Early pregnancy weight gain and fat accrual predict pregnancy outcome in growing adolescent sheep

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Abstract

The competition for nutrients when pregnancy coincides with continuing growth in biologically-immature adolescent girls increases their risk of preterm delivery and low birthweight, and is partly replicated in the overnourished adolescent sheep paradigm. Although overfeeding to promote rapid maternal growth robustly leads to a reduction in average birthweight relative to slow-growing control-fed adolescents of equivalent age, the extent of prenatal compromise is variable. This retrospective analysis of a large cohort of identically managed pregnancies determined whether maternal anthropometry predicts the severity of fetal growth-restriction (FGR) in growing adolescents. Singleton pregnancies were established by embryo transfer in adolescents subsequently control-fed (n=96) or overnourished. The latter pregnancies were classified as nonFGR (n=116) or FGR (n=96) if lamb birthweight was above or below the optimally-fed control mean minus 2SD. A similar approach categorised placental growth-restriction (PlGR) and preterm delivery. Gestation length, placental mass and lamb birthweight were FGR < nonFGR < control (*post hoc* P<0.01). Relative to the nonFGR group, overnourished dams with FGR were marginally leaner and lighter at conception (P=0.023/P=0.014), and had greater gestational weight gain (GWG) during the first-third of pregnancy (P<0.001). GWG during this early period was also higher in PlGR compared with nonPlGR, and in very preterm versus term deliveries (P<0.01). Likewise maternal leptin concentrations (fat accrual biomarker) were FGR > nonFGR by day 60, and changes in leptin throughout pregnancy predicted attenuated fetal cotyledon mass and birthweight (P=0.01 to <0.001). The anthropometric antecedents of FGR in still-growing adolescent sheep originate in early pregnancy coincident with early placental development.

Introduction

Becoming pregnant during adolescence is a well-established risk factor for adverse gestational outcome independent of geographical setting. The hazards are multiple and robustly include a greater likelihood of spontaneous miscarriage, preeclampsia, stillbirth, preterm delivery, low birthweight, neonatal and/or maternal mortality (Malabarey et al.2012; Kozuki et al.2013; Ganchimeg et al.2014; de Azevedo et al.2015; Neal et al.2016; Paul,2018; Marvin-Dowle and Soltani,2020). The degree of risk is particularly high in very young girls (typically <16 years) who are more likely to be gynaecologically and biologically immature (Conde-Agudelo et al.2005; Salihu et al.2006; Leppälahti et al.2013; Torvie et al.2015; Weng et al.2015; Neal et al.2018), and the effect on birthweight is exacerbated if maternal growth *per se* is deemed incomplete (Frisancho et al.1985) or is ongoing during pregnancy (Scholl et al.1997). Maternal anthropometric data from the latter prospective cohort is arguably the most accurate available as maternal growth status was defined on the basis of sequential changes in knee height over a 6-month period from mid-pregnancy to 4-6 weeks post-partum. Girls who continued to grow comprised ~50% of the pregnant population (≤16 years) and were characterised by higher gestational weight-gains (GWG) and increased fat stores. Counterintuitively this was associated with a lower average birthweight and a three-fold greater risk of small-for-gestational-age delivery compared with non-growing adolescents of equivalent age, and mature women (Scholl et al.1994,1997). The effect on birthweight is attributed to a competition for nutrients whereby the mother's growth requirements take priority and conceptus growth is therefore compromised.

Our sheep model was originally developed to explore this alteration in nutrient partitioning in young biologically-immature and still-growing adolescents. We deliberately choose to establish pregnancy using assisted conception procedures, involving adult ewe donor superovulation, embryo recovery and synchronous transfer, in order to avoid several of the known issues linked to attempting to breed adolescent sheep naturally, namely a variation in puberty onset, a transient first breeding season, failure to be mated, poor quality embryos and high embryo loss (Beck et al.1996; Kenyon et al.2014; Edwards et al.2016). Most importantly our approach allowed us to nutritionally manipulate maternal growth-velocity in very young adolescents during a singleton pregnancy. Accordingly, when young adolescent dams were overnourished to promote rapid weight-gain and progressive fat accrual, conceptus development was impaired relative to slow-growing optimally-fed control adolescents of equivalent age. Placental development, uteroplacental blood flows and fetal nutrient supply were negatively impacted and premature delivery of low birthweight lambs followed (Wallace et al. 1996;1997a;2002;2004;2008). These adverse pregnancy outcomes in rapidly-growing dams have proved consistent across multiple studies but the severity of prenatal growth-restriction within studies is variable in spite of an equivalent nutritional manipulation. As the placenta is the root cause of fetal growth-restriction in these pregnancies we hypothesised that subtle differences in maternal live-weight gain and fat accrual during the main period of placental growth may explain the severity of poor pregnancy outcome. In sheep the absolute growth rate of the placenta reaches a maximum near day 55, while the apex in placental mass occurs between day 75 and 80 of gestation (Ehrhardt and Bell,1995), hence our focus was changes in maternal anthropometry spanning the first two-thirds of pregnancy. To test our hypothesis we used a dataset involving >200 overnourished adolescent pregnancies categorised as fetal growth-restricted (FGR) or otherwise, and compared them with ~100 optimally-fed controls.

While external assessment of subcutaneous fat level by body condition scoring is a useful tool for non-invasively assessing adiposity in sheep it is known to be less sensitive mid-scale (Miller et al.2018), and may lack precision in growing adolescents where lean tissue growth is the initial dominant nutrient partitioning priority. Moreover it tells us little about fat accumulation at other potentially important central sites within the body. In contrast, peripheral leptin concentrations reflect the overall quantity of adipose tissue throughout the animal and we have previously reported strong positive correlations between circulating leptin and body-fat percentage measured by dual energy X-ray absorptiometry in growing non-pregnant adolescents (Wallace et al.2020). Thus we further postulate that changes in leptin concentrations in pregnant adolescents may provide a sensitive biomarker of relative differences in maternal fat accrual with the potential to predict the extent of placental and fetal growth-restriction. To test this we used a subgroup of the main cohort involving 55 overnourished and 18 control pregnancies.

Methods

Pregnancy establishment in adolescent sheep

Procedures were licensed under the UK Animals (Scientific Procedures) Act of 1986 and approved by the Rowett's Ethical Review Committee. Animals were housed under natural lighting conditions at 57°N, 2°W in individual open-wide bar pens that facilitated nose-to-nose interactions with adjacent animals. Pregnancies were established by assisted conception procedures precisely as detailed previously (Wallace et al.2020). In brief, adult ewes (Border Leicester x Scottish Blackface) of known reproductive history (third or fourth parity), and in prime breeding condition (mean adiposity score 2.3 units) were superovulated and intrauterine inseminated to act as embryo donors. The resulting embryos were recovered on day 4 after insemination and grade 1 morula, optimum for stage, were synchronously transferred into the hormonally-primed uteri of adolescent recipients (Dorset Horn x Greyface) to generate singleton pregnancies. Adults were preferentially used as embryo donors as earlier studies discovered that embryos from adolescent ewes have an innately low viability following transfer into either an adolescent or adult uterus (Quirke and Hanrahan,1977; McMillan and McDonald,1985). Within individual embryo transfer days the embryos for any given donor ewe were distributed across study groups helping maximise the genetic homogeneity of the resulting conceptus units. The adolescent recipients were selected from a closed flock free from Enzootic abortion and vaccinated against Toxoplasmosis 6 weeks prior to breeding (Toxovax; Intervet UK Ltd.). Embryo transfers were carried out on 33 separate days in 5 different years during the first-half of the natural breeding season (mid-Nov to late-Dec) when the adolescents were ~7.5 months old, peripubertal, and had attained similar initial live-weight (44.3±0.34 kg) and adiposity score (2.3±0.01). The latter is equivalent to 23% body fat (Russel et al.1969), while bodyweight at conception equates to ~70% of the mature bodyweight of primiparous ewes of equivalent genotype at 20 months of age. Approximately one-third of the animals were destined to become optimally-fed controls while the remaining two-thirds were overnourished in the expectation that approximately half of the pregnancies would result in markedly growth-restricted lambs for ongoing developmental programming studies (Adam et al.2011; Wallace et al.2011, 2012, 2014, 2018, 2020).

Nutritional management during pregnancy

Commencing directly after embryo transfer, adolescent recipients were offered either a control or high level of a complete diet providing 12 MJ of metabolisable energy (ME) and 140 g of crude protein per kg. The diet contained 30% coarsely milled hay, 41.5% barley 17.5% Hipro soya, 10% molasses, 0.35% salt, 0.25% limestone, 0.25% dicalcium phosphate and 0.15% of a vitamin-mineral mix and was prepared as required on site (Wallace et al.2006). Fresh food was offered twice-daily at 08:00h and 16:00h. For the optimally-fed controls the dietary level was calculated to preserve the original adiposity level throughout pregnancy and to provide 100% of the estimated ME and protein requirements of the adolescent sheep carrying a singleton fetus according to stage of pregnancy. Practically this is achieved via a target gestational weight-gain of 75g per day for the first two-thirds of gestation followed by individual weekly stepwise increases in rations to meet the evolving nutrient needs of the fetus during the final-third of pregnancy. This facilitates a small degree of maternal growth over the course of the entire pregnancy. In contrast, the high ration was fed *ad libitum* throughout pregnancy. To achieve this rations were increased stepwise over a 10-14 day period until the daily food refusal was ~15% of the amount offered and adjusted twice-weekly thereafter to maintain the daily refusal at this target level. These animals were considered overnourished (~2.25 x control intakes for the first two-thirds of gestation and ~ 1.7 x control intakes thereafter). To facilitate this accurate nutritional management external adiposity score, assessed by a single operator throughout, was measured at ~monthly intervals during the first two-thirds of pregnancy and fortnightly during the final-third. Maternal weight was recorded immediately prior to embryo transfer, and at regular intervals during the first two-thirds of pregnancy. In all cases weight data were available at 27±0.2, 50±0.2, 75±0.1 and 95±0.2 days of gestation (mean±sem). A final pre-delivery weight was measured at day 133±0.1.

Perinatal management, lambing and neonatal measurements

As overnourished adolescent dams consistently deliver early, all ewes were supervised 24h per day during the expected delivery period from day 135 of gestation to the last control birth on day 150. Ewes were allowed to spontaneously labour and lambing assistance matched requirement. After delivery lambs were dried, weighed and girth at the umbilicus measured. Oxytocin (10iu, Intervet UK Ltd.) was administered i.v. to induce milk let-down and the udder stripped by hand to determine the initial colostrum yield. The colostrum was fed back to the lamb by bottle or feeding tube. Where colostrum yield was less than the required 50cc per kg birthweight, supplementary colostrum from a frozen donor pool was used. Following delivery of the fetal component of the placenta it was laid out on a tray, replicating the orientation *in vivo* to check that it was intact, and the fetal cotyledons were then dissected from the membranes and their total weight recorded. Membrane plus cotyledon weight was also recorded (placental weight). Seven ewes either retained the placenta entirely (4 overnourished) or ate part of it before it could be retrieved from the pen (1 control, 2 overnourished). Data pertaining to these individual pregnancies has been excluded leaving full maternal anthropometry, lamb and placenta weight records for 96 control and 212 overnourished pregnancies.

Plasma Leptin Analysis

In a sub-group of the above pregnancies (18 control, 55 overnourished), maternal venous blood samples were collected at ~12 noon on day 0, 30, 60, 90 and 130 of gestation and the resulting plasma analysed for leptin in duplicate (Marie et al.2001). The lower limit of detection was 0.1 ng leptin/ml, and the inter and intra-assay coefficients of variation were <8%

Definitions and data analysis

Data were analysed using Minitab (version 19; Minitab Inc., State College, PA). Normality was confirmed and there was no evidence of outliers for indices of maternal anthropometry or pregnancy outcome parameters using Grubbs' testing at 5% significance. Prenatal growth-restriction and pre-term delivery categories were defined using the mean and standard deviation (SD) for optimal control deliveries. Control birthweight and placental weight were not impacted by year of study (P=0.304 and 0.822, respectively). As control males were heavier than their female counterparts (mean±SD: 5607±717g vs. 5291±746 g, respectively, P=0.037), FGR was defined on a sex-specific basis, and defined as such when birthweight in an overnourished pregnancy was less than the control mean minus 2SD's, thus <4173 g for males and <3799 g for females. The remaining overnourished pregnancies were categorised as nonFGR. A similar approach was used to define placental (cotyledon) growth-restriction. Again the control male total cotyledon weight at delivery was slightly heavier than the female (mean±SD: 156±46.1 vs. 138±39.9 g, respectively, P=0.05) and placental growth-restriction (PlGR) was defined using the sex-specific control mean minus 1.75 SD's, thus <74.5 g for males and <68.2 g for females. A total fetal cotyledon weight above these cut-offs was categorised as nonPlGR. The choice of cut-off in this instance reflected the much greater variance in total cotyledon weight in control pregnancies of both sexes. Gestation length was independent of sex and was 145.2±1.73 days (mean±SD) for controls. Pregnancies were classified as preterm or very preterm delivery if gestation length was less than two or four SD's below the mean control gestation length, respectively, i.e. 140-142 days for preterm and ≤139 days for very preterm.

Maternal anthropometric and pregnancy outcome data for the three groups (control, nonFGR and FGR) were compared by ANOVA and *post hoc* comparisons used Fishers LSD method at 1% (Table 1). Within the overnourished pregnancies ANOVA was also used to separately compare nonFGR with FGR (Table 1), nonPlGR with PlGR (Figure 1c), and early with term delivery (Figure 1d). Categorical data were analysed by Fishers exact test. Multiple regression was used to further interrogate the relationship between indices of maternal anthropometry and key pregnancy outcomes for the cohort as a whole. The former indices included weight and adiposity at baseline, GWG and changes in adiposity score between defined stages during the first two-thirds of gestation, and for pregnancy overall. The same approach was applied to the subgroup where maternal leptin concentrations were determined and confined to the overnourished animals only. In both cases best sets regression was used to identify which aspects could be reasonably omitted to keep the models simple and the predictors retained are detailed in Tables 2 and 3.

Maternal plasma leptin profiles were also analyzed by a mixed-effects repeated-measures model with maternal ID as a random factor and gestational age and prenatal growth category (Control, NonFGR and FGR) as fixed factors in the model together with their interaction (Figure 2a). *Post hoc* comparison between groups at all stages of gestation was by Fishers LSD method. Pearson product-moment correlation analysis was used to explore relationships between variables where indicated and data are presented as correlation coefficients (r).

Results

Maternal anthropometry and pregnancy outcome

By design target GWG during the first two-thirds of pregnancy was achieved and the optimally-nourished control adolescent dams maintained their initial adiposity score from embryo transfer until the final assessment prior to delivery (Table 1). Ninety-seven percent of the control lambs were spontaneously delivered at term ($\geq$143 days) and average gestation length equated the norm for this genotype and maternal age, namely 145 days (Wallace et al.2004). Lamb birthweights in the control group ranged from 3830 to 7650 g and were considered normal as none were classified as FGR using the approach specified in the data analysis section. Similarly placental mass and fetal cotyledon weight in controls provide the optimum growth bench-mark for this genotype (Table 1), and both were positively related to lamb birthweight (r=0.656 and r=0.589, n=96, P<0.001). Relative to these optimally-fed controls and independent of lamb sex, ~45% of overnourished pregnancies were categorised as markedly growth-restricted (FGR): this equated to an average reduction in placental mass, fetal cotyledon weight and lamb birthweight of 55%, 58% and 44%, respectively. Placental mass and fetal cotyledon weight were positively associated with lamb birthweight (r=0.772 and r=0.701, n=96, P<0.001), and 75% of the placentae were defined as markedly growth-restricted (PlGR), based on fetal cotyledon weight. In contrast the remaining overnourished pregnancies (nonFGR) were much less perturbed but average placental mass, fetal cotyledon weight and lamb birthweight were nonetheless lower than in controls (17%, 30% and 12%, respectively P<0.01). Only 11% were defined as PlGR and placental mass and fetal cotyledon weight were again positively associated with lamb birthweight (r=0.691 and 0.644, n=116, P<0.001). The differences in lamb weight remained even after adjusting birthweight to a standard gestation length of 145 days. This adjustment is relevant as two-thirds of overnourished adolescent dams delivered early. The proportion of overnourished pregnancies with preterm delivery at 140-142 days was independent of prenatal growth-category (P=0.487) but there was a greater incidence of very preterm delivery (≤139 days) in the FGR compared with the nonFGR group (P=0.009). Males were heavier than females in both prenatal growth categories (FGR: 3213±95 vs. 2817±89 g, P=0.004, nonFGR: 5038±76 vs. 4535±68 g, P<0.001) but there was no sex difference in the incidence of preterm or very preterm delivery (combined sex ratio shown in Table 1). Colostrum yield immediately after delivery was impacted by gestational intake (control>overnourished) and prenatal growth status (nonFGR>FGR). A similarly high proportion of overnourished dams in the FGR and nonFGR groups failed to produce sufficient colostrum to meet the initial lamb requirement of 50cc per kg birthweight.

With respect to maternal anthropometry in the overnourished dams, the recipients that conceived and went on to have an FGR pregnancy were slightly lighter (P=0.014) and leaner (P=0.023) at the point of embryo transfer than the nonFGR group. Thereafter GWG was higher during the first-third of gestation in the FGR dams (P<0.001, Table 1), equivalent during mid-gestation, and higher over the course of the first two-thirds of pregnancy overall (P=0.006). Figure 1 depicts the inverse relationship between early pregnancy weight-gain and fetal cotyledon weight at delivery on an individual pregnancy basis for all overnourished pregnancies, and the positive relationship between fetal cotyledon mass and lamb birthweight. It also highlights, independent of lamb size, that the most perturbed overnourished pregnancies in terms of both placental growth-restriction and very premature delivery have a relatively higher GWG during early but not mid-pregnancy. Similarly, the regression analysis for the adolescent cohort as a whole reveals that irrespective of nutritional treatment, GWG in the three discrete 25-day periods from conception to day 75 of gestation were all predictive of gestation length, placental mass and lamb birthweight, with the most pronounced effects between day 27 and 50 in all cases (Table 2). All GWG coefficients were negative indicating that the greater the weight-gain the more likely that the lamb would be delivered early and it’s prenatal growth compromised. Further on a group basis, the lower fetal cotyledon and birthweight to maternal weight-gain ratios in the FGR compared with the nonFGR pregnancies (P<0.001) exemplify that the adolescent dams that grow fastest during the first-half of pregnancy transfer a lower proportion of that gain to the developing conceptus (Table 1). Although colostrum yield at parturition was inversely associated with GWG during both early and mid-pregnancy for the cohort as a whole (r=-0.501 and r=-0.479, n=308, P<0.001) no such relationship was evident within the overnourished dams specifically (P>0.65).

Maternal leptin as an index of fat accrual and predictor of pregnancy outcome

Absolute maternal leptin concentrations at intervals throughout gestation in a representative sub-group of animals are shown in Figure 2a. Leptin levels did not vary between conception and late gestation in controls (average birthweight 5596±131 g) in keeping with the nutritional management designed to maintain their initial external adiposity score throughout. In contrast peripheral plasma leptin levels diverged from controls by day 30 of gestation in the overnourished dams, and those that went on to deliver FGR lambs (average birthweight 3149±157 g, n=29) had higher concentrations by day 60 of gestation and thereafter compared with the nonFGR group (average birthweight 5060±129 g, n=26). Figure 2b highlights the inverse relationship between delta maternal leptin concentrations across gestation and lamb birthweight. This association was very weak in control dams (P=0.05) but marked in those who were overnourished (P<0.001). For the latter, a multiple regression model was used to further assess the predictive value of changes in maternal leptin and anthropometry and the main pregnancy outcomes. As detailed in Table 3, delta maternal leptin between all stages of gestation were predictive of both fetal cotyledon mass and birthweight at delivery, with the strongest relationships evident for the change in leptin between day 30 and 60, and between day 90 and 130 of gestation. The change in external adiposity score during the first but not the second or final-third of pregnancy was also strongly associated with these pregnancy outcomes. Similar but less pronounced relationships were evident for gestation length and in this instance it was delta leptin between day 30 and 60 and the change in adiposity during the first-third of pregnancy that had the most pronounced effect. For colostrum yield at parturition there was a significant impact of changes in leptin in 3 of 4 gestational periods but in this instance the delta external adiposity during the first-third of pregnancy did not achieve formal significance (P=0.07). All the aforementioned relationships had negative coefficients indicating that the greater this biomarker of fat accrual then the more likely that the pregnancy would be compromised. In contrast the coefficient for total pregnancy weight-gain, measured between conception and late pregnancy was positive and significant for gestation length, fetal cotyledon weight and birthweight, but notably weight measures in late pregnancy are confounded by the weight of the gravid uterus.

Discussion

Optimum pregnancy outcome in young adolescent sheep

The nutritional management of the control-fed adolescents provided the optimum bench-mark for gestation length, placental growth and lamb birthweight and was key to the approach used to define compromised pregnancies in the present study. By design all ewes gestated a singleton from the outset and a GWG of 75g per day during the first two-thirds of gestation facilitated a small amount of maternal growth, while stepwise increases in dietary intake during the final-third of pregnancy met fetal nutrient requirement while maintaining maternal adiposity, and an average birthweight of 5.4 kg was achieved. This birthweight was comparable to that reported for control-fed adolescents of similar age/weight at conception and housed individually throughout pregnancy following natural conception at a synchronised oestrus (5.1 and 5.3 kg: Peel et al.2012), and exceeded that achieved in adolescents spontaneously ovulating at puberty and managed at pasture (range in average singleton birthweight per study, 4.2 to 4.6 kg: Mulvaney et al.2010a, Corner et al.2013; Pettigrew et al.2019). It also compares favourably with the average singleton birthweight in a large cohort of mature multiparous ewes housed to facilitate appropriate nutritional management in the final-third of gestation (5.5 kg, n=667: Gardner et al.2007). These studies involve a variety of genotypes but importantly our optimum birthweight in control-fed adolescents also matches that achieved in mature primiparous ewes of the same genotype, housed and nutritionally managed in an identical manner (5.2 kg: Wallace et al.2005). The latter study also uniquely provides a comparison for optimum placental growth, and there is striking similarity in both placental weight (477 vs. 442 g) and total fetal cotyledon weight (156 vs. 146 g) at delivery in mature compared with adolescent pregnancies.

Maternal anthropometry and the extent of prenatal growth compromise in growing adolescents

In contrast to the optimally-fed controls, overnourishing adolescents of equivalent age throughout gestation promotes continued maternal growth at the expense of the conceptus. The retrospective analysis of this entire cohort had sufficient power to examine the anthropometric antecedents of FGR within the overnourished group and reveals maternal differences at the point of embryo transfer (conception), and during early pregnancy. The recipients that conceived and went on to have an FGR pregnancy were both lighter and leaner at conception than the contemporaneous nonFGR group. These differences were small (but significant) and are commensurate with the poorer reproductive performance of lighter adolescent (ewe) lambs in terms of reaching natural puberty, conception rate, litter size and offspring weight at weaning (reviewed by Kenyon et al.2014). Although even the smallest of the adolescents studied herein were considered relatively well grown for their age, we have previously reported a decrease in average birthweight of ~500 g in adolescents who were deliberately selected for breeding based on a much larger differential in baseline weight (10 kg) and adiposity (0.5 units) than observed here (Wallace et al.2010). Within the overnourished animals in the current dataset the most striking difference between pregnancies destined to become compromised was a relatively high GWG. On a group basis this divergence was specific to the first third of gestation and applied similarly to pregnancies categorised on the basis of placental mass, lamb birthweight and very preterm delivery (i.e. average differential of 32-38 g per day). Irrespective of degree of prenatal compromise the GWG reported here greatly exceeds that reported in overnourished animals in both pasture based (Morris et al.2005; Kenyon et al.2008; Mulvaney et al.2008, 2010b), and individually housed adolescent studies (Meyer et al.2010; Peel et al.2012), and may in part explain why they fail to report a negative impact on birthweight. None of the aforementioned studies report placental weight at delivery. Herein the implication is a broad threshold of high GWG above which pregnancies are widely compromised but it is noteworthy that the nonFGR pregnancies still have a statistically lower placental weight and lamb birthweight than the optimally-nourished controls. We have long contended that impaired placental growth is the root cause of poor pregnancy outcome in rapidly growing adolescents and the relative importance of the first-third of gestation as highlighted here is in agreement with previous observations of reduced cellular proliferation rates within both the maternal caruncle and fetal cotyledon components of the placenta (Rensick et al.2008), and with attenuated capillary vessel size and density within the fetal cotyledon (Redmer et al.2009), both of which were evident at day 50 of gestation when compared with optimally-fed controls. As placental weight *per se* is not generally significantly perturbed in overnourished dams until 0.7 x gestation the likely severity of placental and/or fetal growth-restriction in individual pregnancies terminated at 0.34 x gestation is impossible to predict but it is notable that when pregnancies were interrupted in late gestation (day 131) placental vascularity in the fetal cotyledon of those with marked FGR was lower than the nonFGR group (Carr et al.2016). Moreover, given that we have identified that rapid GWG during early pregnancy is key to the severity of prenatal growth-restriction it is understandable that studies in other laboratories that began overfeeding adolescents at day 50 of gestation did not influence placental weight and had a very modest effect on birthweight (9% reduction) in a mixed population of single and twin pregnancies (Swanson et al.2008).

As outlined in the introduction it is the youngest girls who are at greatest risk of adverse outcomes including preeclampsia, preterm delivery, and low birthweight, and biological immaturity of the reproductive tract is proposed as a key driver of the presumed underlying placental dysfunction. Brosens and colleagues (2017) propose that the immature uterus requires exposure to regular ovulatory menstrual cycles to prepare for appropriate trophoblast invasion in early gestation. This hypothesis has merit given that the animals studied here were considered peripubertal and overnourished growing dams specifically have delayed and reduced appearance of placental lactogen and pregnancy specific protein-B in the maternal circulation commensurate with impaired trophoblast cell migration (Wallace et al.1997b; Lea et al.2007). No attempt was made to define whether puberty had occurred prior to the start of oestrus synchronisation in the present study as oestrus without ovulation, and conversely ovulation without oestrus, is commonplace at the beginning of the first breeding season (Dyrmundsson,1981; Da Silva et al.2001). The animals were considered peripubertal as there was variable evidence of a prior ovarian cycle (corpus albicans) when the ovary was visualised to confirm ovulation rate and the viability of the corpus luteum at the time of embryo transfer. This was not a factor considered in the allocation of animals to nutritional treatment which was instead based on weight, adiposity and ovulation rate in animals of equivalent chronological age. Nevertheless it is clear that the combination of biological immaturity and rapid GWG during the period of trophoblast proliferation makes these young still-growing adolescents vulnerable to placental insufficiency, leading to FGR. There is a lack of direct data linking differences in maternal growth-velocity, placental growth and pregnancy outcome in human adolescents but high total GWG above recommended levels for individual pre-pregnancy BMI categories have been linked to a two-fold greater risk of low birthweight (<2500 g): 40% of the adolescent participants were 12-15 years old and likely to be still-growing (Samano et al.2018). Trimester-specific weight-gains were not reported in the latter study but the concept that weight-gain during the first trimester plays an important role in setting the fetal-placental growth trajectory (albeit in a different direction) is supported by studies in adult women (Broskey et al.2017; Retnakaran et al.2018).

Maternal leptin as an index of fat accrual and predictor of adverse pregnancy outcome

The early divergence in maternal leptin concentrations between control-fed and overnourished adolescents parallels the wide nutritionally-mediated differences in maternal growth rate and fat deposition, and confirms a previous report (Thomas et al. 2001). Notably leptin levels did not change between conception and late pregnancy in the control group indicating that the nutritional approach to maintain maternal fat stores at a consistent level and thereby meet the evolving fetal nutrient requirement throughout gestation was successfully achieved. In the human, leptin produced by the placenta contributes to higher maternal leptin concentrations as pregnancy progresses and dysregulation of placental leptin is implicated in the aetiology of a number of pregnancy complications including FGR (Kochhar et al.2020). In contrast the ovine placenta does not express significant amounts of leptin mRNA and is unlikely to make a major contribution to peripheral leptin concentrations (Thomas et al.2001; O'Conner et al.2007). The lack of change in maternal leptin in the control group with the greatest placental mass herein further argues against a role for placental leptin involvement in ovine fetal growth. Here we demonstrate for the first time that changes in leptin concentrations beginning during the first-third of pregnancy are a sensitive biomarker of more subtle differences in whole body fat accrual within rapidly growing adolescents exposed to an equivalent nutritional manipulation, and are highly predictive of the degree of prenatal growth-restriction and prematurity recorded at delivery. For this subgroup of the main cohort a greater change in subjective external (subcutaneous) fat score specific to the first-third of gestation was also negatively associated with pregnancy outcome. Thus for this retrospective cohort overall the emerging picture within the overnourished group, is that adolescents with the greatest GWG and/or fat accrual in early pregnancy were more likely to experience placental growth insufficiency leading to reduced fetal growth-velocity and low birthweight. In human adolescents delta leptin between study entry at 17 weeks and 28 weeks gestation was similarly high in girls who continued to grow and associated with greater weight-gain and skinfold thicknesses (Scholl et al.2000). Moreover girls in the upper quartile for leptin had a six-fold higher risk of FGR, and although placental weight at delivery was not reported, alterations in umbilical artery Doppler waveforms consistent with reduced blood flow and thereby attenuated fetal nutrient supply have been reported for this specific adolescent population (Scholl et al.1997). In humans as in sheep, this alteration in the hierarchy of nutrient partitioning is most likely confined to very young and gynaecologically immature adolescents as similarly high GWG, skinfold increases and leptin concentrations in older slow-growing compared with non-growing adolescents (median age 17.8 years, gynaecological age 5 years) was positively associated with birthweight (Jones et al.2010) and did not impact growth or morphology of the placenta (Hayward et al.2011).

Maternal anthropometry and preterm delivery

The putative mechanisms underlying the reduction in gestation length in overnourished compared with optimally-fed control animals have been discussed previously and likely includes attenuated placental reproductive steroid secretion and precocious development and function of the fetal adrenals (Wallace et al. 2004). The retrospective analysis presented here involved sufficient pregnancies to define two categories of early delivery, namely preterm and very preterm, and accordingly it was the FGR pregnancies that were most severely perturbed with 27% of lambs born at or before day 139 of gestation. Moreover these very preterm deliveries were preceded by greater GWG (entire cohort) or fat accrual (subgroup) during the first-half of pregnancy compared with those delivered at term, again indicating the importance of nutrient partitioning priorities established during early pregnancy. Similarly, in the two identical trials of Peel and colleagues (2012) involving exposing singleton bearing adolescents to *ad libitum* intakes throughout pregnancy the trial where maternal weight and adiposity diverged earlier, and to a greater extent, was associated with a 5-day reduction in gestation length similar in magnitude to that reported here. High GWG, greater fat accrual and continued maternal growth are not directly associated with early delivery in human adolescents (Scholl et al. 1997; Jones et al. 2010) but in young girls <16 years, a low but not a higher gynaecological age (≤ or >2 years, respectively) is associated with a two-fold greater risk of spontaneous preterm delivery compared with adult women (Hediger et al.1997), and reinforces the vulnerability of biologically immature adolescents to poor outcomes.

In humans, fetal sex influences a number of pregnancy outcomes with males being disadvantaged with respect to increased risk of preterm delivery and term-SGA birth following natural conception (Al-Qaraghouli & Fang, 2017), but advantaged in terms of their representation in newborns after blastocyst transfer following assisted conception (Ding et al. 2018). However in the present study we found no imbalance in the sex ratio of lambs following embryo transfer in the study overall and no difference in the sex ratio within early delivery or prenatal growth categories. Males were in fact heavier than females in the control, nonFGR and FGR groups, and the differential in birthweight between sexes in the control and overnourished groups (316 and 326 g, respectively) closely aligns with the extra 363 g attributed to male sex in a large cohort of multiparous ewes with variable litter size (Gardner et al. 2007).

In summary, we deliberately established a major competition for nutrients between maternal and conceptus growth in biologically immature adolescent sheep to replicate the scenario observed in still-growing very young girls. This retrospective analysis reveals that the degree of premature delivery and prenatal growth-restriction within these overnourished pregnancies is dependent on high GWG and fat accrual during early pregnancy. This coincides with the main period of placental growth and a key phase of vascular development, which in turn lays the haemodynamic foundations for nutrient supply to the fetus. Reduced placental nutrient supply has been implicated in the pathway to reduced fetal growth in adolescents who have not achieved their predicted height based on the height of their parents (Frisancho et al.1985), but there is a paucity of placental data in relation to measured growth in gynaecologically immature human adolescents, and this should be a focus in future prospective studies.

Declaration of interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

JW developed the animal model, performed experiments, analysed the data and wrote the paper. RA and JM performed experiments and conducted the laboratory analysis.

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Figure Legends

Figure 1

Relationship between (a) gestational weight gain during early pregnancy and fetal cotyledon weight at delivery, r=-0.319, P<0.001, and between (b) fetal cotyledon weight and lamb weight at birth, r=0.804, P<0.001 in overnourished adolescent pregnancies (n=212). Gestational weight gain (GWG) of overnourished dams per defined period in early to mid-gestation is shown in relation to whether (c) the placenta was categorised as growth-restricted (PlGR) or nonPlGR and (d) the delivery was categorised as very premature ($\leq$139 days), premature (140-142 days) or term ($\geq$143 days). In (c) **P=0.002, ***P=0.001, ns= not significant, and in (d) for GWG in early pregnancy the overall effect of delivery category was P=0.016 and where superscript letters differ categories differ at P<0.01.

Figure 2

Maternal plasma leptin concentrations throughout gestation in control (open circles, n=18) and overnourished pregnancies categorised as FGR (black squares, n=29) or nonFGR (grey squares, n=26) based on lamb birthweight (a), and the relationship between the change in maternal leptin concentrations between day 0 and 130 of gestation and lamb birthweight (b). For (a) stage of gestation, prenatal growth category and their interaction were significant P<0.001, control differed from both overnourished groups from day 30 to 130 inclusive (α) and FGR differed from nonFGR at

60, 90 and 130 days gestation (*), $P<0.05$. For (b) $r=-0.455$, $P=0.05$ for control (open circles), and $r=-0.643$, $P<0.001$ for overnourished pregnancies (black diamonds).

Table 1. Maternal anthropometry and pregnancy outcome in adolescent sheep offered an optimum control intake (n=96) or overnourished (n=212) throughout gestation and categorised according to fetal growth status after spontaneous delivery[¥]. Values are mean ± sem.

Gestational intake	Control	Overnourished	Overnourished	P-value	
Lamb growth category[β]	Normal	NonFGR	FGR	Normal vs NonFGR vs FGR	NonFGR vs FGR
Male:female	45:51	58:58	55:41	0.331	0.289
Wt. at conception (kg)	44.2±0.39^{ab}	45.3±0.64^{a}	43.0±0.63^{b}	**0.022**	**0.014**
GWG, ET to d 50 (g/day)	48±3.2^{a}	254±6.0^{b}	286±6.4^{c}	**<0.001**	**<0.001**
GWG, d50 to d 95 (g/day)	109±2.9^{a}	315±5.4^{b}	319±6.3^{b}	**<0.001**	0.655
GWG, ET to d 95 (g/day)	76±2.3^{a}	283±4.6^{b}	302±4.9^{c}	**<0.001**	**0.006**
Weight pre-delivery, d 133 (kg)	62.2±0.39^{a}	84.7±0.65^{b}	81.9±0.72^{c}	**<0.001**	**0.004**
*Adiposity at conception	2.3±0.01	2.3±0.02	2.3±0.03	**0.028**	**0.023**
Δ adiposity, ET to d 50	0±0^{a}	0.2±0.01^{b}	0.2±0.02^{b}	**<0.001**	0.079
Δ adiposity, d 50 to 95	0±0^{a}	0.3±0.01^{b}	0.3±0.02^{b}	**<0.001**	0.808
Δ adiposity, ET to d 95	0±0^{a}	0.6±0.02^{b}	0.6±0.02^{b}	**<0.001**	0.204
Adiposity pre-delivery	2.3±0.01^{a}	3.2±0.02^{b}	3.1±0.03^{b}	**<0.001**	0.335
Gestation length (days)	145.2±0.18^{a}	141.6±0.18^{b}	140.8±0.23^{c}	**<0.001**	**0.004**
[¥]Preterm delivery, n (%)	3 (3.1%)^{a}	66 (56.9%)^{b}	50 (52.1%)^{b}	**<0.001**	0.483
[¥]Very preterm delivery, n (%)	0 (0%)^{a}	15 (12.9%)^{b}	26 (27.1%)^{c}	**<0.001**	**0.009**
Sex ratio for early deliveries	1M, 2F	37M, 44F	41M, 35F	0.500	0.300
Birth weight (g)	5439±76^{a}	4787±56^{b}	3044±69^{c}	**<0.001**	**<0.001**
[ϒ]Adjusted birth weight (g)	5416±72^{a}	5007±55^{b}	3205±70^{c}	**<0.001**	**<0.001**
Girth at umbilicus (mm)	40.1±0.31^{a}	39.1±0.23^{a}	33.0±0.37^{b}	**<0.001**	**<0.001**
Placental weight (g)	442±11.5^{a}	369±8.7^{b}	241±6.3^{c}	**<0.001**	**<0.001**
Fetal cotyledon weight (g)	146.3±4.4^{a}	101.8±2.7^{b}	61.5±2.1^{c}	**<0.001**	**<0.001**
[§]Placental growth-restriction, n (%)	0 (%)^{a}	14 (12.1%)^{b}	71 (74.0%)^{c}	**<0.001**	**<0.001**
Sex ratio for placental growth-restriction	n/a	9M, 5F	38M, 33F	n/a	0.563
Colostrum yield (ml)	492±39.8^{a}	202±13.6^{b}	116±10.4^{c}	**<0.001**	**<0.001**
[α]No. with inadequate colostrum/kg fetus	24 of 90^{a}	76 of 116^{b}	60 of 87^{b}	**<0.001**	0.605
Birth wt: cotyledon wt	39.6±1.00^{a}	49.9±1.15^{b}	52.1±1.13^{b}	**<0.001**	0.162
Birth wt: Maternal wt. gain ET to d 95	863±40.2^{a}	184±4.3^{b}	110±3.4^{b}	**<0.001**	**<0.001**
Cotyledon wt: Maternal wt. gain ET to d 95	23.5±1.34^{a}	3.9±0.14^{b}	2.2±0.09^{b}	**<0.001**	**<0.001**

$^{\beta}$ Lambs from overnourished dams classified as fetal growth restricted (FGR) if birthweight was less than two standard deviations below the mean sex-specific birthweight of the optimally nourished control group, i.e. <3799 g for females and <4173 g for males. *Based on external body condition score (5-point scale where 1 =emaciated and 5=morbidly obese, Russell et al. 1969) and assessed by a single operator throughout. $^{¥}$Classified as preterm or very preterm delivery if gestation length was less than two or four standard deviations below the mean control gestation length, respectively, i.e. 140-142 days for preterm and $\leq$139 days for very preterm. $^{\Upsilon}$ Individually adjusted to a standard gestation length of 145 days according to the formula; adjusted birthweight = weight at birth/1.01305 per day of gestation. §Pregnancies classified as major placental growth restriction (PlGR) if total fetal cotyledon weight was less than 1.75 x standard deviations below the mean sex-specific cotyledon weight of the optimally nourished control group, i.e. <68.2 g for females and <74.5 g for males. $^{\alpha}$ Defined based on requirement of 50ml/kg fetal weight, missing data for 6 control, 7 non-FGR and 3 FGR pregnancies
3-way comparison by ANOVA followed by Fishers LSD method. Within rows where superscripts differ, P<0.01. Categorical data by Fisher's exact test.
GWG= gestational weight gain, ET= single embryo transfer at day 4.

Table 2. Pregnancy weight gain as a predictor of gestation length, fetal cotyledon weight and birthweight at delivery in adolescent sheep (n= 308) carrying a single fetus.

	Gestation length, days		Fetal cotyledon weight, g		Birthweight, g	
	B coefficient (SE)	P-value	B coefficient (SE)	P-value	B coefficient (SE)	P-value
Weight at ET	-0.041 (0.021)	**0.05**	1.160 (0.368)	**0.002**	15.9 (10.2)	0.118
GWG ET to D 27	-0.005 (0.001)	**0.001**	-0.059 (0.026)	**0.027**	-1.525 (0.832)	**0.038**
GWG D 27 to D 50	-0.007 (0.002)	**<0.001**	-0.104 (0.028)	**<0.001**	-2.940 (0.783)	**<0.001**
GWG D 50 to D 75	-0.005 (0.001)	**0.001**	-0.100 (0.025)	**<0.001**	-2.112 (0.685)	**0.002**
GWG D 75 to D 95	-0.000 (0.001)	0.734	-0.005 (0.021)	0.818	0.351 (0.576)	0.543

Weight in Kg, ET= embryo transfer at day 4, gestational weight gain (GWG) g/day.

Table 3. Predictors of fetal cotyledon weight, birthweight at delivery, gestation length and colostrum yield in fifty-five overnourished adolescents.

	Fetal cotyledon weight, g		Birthweight, g		Gestation length, days		Colostrum yield, g	
	B coefficient (SE)	P-value	B coefficient (SE)	P-value	B coefficient (SE)	P-value	B coefficient (SE)	P-value
Δ leptin D 0-30	-3.93 (1.27)	**0.003**	-137.5 (34.8)	**<0.001**	-0.100 (0.061)	0.111	-14.20 (3.87)	**0.001**
Δ leptin D 30-60	-4.68 (1.20)	**<0.001**	-185.1 (33.1)	**<0.001**	-0.252 (0.058)	**<0.001**	-9.52 (3.74)	**0.014**
Δ leptin D 60-90	-3.66 (1.36)	**0.010**	-139.2 (37.5)	**0.001**	-0.076 (0.066)	0.257	-6.96 (4.17)	0.102
Δ leptin D 90-130	-3.88 (1.05)	**0.001**	-125.8 (29.0)	**<0.001**	-0.107 (0.051)	**0.044**	-7.00 (3.28)	**0.038**
Δ adiposity D 0-49	-187.5 (52.8)	**0.001**	-4120 (1430)	**0.006**	-10.92 (2.39)	**<0.001**	-295 (159)	0.070
Δ adiposity D 49-96	-36.1 (40.6)	0.378	-1090 (1070)	0.314	-3.05 (1.89)	0.114	28 (122)	0.820
Δ adiposity D 96-132	-18.3 (29.7)	0.541	-756 (784)	0.340	-3.12 (1.39)	**0.030**	-110 (95.2)	0.254
Pregnancy wt. gain	3.45 (1.13)	**0.004**	107.3 (35.5)	**0.004**	0.163 (0.068)	**0.021**	4.25 (4.12)	0.309

Leptin ng/ml; Adiposity score 1 to 5; Pregnancy weight gain Kg.

Figure 1

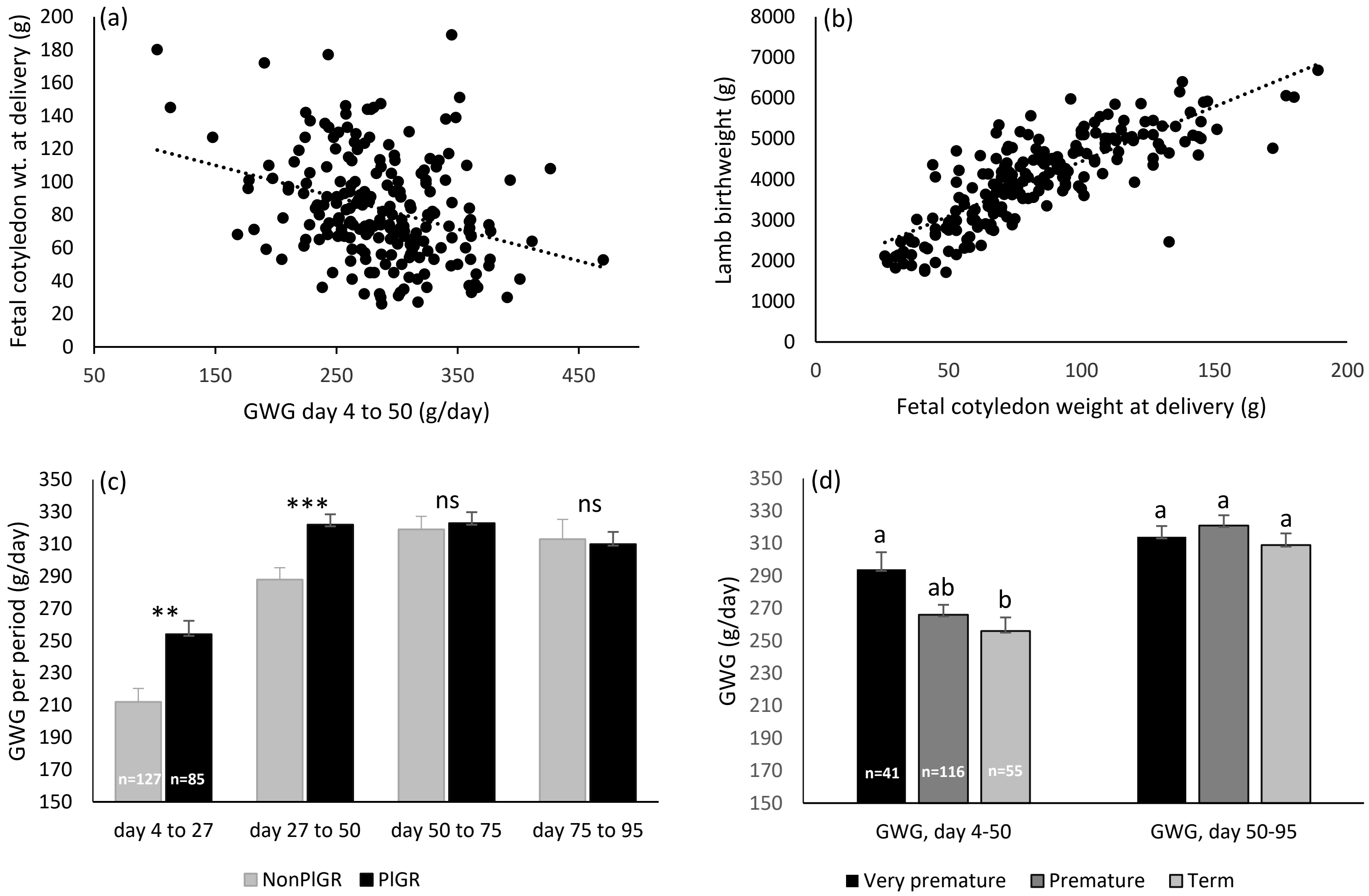
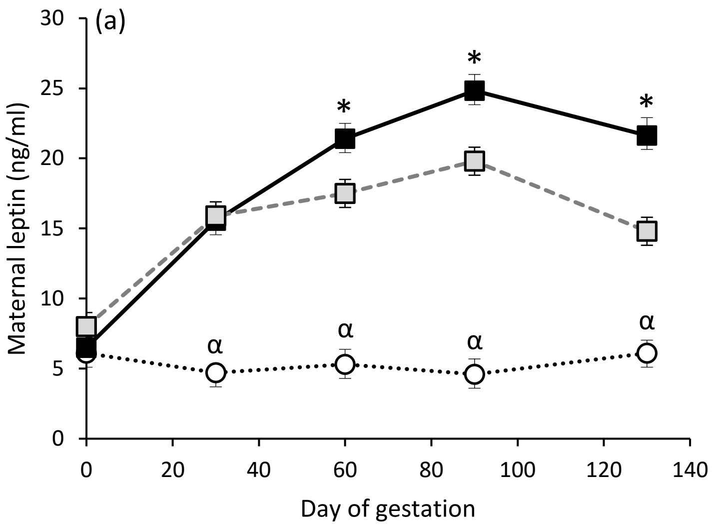
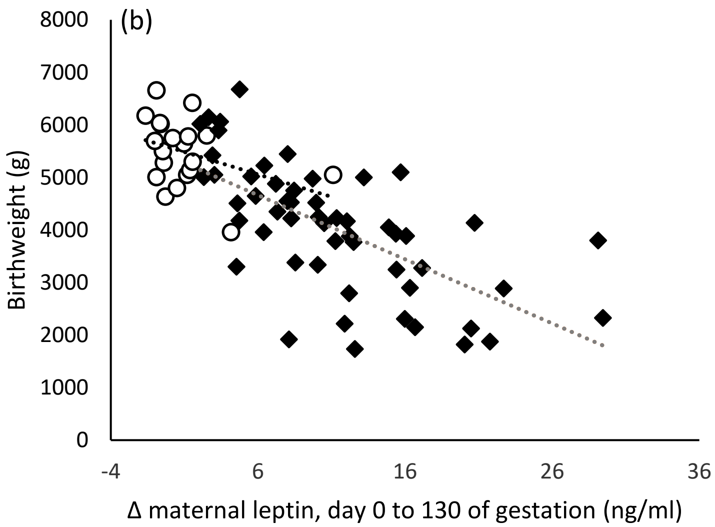

(a)
Fetal cotyledon wt. at delivery (g)
GWG day 4 to 50 (g/day)
(b)
Lamb birthweight (g)
Fetal cotyledon weight at delivery (g)
(c)
GWG per period (g/day)
** *** ns ns
n=127 n=85
day 4 to 27
day 27 to 50
day 50 to 75
day 75 to 95
NonPlGR PlGR
(d)
GWG (g/day)
a ab b a a a
n=41 n=116 n=55
GWG, day 4-50
GWG, day 50-95
Very premature Premature Term

Figure 2



(a)
Maternal leptin (ng/ml)
Day of gestation
0
5
10
15
20
25
30
0
20
40
60
80
100
120
140
*
α



(b)
Birthweight (g)
Δ maternal leptin, day 0 to 130 of gestation (ng/ml)
0
1000
2000
3000
4000
5000
6000
7000
8000
-4
6
16
26
36