PROGRAMMING OF GLUCOSE-INSULIN METABOLISM IN ADULT SHEEP
AFTER MATERNAL UNDERNUTRITION

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Running title: Gardner et al: Prenatal undernutrition and adult glucose
metabolism

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Abstract

The present study examines the effects of late vs. early gestation undernutrition on adult glucose-insulin homeostasis in sheep, and investigates whether the lower birth weight of twins alters glucose-insulin handling in adult life. Pregnant sheep were fed to requirement (100% intake) from day 0 of gestation to term (~147 dGA, control singles [CS] n=5; control twins [CT] n=5) or to 50% requirement from day 0-30 dGA (NRE, n=5) or day 110-term (NRL, n=4). At all other times NR sheep received 100% intake. All sheep lambed naturally; offspring were weaned at 10 weeks, and reared on pasture until 1 year of age. At this time, indwelling catheters were inserted and 2-4 days later basal metabolic and endocrine status and responses to an IVGTT and feeding were assessed. Adipose and skeletal muscle were then sampled after humane euthanasia and analysed for expression of insulin signalling proteins and GLUT4. Between groups, birth weight of singletons was similar and increased relative to twins. At one year of age, weights were similar between groups. The AUC for glucose and insulin during the IVGTT were greater in NRL vs. other groups, indicating glucose intolerance. This was associated with reduced adipose, but not muscle, GLUT4 and increased adipose tissue mass. Adult glucose-insulin homeostasis in sheep was unaffected by fetal number. In conclusion prenatal undernutrition, specifically during late gestation, affects adult offspring intermediary metabolism, in particular, glucose-insulin homeostasis.

Keywords: Type 2 diabetes, Maternal Nutrition, Adipose, GTT, Sheep, Leptin,
Abbreviations:

IVGTT, Intravenous Glucose Tolerance Test

NR, Nutrient restriction

NRE, Nutrient restriction during early gestation

NRL, Nutrient restriction during late gestation

NEFA, Non-esterified fatty acids
**Introduction**

Type 2 diabetes (T2D) and related risk factors such as obesity, insulin resistance and glucose intolerance have all increased in frequency in westernised populations over recent years and are predicted to continue increasing unabated (11). The rapid progression of these non-communicable metabolic disorders within a generation suggests an environmental rather than genetic aetiology. In the last 10 years it has been suggested, and since corroborated by retrospective human and prospective animal trials, that the risk of developing ‘metabolic syndrome’ in middle age has its origins early in life; specifically during fetal and early postnatal development (5; 22).

Some of the most compelling data regarding the developmental programming of obesity and related metabolic disorders has come from retrospective studies examining a proportion of the Dutch population exposed to famine during World War II – the ‘Dutch Hunger Winter Famine’. Very early on it was realised that the period of famine exposure (3-4 months) produced different outcomes in the adult offspring, dependent upon when the famine conditions were endured during pregnancy (36). For example, exposure during the first trimester of pregnancy only, gave rise to offspring with increased risk of coronary heart disease (CHD odds ratio, 8.8%), compared to exposure during mid-, late-gestation or non-exposed individuals (mid, 0.9%; late, 2.5%; non-exposed, 3.0% (37)). In contrast, famine exposure during late gestation only tended to impact upon intermediary metabolism, in particular, glucose-insulin homeostasis (35). Such effects can be readily explained when one considers the chronological development of system/organ growth in the fetus; cardiovascular growth has a priority early in
gestation, with adipose and muscle growth (highly insulin sensitive tissues) occurring relatively late.

Animal studies have, in part, replicated these original hypotheses and shown that undernutrition confined to early gestation may impact upon adult cardiovascular function (19; 27), whereas undernutrition throughout gestation and/or lactation produces both hypertensive (26) and insulin resistant (16; 32) offspring. Surprisingly, to date, no animal study has investigated adult glucose-insulin metabolism after prenatal undernutrition confined to late gestation alone, nor to have directly compared the effect of early vs. late undernutrition on aspects of glucose-insulin intermediary metabolism in the resultant adult offspring. The current study was therefore established to directly address the hypothesis that late, rather than early, gestation undernutrition has the greatest impact on adult glucose-insulin homeostasis.

In addition, one of the many criticisms levelled at the developmental origins of adult disease hypothesis concerns the incidence of non-communicable disease in twins; which are generally of lower weight at birth (7), but do not seem to exhibit a higher risk for either cardiovascular or metabolic disease (4; 8; 9; 23). While aspects of intrauterine development, predominantly endocrinological (13; 18), are clearly different between singletons and twins, there is little evidence to support a programmed effect on cardiovascular function after maternal undernutrition (19). Follow up studies in humans have shown that irrespective of birth weight twins have a reduction in insulin sensitivity (24) as well as differences in blood pressure regulation (24). The use of twins as a model for investigating the effect of uterine
growth retardation on the developmental origins of later disease is therefore contentious. However, as long term nutritional programming effects can be observed in the absence of any effect on birth weight (6) twin studies can be useful in determining the relative impact of size at birth as opposed to a reduction in maternal food intake. In this regard there is very little experimental information on adult intermediary metabolism in comparable singletons and twins and therefore, in the current study, we include a subgroup of twins born to nutritionally replete mothers to test the hypothesis that programming of adult glucose-insulin handling and sensitivity is independent of fetal number and thus reduced birth weight.
Materials and methods

Animals. All procedures were performed under the UK Animals (Scientific Procedures) Act, 1986 and the general principles of laboratory animal care were strictly followed (29). Nineteen blue-faced Leicester x Swaledale (Mule) sheep from the University of Nottingham’s commercial flock were used in the study. Sheep were mated during their natural breeding season without the use of ovarian stimulants. After mating sheep were individually housed and randomly assigned to receive one of three diets during pregnancy; 1) 100% metabolisable energy (ME) requirements as defined by the Agricultural and Food Research Council (AFRC; (1)) throughout gestation to term (Controls; n=10); 2) 50% AFRC ME requirement from day 1-30 gestation (nutrient restriction – early; NRE, n=5) or 3) 50% AFRC ME requirement from day 110-term (nutrient restriction – late; NRL, n=4). All NR sheep received 100% ME requirement at all other times during gestation. Of the ten C sheep five bore singletons and five bore twins. Thus four groups were formed in total 1) control singletons (CS, 46.1 ± 1.8 kg ewe weight at mating), 2) control twins (CT, 44.8 ± 1.5 kg), 3) early nutrient restricted singles (NRE, 45.9 ± 1.9 kg) and 4) late nutrient restricted singles (NRL, 58.4 ± 2.2 kg). NRE and NRL all bore singletons. The diet for all control and NR sheep contained an adequate amount of vitamins, with minerals provided in the form of mineral blocks and comprised 75% chopped hay (ME, 8.5 MJ/kg dry matter (DM) and a crude protein content of 85 g/kg DM) and 25% concentrate pellets (ME, 8.9 MJ/kg DM; protein, 218 g/kg DM). The nutritional regimen for each ewe was adjusted accordingly throughout gestation to allow for ewe weight gain, fetal number and conceptus growth. At term (~147 days gestation), lambs were delivered naturally and birth weights recorded. Any twin lambs delivered were reared as singletons with the ewe to standardise
postnatal nutritional status. The sex ratio for each group was (numbers of male/female): 1) CS (3/2), 2) CT (2/3), 3) NRE (1/4), 4) NRL (2/2). The offspring were ewe reared until weaning at 3 months of age and thereafter grass-fed until one year of age. During this time, the weights and growth of individual lambs were recorded monthly.

At 11 months of age, all sheep were brought indoors in preparation for the surgical implantation of catheters and experimental studies. For 24 h prior to surgery all food, but not water, was withdrawn from the animals. Surgical details and post-operative handling of these animals were as described previously (19). In brief, carotid and jugular catheters were inserted under anaesthesia (1-2% halothane in 50:50 O2/N2O) with appropriate antibiotic (15 mg.kg⁻¹ I.M. amoxycillin, ‘Duphamox’; Fort Dodge Animal Health Ltd, Southampton, UK) and analgesic coverage (1 mg.kg⁻¹ flunixin meglumine; ‘Finadyne’; Shering-Plough, Kenilworth, UK) post-operatively. Catheters were kept patent by daily flushing with heparinized saline (50 I.U. heparin.ml⁻¹).

**Experimental protocols.** No experiment was performed until after 2-4 days post-operative recovery. The investigator was blinded to the dietary origin of the sheep prior to any experiment being performed. In all sheep 2 experiments were conducted on separate days:

Experiment 1. *Metabolic response to feeding*. Both hay and concentrate were withheld from the sheep for a period of 24 hr. The following morning two baseline blood samples (2ml; -10 and –5 min) were taken prior to feeding then subsequently at 5, 15, 30, 45 and 60 min after feed was given at time zero which coincides with the rapid switch from a dependence on lipid to carbohydrate metabolism in
ruminants (38). All concentrate feed for all animals was consumed within 10 min and blood samples handled as previously described for experiment 1. The combined 45 and 60 min sample was used for a paired comparison of fasted i.e. mean of baseline samples (-10 and –5 min) vs. fed state (45 and 60 min sample).

Experiment 2. *Intravenous Glucose Tolerance Test* (IVGTT). Both hay and concentrate were withheld from the sheep for a period of 24 hr. The following morning two baseline blood samples (2ml; at –10 and –5 min) were collected into chilled heparinised tubes prior to feeding, then subsequently at 2, 5, 10, 15, 20, 30, 40, 60, 80, 120, 150, 180, 210 and 240 min after 0.5g/kg glucose was administered (I.V. in 0.9% NaCl) within 2 min, through the jugular catheter at time zero. All blood samples were centrifuged immediately for 5min at 4°C and, after decanting of the supernatant plasma, were stored at –20°C for further analysis of plasma glucose, insulin, and non-esterified fatty acids (NEFA) concentrations. In two sheep, a similar volume (~45ml) of 0.9% NaCl only was administered as a control. Values for glucose, insulin and NEFA remained unchanged from baseline in these sheep (data not shown).

**Biochemical analyses.** Plasma concentrations of glucose and NEFA were measured enzymatically as described by Symonds *et al* (39) whilst plasma triglycerides were determined using a commercial colormetric kit (“GPO-PAP”; TR212 Randox Laboratories, Crumlin, Co Antrim, UK) adapted for use with 96-well plates. The intra- and interassay coefficients of variation were 6.4 and 9.6% (n=10 and 8). Plasma cortisol and leptin were both determined by RIA as previously described in detail (6; 10). *Insulin* – 50µl plasma was assayed in duplicate using a commercially available coated-tube assay (“INSI-CTK IRMA”; Diasorin, Wokingham,
UK). The assay uses human insulin as the reference standard and has a minimum detection limit of 4.3 pmol L⁻¹. The intra-assay variation was <5%. All samples for insulin were assayed at the same time with the same standard curve.

**Molecular analyses.** At the end of all experimental protocols the one-year old sheep were humanely euthanased with a lethal dose of sodium pentobarbitone (Euthatal; 100mg/kg). All major organ weights were recorded and tissue samples flash frozen in liquid nitrogen and stored at -80°C. A portion of perirenal fat and skeletal muscle were also collected for western blotting as previously described for the rat (16) but with the following modifications for sheep: the cleared protein lysates from each animal were standardised to a final concentration of 0.5 mg/ml in Laemmlli's sample buffer and equal amounts of protein for each animal (10 μg) were loaded onto 10 % SDS polyacrylamide gels for separation by electrophoresis. The ovine antibodies used in this study were to insulin receptor βsubunit (Irβ; Santa Cruz sc-711; Autogen Bioclear, UK); PI 3-kinase p110 beta subunit (p110β; Santa Cruz); PI 3-kinase p85 alpha subunit (p85α; Upstate Biotech); GLUT4 (Abcam Ltd, Cambridgeshire, UK). Secondary antibodies and an antibody binding (ECL) kit were both obtained from Amersham, UK.

**Calculations.** For the IVGTT a glucose and insulin curve was plotted. From this plot of the rapid increases and gradual decay of glucose, and subsequently insulin, a value for area under the curve was derived using the GraphPad Prism software (Version 3, Graphpad). Variables derived were: 1) the maximum change in glucose/insulin – calculated as the maximum value achieved minus the average baseline value (Glu/Ins Δmax), 2) time to restore fasting baseline glucose/insulin –
calculated from the intercept of the slopes for the glucose/insulin curves with
fasting glucose/insulin concentration (Glu/Ins_{t\text{base}}), 3) Glucose tolerance - measured
as the area above fasting plasma glucose under the plasma glucose curve (Glu_{auc};
min.mmol.L^{-1}), 4) Absolute insulin secretion - measured as the area above fasting
plasma insulin under the plasma insulin curve (Ins_{auc}; \text{min.nmol.L^{-1}}), 5) Relative
insulin secretion (nmol/mmol.L^{-1}) – calculated as the absolute insulin secretion
divided by glucose intolerance 6) Glucose clearance rate (mmol.min^{-1}) – calculated
as (t_{\text{base}} \text{ minus time at peak glucose}) divided by the peak glucose achieved
(Glu_{\text{clearance}}).

**Statistical analyses.** All data are expressed as Means ± S.E.M. unless otherwise
stated. The effects of nutritional group on measures of postnatal growth rates,
glucose homeostasis, insulin secretion and plasma metabolites or hormones were
analysed by univariate general linear model with group and sex as fixed effects
using SPSS version 11.5.2 (SPSS Inc, Chicago, IL). Relationships between pairs of
variables were analysed by Pearson’s correlation across all animals (SPSS v11.5).
Descriptive data for maternal weight, food intake, and birth weight were analysed
by one-way analysis of variance (SPSS v11.5). For a comparison between hormone
concentrations in the ‘fed’ and ‘fasted’ state a paired \textit{t}-test was employed. For all
comparisons, statistical significance was accepted when \textit{P}<0.05.
Results

**Gestation and postnatal growth:** Control sheep consumed from 7.6±0.3 MJ/day at week 1 of pregnancy to 15.8±0.9 (CS, carrying singles) and 18.9±0.8 (CT, carrying twins) MJ/day at week 21 of pregnancy. NR1-30 and NR 110-140 sheep consumed 50% of that amount for the first 30 days (3.61±0.11 MJ/day) or days 110-140 (8.67±0.15 MJ/day), respectively. At all other times, the undernourished groups consumed 100% of control intake. Birth weights were similar between singleton lambs in all groups (CS, 4.4±0.4 kg; NRE, 4.2±0.3 kg; NRL 4.9±0.3 kg) but control-fed twin lambs were significantly smaller at birth (CT, 3.4±0.2 kg). There was no difference in the growth rates (current weight-birth weight/time) of lambs in each group during the first 3 months (0-3 months), second 3 months (4-6 months) and between 7-12 months: CS, 1.66±0.31, 1.27±0.15, 0.92±0.07 kg/week respectively; CT, 1.99±0.16, 1.47±0.09, 0.89±0.11; NRE, 1.62±0.18, 1.32±0.08, 0.88±0.09; NRL, 2.62±0.19, 2.01±0.08, 1.02±0.09. The rates of growth in all groups slowed over time ($P<0.001$). At one year of age, when the metabolic studies were undertaken, there was no difference in body weight between the groups of sheep (CS, 47.1±4.0; CT, 50.2±5.9; NRE, 49.8±4.3; NRL, 58±5 kg).

**Fed vs fasted metabolite and leptin concentrations:** The fed and fasted plasma concentrations of metabolites and leptin are given in Table 1. Fasted plasma glucose concentration was similar between groups and values did not change significantly during feeding. In contrast, plasma NEFA significantly decreased from fasted values during feeding in CS ($P=0.01$) and NRE ($P=0.002$) but no effect was observed in NRL. There was no effect of fasting or feeding on plasma triglycerides or leptin. Fasted values for plasma leptin and triglycerides showed a strong positive
correlation with each other (r=0.74, P<0.01). Gender had no effect on any metabolite or hormone values as assessed using a univariate general linear model with sex as a fixed influence. Similarly basal plasma cortisol concentration was unaffected by any of the variables assessed (e.g. CS, 31.6±7.5; CT, 29.5 ± 5.5; NRE, 42.7 ± 7.0; NRL, 35.7 ± 12.7 nmol.L⁻¹).

**Intravenous glucose tolerance test (IVGTT):** Baseline plasma glucose (CS, 5.11±0.43; CT, 5.35±0.35; NRE, 5.42±0.52; NRL, 5.25±0.66 mmol.L⁻¹) and insulin (CS, 100±18; CT, 113±25; NRE, 76±8.3; NRL, 148±41 nmol.L⁻¹) concentrations were similar between dietary groups. Infusion of an I.V. glucose load significantly increased plasma glucose (Figure 1) and insulin (Figure 2) concentration in all groups of sheep with the peak values achieved being similar (CS, 21.7±1.0 and 1146±196; CT, 21.7±0.5 and 990±102; NRE, 22.6±0.5 and 803±132; NRL, 24.5±1.8 mmol.L⁻¹ and 1165±129 nmol.L⁻¹ for glucose and insulin, respectively). There was no significant effect of diet or fetal number on the maximal change in plasma glucose (Gluₐₘₐₓ), or insulin (Insₐₘₐₓ), or time to restore plasma glucose to baseline (Gluₜₐₜ); however, the time taken for plasma insulin to return to baseline after the IVGTT was significantly increased in NRL relative to CS (Table 2, P=0.02). In addition, glucose tolerance as defined by glucose area under the curve was decreased (P=0.054) in NRL vs. CS and CT, despite significantly increased (P=0.01 vs. NRE) absolute insulin secretion (Insₐᵤᶜ; Table 2). While the relative insulin secretion rate was higher in both CT and NRL relative to NRE (P=0.05 and 0.039, respectively), the glucose clearance rate (i.e. glucose AUC/(time to peak minus Gluₜₐₜ)) was similar between groups (Table 2). Gender had no significant effect on glucose tolerance (Figure 3a) or insulin response to a glucose load (Figure 3b).
Infusion of glucose completely suppressed plasma NEFA in all sheep, with the rapidity of decrease being similar between groups (data not shown).

**Protein expression of insulin signalling molecules.**

*Perirenal Fat:* Western blot analysis indicated a significant increase in the protein for the beta subunit of the insulin receptor (Irß; Figure 4a) and p110ß subunit of PI-3 kinase (Figure 4b), but significant decrease in GLUT4 protein expression (Figure 4c) in NRL relative to the other nutritional groups (P<0.01 all cases). There was no effect of nutritional group or fetal number on the expression of the p85α subunit of PI-3 kinase (CS, 20833±1251; CT, 23089±1148; NRE, 18755±2438; NRL, 24074±1339 arbitrary units).

*Muscle:* Western blot analysis indicated no differences in protein for the p85α subunit of PI-3 kinase (CS, 21157±2052; CT, 22916±691; NRE, 22737±1968; NRL, 21156±2677 arbitrary units) or GLUT4 (CS, 38002±3694; CT, 29004±7304; NRE, 31846±4499; NRL, 24017±7619 arbitrary units) protein expression in the muscle. There was, however, a significant effect of group on Irß protein expression with NRE having increased levels compared to the other groups (CS, 17516±2488; CT, 13137±2315; NRE, 25418±2000; NRL, 19516±3492 arbitrary units; P=0.049 CT vs. NRE). Interestingly, the expression of p110ß protein in muscle was <10% the expression in perirenal fat (1072±593 vs. 16366±3388 arbitrary units; P<0.0001).

**Determinants of glucose-insulin handling:** There was no correlation between birth weight and glucose-insulin handling or insulin signalling proteins at one year of age in the current cohort of sheep. Higher adipose (r=0.56, P=0.01) and muscle
(r=0.54, \(P=0.01\)) p110\(\beta\) and Ir\(\beta\) (r=0.45, \(P=0.057\)) but lower GLUT4 expression (r=-0.48, \(P=0.049\)) predicted worse glucose tolerance (i.e. increased Glu\textsubscript{AUC} of the offspring). However, when current weight was controlled for in a partial correlation analysis, the relationship with GLUT4 became non-significant (r=-0.32, \(P=0.22\)) and the significance of the relationships with p110\(\beta\) and Ir\(\beta\) were weakened (r=0.56, \(P=0.06\); r=0.44, \(P=0.07\), respectively). The positive relationship between muscle Ir\(\beta\) and Glu\textsubscript{AUC} remained after controlling for current weight. Fasting plasma insulin and insulin AUC (Ins\textsubscript{AUC}) correlated negatively with muscle (r=-0.56, \(P=0.01\)) and adipose GLUT4 expression (r=-0.61, \(P=0.01\)), respectively. Allowing for current weight weakened the relationship between Ins\textsubscript{AUC} and GLUT4 (r=-0.38, \(P=\text{NS}\)) but had no effect on the relationship between fasting plasma insulin and muscle GLUT4. There was a strong positive correlation between Ins\textsubscript{AUC} and p110\(\beta\) expression in adipose (r=0.71, \(P<0.001\)), which remained after controlling for current weight.

**Sheep biometry at one year of age**: There were no differences between dietary groups in the weights of any organ measured. However the proportion of adipose tissue in central (omentum) and peripheral (perirenal) depots was significantly (\(P=0.01\)) greater in NRL relative to other groups of sheep (Table 3).
Discussion

In this study it has been shown that undernutrition confined to late gestation, during the period of maximal fetal growth, impacts negatively upon the subsequent young adults glucose-insulin homeostasis i.e. they show evidence of glucose intolerance (increased $\text{Glu}_{AUC}$) and insulin resistance (increased $\text{Ins}_{AUC}$). Importantly, no major change in glucose tolerance and/or insulin sensitivity was observed in the young adult offspring of sheep undernourished for an equivalent period during very early gestation. The underlying mechanism for disturbed glucose-insulin homeostasis in these late-gestation undernourished offspring appears to be altered tissue (specifically adipose rather than muscle) glucose uptake, through reduced cellular expression of GLUT4. In addition, the study has clearly shown that the lower birth weight of twins has no bearing on their future metabolic competence, at least in terms of their glucose-insulin handling.

Retrospective data from the ‘Dutch Hunger Winter Famine’ has shown quite clearly that specific periods of famine exposure may impact upon specific physiological control systems in adult life. For example, exposure during early gestation influenced the cardiovascular system, clinically reflected as an increased risk of coronary heart disease (37); whereas exposure during late gestation tended to affect intermediary metabolism, in particular, glucose-insulin homeostasis, and was clinically reflected as an increased risk of T2D (35). Subsequent animal studies established to model these epidemiological data have recognised early programming of the metabolic syndrome, and have utilised a similar degree of
undernutrition i.e. 50% normal intake (protein and/or energy) (for review see (3; 30)). However, in general, the diet in these studies has been restricted either throughout gestation or throughout gestation and lactation thereby covering the entire period of embryonic, fetal and postnatal growth. This is important when one further considers the relatively greater nutritive demands of the small compared with large animal species used in these studies (28). Nevertheless, in larger farm animal species programming of cardiovascular and metabolic function have been described although the magnitude of adaptation is attenuated compared to that described in small laboratory species. These studies have focussed on the impact of either birth size (33; 34) or excess glucocorticoid exposure (12) rather than the plane of maternal nutrition. Two recent studies have now directly examined the effect of a specific period of maternal undernutrition (early/early-mid gestation) on adult physiological function in a larger species and have shown programming of cardiovascular control (19; 21), in common with the observations after early famine exposure in the ‘Dutch Hunger Winter Famine’. In the current study these observations are extended to include a further group of late gestation undernourished animals for comparison, and to an examination of metabolic function in the young adult offspring of a large animal species; two hitherto unknown endpoints.

When the current study is considered alongside a contemporaneous study (19), it is clear that late gestation undernutrition has a more profound impact upon intermediary metabolism whereas early gestation undernutrition affects cardiovascular control (19); two observations that replicate the Dutch famine data. Furthermore, we have shown that despite the lower birth weight and relatively
increased early growth rate of twins, their intermediary metabolism (current study) and cardiovascular control (19) are not compromised. Thus developmental programming is not related to birth weight *per se*, which in multiple births is largely due to maternal constraint, but is apparent only when the intra/extrauterine environment is specifically challenged or inappropriate at that point in time e.g. through nutritional imbalance (under/over nutrition). Twins appear to have specific adaptations to accommodate their ‘compromised’ prenatal environment (13; 18) but these adaptations appear to confer no further disadvantage in later life. In contrast, brief periods of maternal undernutrition elicit changes in the developmental pattern and have further consequences as the individual ages.

The reduced glucose tolerance in NRL vs. other groups in the current study is very clear despite the relatively low *n* in this group. This glucose intolerance is related to insulin resistance and not insulin deficiency as indicated by the increased insulin AUC during the glucose tolerance test. As in human diabetes, the molecular basis of this insulin resistance appears to lie downstream of the insulin receptor (25) given the increase in expression of the adipose insulin receptor in the NRL group. However GLUT4, the major insulin responsive glucose transporter, was substantially reduced in adipose, but not muscle, tissue from the NRL group suggesting that impaired glucose tolerance was related to the ability of adipose tissue to take up glucose in an insulin responsive manner. Indeed, expression of adipose specific GLUT4 is essential for whole body glucose disposal (41) and insulin resistance has been shown to be related to adipose tissue specific reductions in GLUT4 expression. In the current study, there is no effect of prenatal diet on the p85α subunit of PI-3 kinase and the increase in adipose p110β appears more related to current weight
than prenatal diet; however, the interaction between these two subunits may be deficient (16). In contrast to the present study, in low birth weight humans (31) and in low protein exposed rats (16) p110ß is actually reduced, perhaps reflecting the reductions in birth weight in these studies as oppose to the current study where birth weight was unaffected by the dietary regimen.

While we cannot separate cause or effect in relation to the increased adiposity, alteration in expression of insulin signalling components and reduced glucose tolerance of NRL in the current study, it has previously been shown that the subcellular insulin signalling proteins downstream of the receptor may be programmed prior to development of increased adiposity and glucose intolerance (16). Thus it is conceivable that late gestation undernutrition provokes a compensatory increase in adipose insulin receptor in response to the reduction in maternal and/or fetal glucose concentrations (50% reduction with NRL dietary regimen (14)). While the NRL regimen may reduce term fetal adipose mass (40), irreversibly increased adipose insulin receptor may sustain a postnatal anabolic drive toward increased adipose deposition. The underlying mechanism for reduced GLUT4 following late gestation undernutrition is unclear, however it has been shown that hyperinsulinaemia during late gestation in the ovine fetus produces tissue specific (i.e. in insulin sensitive tissues such as myocardium, adipose) insulin resistance and reduced GLUT4 expression (2).

It is clear, therefore, that the period of exposure to a dietary imbalance during gestation may have different effects on the offspring depending on when the
imposition occurs. The extent to which these effects may be amplified or ameliorated by the postnatal environment i.e. the ‘thrifty phenotype’ cannot be determined in the current study – all sheep were treated similarly postnatally – but the potential is obvious (15). In addition, it is clear from studies in rats that the age-related decline in glucose tolerance in prenatally protein restricted offspring is sex-specific i.e. it is delayed in female, compared to male, rats (16). When included as an independent co-variate, the sex of the offspring did not influence any of the outcome measures in the current study; however, it is recognised that we do not have sufficient statistical power to draw firm conclusions regarding sex-specific effects on adult glucose metabolism after prenatal undernutrition in sheep. In addition, future studies should consider longitudinal assessments of glucose tolerance and insulin sensitivity of prenatally programmed male and female sheep to determine whether the observed decline with age (20) is exacerbated by a poor prenatal environment, specifically during functional development of fetal pancreas i.e. mid-late gestation (17).
Acknowledgements

The authors wish to acknowledge the Joint Animal Breeding Unit, University of Nottingham for the routine care of the animals used in this study. This work was supported by the British Heart Foundation (BS/03/001), Diabetes UK (RD03/0002677) and a Clinical Endocrinology Trust Medical Student award (B.W.M.V.B).


6. **Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Broughton-Pipkin F, Stephenson T and Symonds ME.** Maternal endocrine adaptation throughout pregnancy to nutritional manipulation:


13. **Edwards LJ and McMillen IC.** Impact of maternal undernutrition during the periconceptional period, fetal number, and fetal sex on the development of the


27. **Langley-Evans SC, Welham SJ, Sherman RC and Jackson AA.**


Legends

Table 1. Fed and fasted plasma concentrations of glucose, non-esterified fatty acids (NEFA), triglycerides and leptin in control and nutrient restricted sheep at one year of age.
Values are means ± SEM for control singles (CS; n = 5), control twins (CT; n = 5), early nutrient restricted singles (NRE; n = 5) and late nutrient restricted singles (NRL; n = 4) sheep. The fasted concentrations reflect the mean value for 2 baseline samples and the fed sample the mean value for 2 samples collected between 45-60 min after feeding. Data were statistically tested using paired t-test and differences are: a, P<0.05 fasted vs. fed.

Table 2. Derived values from the IVGTT in control and nutrient restricted sheep at one year of age.
Values are means ± SEM for control singles (CS; n = 5), control twins (CT; n = 5), early nutrient restricted singles (NRE; n = 5) and late nutrient restricted singles (NRL; n = 4) sheep. Values were derived as described in Methods. Values arranged in individual rows with differing superscripts are statistical different at P<0.05 (1-way ANOVA).

Table 3. Organ weights and fat mass of control and nutrient restricted sheep at one year of age.
Values are means ± SEM for control singles (CS; n = 5), control twins (CT; n = 5), early nutrient restricted singles (NRE; n = 5) and late nutrient restricted singles (NRL; n = 4) sheep. Values arranged in individual rows with differing superscripts
are statistical different at P<0.05 (1-way ANOVA). Other superscripts are: c, \( P=0.07 \) NRL vs. CS; d, \( P=0.053 \) NRL vs. CS.

**Figure 1. Plasma glucose after an IVGTT in control and nutrient restricted sheep at one year of age.**

Values are means ± S.E.M. for control singles (CS), control twins (CT), early nutrient restricted singles (NRE) and late nutrient restricted singles (NRL) sheep. Glucose (0.5g/kg in 0.9% NaCl) was administered as a bolus over 2min. Blood samples were taken during baseline at −10 and −5 min and subsequently at 2, 5, 10, 15, 20, 30, 40, 60, 80, 120, 150, 180, 210 and 240 min after the glucose bolus.

**Figure 2. Plasma insulin after an IVGTT in control and nutrient restricted sheep at one year of age.**

Values are means ± S.E.M. for control singles (CS), control twins (CT), early nutrient restricted singles (NRE) and late nutrient restricted singles (NRL) sheep. Glucose (0.5g/kg in 0.9% NaCl) was administered as a bolus over 2min. Blood samples were taken during baseline at −10 and −5 min and subsequently at 2, 5, 10, 15, 20, 30, 40, 60, 80, 120, 150, 180, 210 and 240 min after the glucose bolus.

**Figure 3. Plasma glucose (A) and insulin (B) after an IVGTT in male and female control sheep at one year of age.**

Values are means ± S.E.M. for male (○, n=5) and female (●, n=5) sheep. Glucose (0.5g/kg in 0.9% NaCl) was administered as a bolus over 2min. Blood samples
were taken during baseline at –10 and –5 min and subsequently at 2, 5, 10, 15, 20, 30, 40, 60, 80, 120, 150, 180, 210 and 240 min after the glucose bolus.

**Figure 4.** Protein expression of insulin signalling molecules in perirenal fat of control and nutrient restricted sheep at one year of age.

Values are means ± S.E.M. for control singles (CS), control twins (CT), early nutrient restricted singles (NRE) and late nutrient restricted singles (NRL) sheep. Tissue lysates were prepared for SDS-PAGE and then immunoblotted with antibodies to Irβ, p110β and GLUT 4 as described in the methods. Statistical differences are *, P<0.05 (1-way ANOVA).
Table 1. Fed and fasted plasma concentrations of glucose, non-esterified fatty acids (NEFA), triglycerides and leptin in control and nutrient restricted sheep at one year of age.

<table>
<thead>
<tr>
<th></th>
<th>CS  (n=5)</th>
<th>CT  (n=5)</th>
<th>NRE (n=5)</th>
<th>NRL (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glucose (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fasted</td>
<td>5.59 ± 0.55</td>
<td>4.88 ± 0.17</td>
<td>5.42 ± 0.54</td>
<td>5.25 ± 0.51</td>
</tr>
<tr>
<td>fed</td>
<td>4.94 ± 0.60</td>
<td>6.08 ± 0.91</td>
<td>7.28 ± 1.28</td>
<td>7.14 ± 1.82</td>
</tr>
<tr>
<td><strong>NEFA (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fasted</td>
<td>0.63 ± 0.13</td>
<td>0.45 ± 0.11</td>
<td>0.95 ± 0.12</td>
<td>0.45 ± 0.11</td>
</tr>
<tr>
<td>fed</td>
<td>0.12 ± 0.01 a</td>
<td>0.15 ± 0.04</td>
<td>0.13 ± 0.04 a</td>
<td>0.42 ± 0.19</td>
</tr>
<tr>
<td><strong>Triglycerides (mmol.L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fasted</td>
<td>0.18 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>0.23 ± 0.03</td>
<td>0.31 ± 0.07</td>
</tr>
<tr>
<td>fed</td>
<td>0.21 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>0.24 ± 0.03</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td><strong>Leptin (ng.ml⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fasted</td>
<td>0.99 ± 0.10</td>
<td>1.36 ± 0.28</td>
<td>0.93 ± 0.19</td>
<td>3.27 ± 1.52</td>
</tr>
<tr>
<td>fed</td>
<td>2.60 ± 0.73</td>
<td>1.84 ± 0.54</td>
<td>2.20 ± 1.23</td>
<td>1.49 ± 0.52</td>
</tr>
</tbody>
</table>
Table 2. Derived values from the IVGTT in control and nutrient restricted sheep at one year of age.

<table>
<thead>
<tr>
<th></th>
<th>CS (n=5)</th>
<th>CT (n=5)</th>
<th>NRE (n=5)</th>
<th>NRL (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peak change in glucose:</strong></td>
<td>16.6 ± 1.2</td>
<td>16.4 ± 0.7</td>
<td>17.2 ± 0.3</td>
<td>19.3 ± 1.4</td>
</tr>
<tr>
<td>(Glu ( \Delta_{\text{max}} ); mmol.L(^{-1}))</td>
<td>1045 ± 194</td>
<td>876 ± 112</td>
<td>726 ± 131</td>
<td>1134 ± 36</td>
</tr>
<tr>
<td><strong>Time to baseline (glucose):</strong></td>
<td>175 ± 23</td>
<td>137 ± 14</td>
<td>163 ± 20</td>
<td>200 ± 10</td>
</tr>
<tr>
<td>(Glu_{\text{base}}; min)</td>
<td>137 ± 7 (^a)</td>
<td>172 ± 18 (^{ab})</td>
<td>149 ± 20 (^{ab})</td>
<td>225 ± 9 (^b)</td>
</tr>
<tr>
<td><strong>Glucose tolerance:</strong></td>
<td>1111 ± 54 (^a)</td>
<td>964 ± 109 (^a)</td>
<td>1340 ± 206 (^{ab})</td>
<td>1619 ± 170 (^b)</td>
</tr>
<tr>
<td>(Glu_{\text{auc}}; min.mmol.L(^{-1}))</td>
<td>69.5 ± 9.0 (^a)</td>
<td>69.5 ± 6.9 (^a)</td>
<td>53.0 ± 10.6 (^a)</td>
<td>115.6 ± 22.0 (^b)</td>
</tr>
<tr>
<td><strong>Insulin secretion:</strong></td>
<td>63.2 ± 9.2 (^a)</td>
<td>73.6 ± 6.2 (^b)</td>
<td>39.2 ± 4.3 (^a)</td>
<td>80.6 ± 15.9 (^b)</td>
</tr>
<tr>
<td>(Ins_{\text{rel}}; nmol/mmol.L(^{-1}))</td>
<td>10.6 ± 1.3</td>
<td>8.1 ± 1.2</td>
<td>8.4 ± 0.6</td>
<td>10.3 ± 0.9</td>
</tr>
</tbody>
</table>
### TABLE 3. Sheep biometry at one year of age

<table>
<thead>
<tr>
<th>Tissue mass</th>
<th>CS</th>
<th>CT</th>
<th>NRE</th>
<th>NRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain weight (g)</td>
<td>96.7 ± 3.5</td>
<td>99.7 ± 2.8</td>
<td>93.5 ± 1.5</td>
<td>94.3 ± 3.5</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>178 ± 17</td>
<td>195 ± 21</td>
<td>213 ± 14</td>
<td>225 ± 22</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>633 ± 32</td>
<td>688 ± 107</td>
<td>669 ± 99</td>
<td>672 ± 72</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>111 ± 9</td>
<td>116 ± 6</td>
<td>119 ± 6</td>
<td>172 ± 35</td>
</tr>
<tr>
<td>Adrenal weight (g)</td>
<td>2.90 ± 0.64</td>
<td>3.17 ± 0.40</td>
<td>3.17 ± 0.20</td>
<td>2.66 ± 0.66</td>
</tr>
<tr>
<td>Spleen weight (g)</td>
<td>111 ± 19</td>
<td>110 ± 10</td>
<td>92 ± 15</td>
<td>145 ± 33</td>
</tr>
<tr>
<td>Lung weight (g)</td>
<td>475 ± 19</td>
<td>476 ± 21</td>
<td>464 ± 42</td>
<td>478 ± 40</td>
</tr>
<tr>
<td>Pancreas weight (g)</td>
<td>42.5 ± 3.5</td>
<td>49.0 ± 10.1</td>
<td>43.3 ± 7.8</td>
<td>29.0 ± 3.4</td>
</tr>
<tr>
<td>Perirenal adipose tissue (g)</td>
<td>104 ± 25a</td>
<td>189 ± 33a</td>
<td>151 ± 27a</td>
<td>361 ± 65b</td>
</tr>
<tr>
<td>Omental adipose tissue (g)</td>
<td>259 ± 96</td>
<td>295 ± 41</td>
<td>273 ± 31</td>
<td>558 ± 132c</td>
</tr>
<tr>
<td>Relative fat mass (g/kg)</td>
<td>8.09 ± 1.89</td>
<td>11.24 ± 1.36</td>
<td>9.72 ± 0.60</td>
<td>17.52 ± 4.33d</td>
</tr>
</tbody>
</table>
Figure 1

Glucose (mmol.L\(^{-1}\))

- CS (n=5)
- CT (n=5)
- NRE (n=5)
- NRL (n=4)
Figure 3a

Glucose (mmol.L\(^{-1}\))

- Males (n=5)
- Females (n=5)

Figure 3b

Insulin (nmol.L\(^{-1}\))

- Males
- Females
Figure 4

A  IRß (arbitrary units)

B  p110ß (arbitrary units)

C  GLUT-4 (arbitrary units)