole \( M. \) semitendinosus areas. \( \square = T1 \) (Control); \( \square = T2; \square = T3; \square = T4 \). Numerical values are expressed as arithmetic means \( ± \) s.e.; \( n = 10/treatment \). Within each muscle area, means without a common letter are significantly different (\( P < 0.05 \)).
particularly significant in T2, in which the mean absolute number of IIA fibres is also significantly increased above control in the deep portion and whole muscle area. Pilot studies have shown that at 80 to 100 dg, the heavyweight foetus contains more IIA fibres in both deep and superficial areas (Ashton, 2002). Here we have shown that at 6 months of age, elevated maternal nutrition between 25 and 50 dg significantly increases the proportion of IIA fibres in the *M. semitendinosus* in the offspring. Similarly, the oxidative fibre proportion and number is significantly increased above the control treatment in the deep portion, and the whole muscle in T2. No treatment effects are seen on the proportion and number of Type I fibres, and therefore the oxidative fibre number, and hence oxidative capacity is increased in the *M. semitendinosus* due to an increase in Type IIA fibres and not Type I fibres.

The number of Type I fibres was significantly reduced in the superficial portion in all treatments. In conjunction with a reduced percentage area, the superficial portion showed no change in TNF or P fibre number, but a significant increase in IIA fibre proportion. The decreased number of Type I fibres and increase in Type IIA fibres explains why no overall change in oxidative fibre type proportion was seen.

Primary fibre number is believed to be a fixed, genetic effect (Wigmore and Stickland, 1983). The S : P ratio is used to measure the effect of nutrition on S fibres, excluding the genetic component associated with P fibres. The ratio was not affected by increased maternal nutrition in this study. This is inconsistent with earlier findings (Dwyer et al., 1994) where a significant increase in the S : P ratio was found due to increased maternal nutrition from 25 to 50, 50 to 80 and 25 to 80 dg. These findings were explained by an increased number of S fibres, with no effect on P fibre number seen. However, in this study, there was also no significant effect on TNF, muscle weight or muscle and fibre CSA, and hence a significant effect on S : P ratio would not be expected.

The results of this study suggest that as increased maternal nutrition enhances the oxidative capacity of the *M. semitendinosus*, the lipid content within the fibres is also increased. This is evident in the correlation between fibres that stained positive histochemically for Oil Red O (lipid) and SDH activity (oxidative capacity). The more oxidative fibre types (Type I and IIA) have an increased lipid content compared to less oxidative fibre types (Type IIX and IIB). It has been shown that metabolic phenotype can be altered by stimuli other than increased maternal nutrition. Endurance training (Fluck and Hoppeler, 2003) and electrical stimulation (Sutherland et al., 2006) both increase the oxidative metabolism of muscle. Fast to slow MyHC isoform transitions in the order MyHC-IIIb, -IIX, -Ia, to MyHCα occur in parallel with increases in oxidative activity, due to an increase in the number of mitochondria. With endurance training, fat within individual muscle fibres increases in conjunction with the increase in oxidative metabolism as an adaptation of the body to acquire more readily available fuel (Hoppeler, 1999). The underlying mechanisms occurring in these studies may also be acting in the model used in this study, in order to achieve an increased oxidative metabolism.

As with lipid content, more oxidative fibres (Type I and IIA) have a greater capillary density than less oxidative fibres (Type IIX and IIB) (Hudlicka et al., 1992). Angiogenesis and oxidative capacity are independently regulated (Egginton and Hudlicka, 2000). Capillary density, and to a lesser extent C : F ratio, are dependent upon fibre CSA (Deveci et al., 2001). Where an increase in fibre CSA is evident, an increase in capillary number also occurs. Muscle fibre CSA was not significantly affected by increased maternal nutrition. Accordingly, maternal nutrition had no effect on capillary number. If no change in fibre CSA is seen, as in this study, the C : F ratio provides a good indication of angiogenesis. However, the C : F ratio was also unaffected by increased maternal nutrition, but as no change in the TNF or capillary number was seen, a significant effect on the C : F ratio would not be expected. Therefore, although an increase in oxidative metabolism is seen via an increase in the number of Type IIA fibres present and an increased deep area of the muscle, this is not paralleled with an alteration in the capillary supply.

Increasing maternal feed intake from 25 to 80 dg (T4) appears to have no more beneficial effect than increasing it from 25 to 50 dg on the percentage deep area, S fibre number (S : P ratio), and oxidative and IIA fibre proportion or number. In fact, although it encompasses the critical initial time period of 25 to 50 dg, T4 is not as effective at influencing muscle phenotype as T2. The reasons for this are unknown. Although contradictory to the results found in our study, a study by Nissen et al. (2003) suggests that increased maternal feeding can have disadvantageous effects on muscle phenotype at slaughter weight. Therefore, it can be suggested that in our study, prolonging increased feeding from 50 to 80 dg may decrease the increases seen in T2 back to control, having a balancing or ‘negative’ effect. Increased feeding from 50 to 80 dg (T3) has no significant influence on skeletal muscle phenotype, supporting this idea. However, it must be stressed that this is purely speculation.

From these data, it is concluded that increased maternal nutrition alters skeletal muscle phenotype proportions in the offspring, with a resulting increase in oxidative capacity. The percentage area comprising the deep, more oxidative portion of the *M. semitendinosus* was significantly increased, as well as the oxidative and IIA fibre type proportion, and S : P ratio. These findings were most significant in T2 and are consistent with other studies which find the time period between 25 to 50 dg as most sensitive to the effects of the plane of nutrition (Dwyer et al., 1994; Gatford et al., 2003). This is the most critical time for stimulating the proliferation of presumptive secondary myoblasts, providing the potential for manipulation of overall muscle fibre type composition. Hence, nutrition level in the period immediately before the appearance of secondary myotubes may be more important than that during the actual appearance of muscle fibres, as proposed earlier (Dwyer et al., 1994), and may act to exert an effect on the...
population of foetal myoblasts available for the development of S fibres.

The mechanisms underlying the altered oxidative metabolism in the M. semitendinosus of slaughter weight offspring due to increased maternal nutrition are as yet unknown. A number of studies have implicated various growth- and transcription-factors in the regulation of oxidative metabolism and the oxidative fibre types (Chin et al., 1998; Hughes et al., 1999; Luquet et al., 2003; Gregorevic et al., 2004; Khorram et al., 2007), some of which are shown to be influenced by nutritional status. Altered organ or organ system structure during organogenesis could permanently affect an organism’s metabolism. This developmental ‘programming’ could be explained by the ability of individual cells to generate and respond to external signals within the organism. The precise molecular mechanism(s) by which these effects occur are being pursued.

This study shows changes at the muscle tissue level but not at the whole animal level (Lawlor et al., 2007). This suggests that the pigs may have been pushed to their limit in terms of factors such as birth weight and weaning weight, as a result of the high level of genetic selection. With regard to phenotype, the TNF is also unaffected by increased maternal energy. The changes that do occur in the muscle are associated with muscle fibre type composition. This indicates that the fibre type proportions are still malleable, but the TNF and whole animal growth factors may have reached their limit and cannot be increased any further by nutrition. In order to view differences at the whole animal level, it may be necessary to use less selected pigs. Limitations to this study include not controlling for the quantity of milk received by the piglets during the month of nursing, following farrowing and prior to weaning. Differences in intake during this time may have contributed towards the results seen.

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