Altered arterial concentrations of placental hormones during maximal placental growth in a model of placental insufficiency

T R H Regnault¹, R J Orbus², F C Battaglia¹, R B Wilkening¹ and R V Anthony^{1,2}

¹Perinatal Research Facility, Department of Pediatrics, Division of Perinatal Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262, USA

²Department of Physiology, Colorado State University, Fort Collins, Colorado 80523, USA

(Requests for offprints should be addressed to T R H Regnault, University of Colorado Health Sciences Center, Department of Pediatrics, Division of Perinatal Medicine, Fitzsimons, Mail Stop F441, P O Box 6508, Aurora, Colorado 80045, USA)

Abstract

Pregnant ewes were exposed chronically to thermoneutral (TN; $20 \pm 2^\circ\text{C}$, 30% relative humidity; $n=8$) or hyperthermic (HT; $40 \pm 2^\circ\text{C}$ 12 h/day, $35 \pm 2^\circ\text{C}$ 12 h/day, 30% relative humidity, $n=6$) environments between days 37 and 93 of pregnancy. Ewes were killed following 56 days of exposure to either environment (days in treatment (dit)), corresponding to 93 ± 1 day post coitus (dpc). Maternal core body temperatures (CBT) in HT ewes were significantly elevated above the TN ewes (HT; $39.86 \pm 0.1^\circ\text{C}$ vs TN; $39.20 \pm 0.1^\circ\text{C}$; $P<0.001$). Both groups of animals displayed circadian CBT, though HT ewes had elevated amplitudes (HT; $0.181 \pm 0.002^\circ\text{C}$ vs TN; $0.091 \pm 0.002^\circ\text{C}$; $P<0.001$) and increased phase shift constants (HT; 2100 h vs TN; 1800 h; $P<0.001$). Ewes exposed to chronic heat stress had significantly reduced progesterone and ovine placental lactogen (oPL) concentrations from 72 and 62 dpc respectively ($P<0.05$), corresponding to approximately 30 dit. However, when compared with the TN ewes, HT cotyledonary tissue oPL mRNA and protein concentrations were not significantly different ($P>0.1$). Prolactin concentrations rose immediately upon entry into the HT environment, reaching concentrations approximately four times that of TN ewes, a level maintained throughout the study (HT; 216.31 ± 32.82 vs TN; 54.40 ± 10.0 ; $P<0.0001$). Despite similar feed intakes and euglycemia in both groups of

ewes, HT fetal body weights were significantly reduced when compared with TN fetuses (HT; 514.6 ± 48.7 vs TN; 703.4 ± 44.8 ; $P<0.05$), while placental weights (HT; 363.6 ± 63.3 vs TN; 571.2 ± 95.9) were not significantly affected by 56 days of heat exposure. Furthermore, the relationship between body weight and fetal length, the ponderal index, was significantly reduced in HT fetuses (HT; 3.01 ± 0.13 vs TN; 3.57 ± 0.18 ; $P<0.05$). HT fetal liver weights were also significantly reduced (HT; 27.31 ± 4.73 vs TN; 45.16 ± 6.16 ; $P<0.05$) and as a result, the brain/liver weight ratio was increased. This study demonstrates that chronic heat exposure lowers circulating placental hormone concentrations. The observation that PL mRNA and protein contents are similar across the two treatments, suggests that reduced hormone concentrations are the result of impaired trophoblast cell development, specifically trophoblast migration. Furthermore, the impact of heat exposure during maximal placental growth is great enough to restrict early fetal development, even before the fetal maximal growth phase (100 dpc–term). These data highlight that intrauterine growth retardation (IUGR) may result primarily from placental trophoblast cell dysfunction, and secondarily from later reduced placental size.

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Introduction

In sheep, fetal membranes attach to the maternal uterine mucosa at 15–20 days post coitus (dpc) (Nephew *et al.* 1989), and by 35–40 dpc, trophoblastic interdigitation of developing placenta is well underway (Boshier 1969). The ruminant placenta develops through a non-invasive trophoblast attachment to specialized zones within the uterus, fusing with the maternal epithelium (Wooding 1982). Trophoblast cells are responsible for the synthesis

and secretion of placental steroid and protein hormones (Strauss *et al.* 1996, Wooding *et al.* 1997). However, the interactions of the various hormones, angiogenic growth factors and receptors responsible for this active phase of placental growth and development are still to be fully documented. Placental growth proceeds rapidly from 40 dpc to an apex at 75–80 dpc, with maximal accretion rates for protein and dry matter contents at approximately 55 dpc (Ehrhardt & Bell 1995). Associated with this phase of rapid placental growth are rapid increases in DNA

synthesis and tissue mass, with the period of proliferative growth ceasing at approximately 70–80 dpc (Ehrhardt & Bell 1995).

Intrauterine growth retardation (IUGR) occurs in pregnant ewes exposed to high ambient temperature during the middle and last third of gestation (Bell *et al.* 1989, Dreiling *et al.* 1989, Thureen *et al.* 1992), after the period of maximal placental growth and development. The successful development and subsequent growth of the placenta is required for the desirable outcome of pregnancy, and modulators of placental development and growth include steroids and peptide hormones. Alterations in steroid and protein hormones of placental origin have been shown to occur in IUGR pregnancies, specifically progesterone and placental lactogen (PL) (Nisbet *et al.* 1982, Westergaard *et al.* 1984, Yanaihara *et al.* 1984, Laurin *et al.* 1987). Changes in hormonal profiles, placental functional capacities, and IUGR have been associated with changes in placental mass (Brown *et al.* 1977, Bell *et al.* 1987, 1989, Thureen *et al.* 1992). The underlying associations made from such studies have been that these hormonal and fetal alterations are a result of reduced placental growth. An alternative hypothesis is that the mechanisms involved in steroid and protein hormone synthesis and secretion may have been compromised earlier in gestation and it is this factor that results in reduced placental development and growth.

The relationship between placental and fetal growth implies that placental growth must precede fetal growth, and potential deficiencies in placental growth will interfere with subsequent fetal development. These observations highlight that the period of maximal placental development and growth, 40–80 dpc, would be an important time frame in which to investigate the effects of heat on placental hormone production and associated IUGR. We consider that the mechanisms responsible for IUGR following heat exposure during late gestation are different from those that occur following exposure during maximal placental growth. Heat exposure earlier in gestation potentially involves alterations in placental trophoblastic capacity. In addition to studying progesterone and ovine placental lactogen (oPL) concentrations, prolactin, a stress related hormone, was also measured. The objective of this study was to determine the effects of chronic heat stress through the period of maximal placental growth, 40–80 dpc, on placental endocrine function, specifically focusing on alterations in progesterone, oPL and prolactin concentrations, and consequences on placental and fetal growth.

Materials and Methods

Experimental protocol

This study was approved by the University of Colorado Health Sciences Center Animal Care and Use Commit-

tee. Fourteen time-mated 2–3-year-old Columbia–Rambouillet ewes pregnant with a single fetus were used for these studies. Litter size was determined by real-time vaginal ultrasonic scanning at approximately 32 dpc and confirmed at surgery. Surgery was performed at 32–37 dpc, following a 48-h fast with free access to water. At surgery the ewe was sedated with intravenous ketamine (0.01 ml/kg body weight). After midline laparotomy, the retro-peritoneal adipose deposit was exposed, the membrane cut and a temperature transmitter (Mini-Mitter, Sunriver, OR, USA) was inserted. The membrane incision was sutured and the tissue returned to its pre-operative position. The midline was sutured and a polyvinyl sampling catheter was inserted in the maternal femoral artery, which was tunneled subcutaneously to a maternal flank pouch. Antibiotics were given pre- and post-operatively for 2 to 3 days. Post-operatively, all animals were kept in individual carts and given water and alfalfa pellets and allowed to feed *ad libitum*. Following surgery transmitter signal was determined and core body temperature (CBT) recording commenced (DataCol5, Mini-Mitter), recording CBT every 15 min.

Approximately 2–4 days after surgery, once CBT was 39.2 °C, ewes were weighed and allocated to either the thermoneutral (TN) or hyperthermic (HT) treatment. A total of eight ewes were maintained in the TN regime, in individual pens (2.0 × 2.5 m) at a constant ambient temperature 20 ± 2 °C 24 h; 30% relative humidity. Six ewes were moved to individual pens (1.3 × 1.7 m) in a HT treatment chamber (Bainbridge, Marcellus, MI, USA). The HT treatment was characterized as being 40 °C 12 h, 35 °C 12 h; 30–40% relative humidity. Lighting in the TN room was 0600–1800h, 100% light, 1800–2100h, 66% light, 2100–2300h, 33% light and 2300–0600h, 0% light. The HT room was 12 h 100% light (0700–1900h) and 12 h 0% light.

Ewes in the HT environment had their feed and water intake recorded daily. Ewes were fed a diet of alfalfa hay pellets, containing 90.4% dry matter, crude protein of 20.8%, and an acid detergent fiber content of 25.9%. Calculated metabolizable energy content was 9.7 MJ/kg. Gestational age-matched TN ewes were fed the average of the HT ewes intake, never falling below 1 kg/day (9.75 MJ), a level in excess of required metabolizable energy intakes for ewes at this stage of gestation (Subcommittee on Sheep Nutrition 1998). Water consumption of TN ewes was also measured daily and for both groups, water was changed daily. Maternal arterial blood samples (6 ml), were collected twice weekly, including two pre-treatment samples collected approximately 2 days after surgery, with the first treatment sample taken 12–24 h after entry into treatment environment. Sampling occurred between 0700 and 0900h, prior to feeding, and resulting plasma was stored at –80 °C until assay. In addition to hormone sample collection, plasma glucose concentrations were determined in duplicate with a

glucose/lactate analyzer (YSI Model 2700 Select and Dual Standard). Ewe breaths per minute (bpm) were also recorded, consisting of four, 15-s observation periods.

Tissue collection

Ewes were killed at 93 ± 1 dpc, corresponding to approximately 56 days in treatment (dit). The gravid uterus was removed and dissected into placental and fetal components. Placentomes were trimmed of endometrium and fetal membranes and weighed, placental weight being recorded as the sum of the placentomes. An average placentome weight was recorded as the total placentome weight divided by the total placentome number. Placentome cotyledonary tissue was manually separated from caruncular tissue, rinsed in 4 °C physiologic saline, frozen in liquid N₂, and stored at -80 °C until RNA isolation. Fetuses were towel dried, weighed, and crown-rump length (CRL) measured (Mellor & Matheson 1979). Fetal liver, heart and brain were removed and weighed. From these measurements fetal brain/liver index (brain weight (g)/liver weight (g)) and fetal ponderal index (fetal weight (g)/CRL (cm³)) were calculated.

Hormone assays

Blood samples were analyzed for progesterone, PL and prolactin. Progesterone concentrations were determined using a validated (Bevers *et al.* 1989) Coat-A-Count progesterone RIA kit (DPC, Los Angeles, CA, USA), with interassay and intra-assay coefficients of variation (CV (%)) for the low quality control (QC) being 9.23 and 6.02% respectively, and for the high QC, 6.68 and 3.44% respectively.

oPL concentrations were determined as previously described (Kappes *et al.* 1992), with changes in sample volume to take into account for the early gestational age of the samples and advancing gestational age. Interassay and intra-assay CV values were 8.25 and 6.15%, respectively for a low QC, 3.25 and 2.35% for a medium QC, and 3.67 and 3.45% for a high QC, over three assays.

Ovine prolactin concentrations were determined using a double antibody RIA with ovine prolactin standards (oPRL NHPP-AFP-10789B) and anti-ovine prolactin antisera (anti-oPRL NHPP-AFP-C358106) following methods previously described (Nett *et al.* 1975). Interassay and intra-assay CV values were 8.64 and 8.13% respectively for a low QC, 3.07 and 3.07% for a medium QC, and 7.47 and 5.53% for a high QC, over three assays.

Cotyledonary mRNA extraction and Northern blot analysis

Total cellular RNA was isolated from cotyledonary tissue and Northern analysis performed as previously described (Warren *et al.* 1990a, Kappes *et al.* 1992). Cotyledonary DNA was determined in tissue samples extracted for RNA

by the fluorometric assay as previously described (Labarca & Paigen 1980). A total of 20 µg total cellular RNA was analyzed using standard Northern blot and hybridization techniques (Kappes *et al.* 1992). A 866 bp oPL cDNA fragment was isolated from EcoRI sites in its pBluescript SK(-) vector, as described previously (Kappes *et al.* 1992). The oPL cDNA and a 250 bp oGAPDH cDNA were random prime labeled with [³²P]deoxy-CTP to a specific activity of 1.5×10^8 c.p.m./µg DNA. Autoradiographs were obtained by exposure to film. Densitometry was performed using a scanner and National Institutes of Health Image software.

Western blot analysis

Cotyledonary tissue was homogenized on ice in a tissue homogenate buffer (25 mM Tris(hydroxymethyl) amino-methane (Tris) HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 1 mM PMSF, 2 µM leupeptin and 1 µM pepstatin A). The homogenate was centrifuged at 1500 g at 4 °C for 10 min to remove cell debris. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on 20 µg aliquots homogenate protein with a 15% (wt/vol) polyacrylamide gel. Prior to loading, samples were mixed with Laemmli sample buffer (1:2), heated at 95 °C for 5 min and then centrifuged briefly. Proteins were transferred to nitrocellulose membrane using an electrophoretic transfer cell. The blot was blocked in TBS-T (50 mM Tris HCl pH 7.4, 150 mM NaCl, 5% (vol/vol) bovine serum albumin (BSA), and 0.1% (vol/vol) Tween-20) for 1 h at room temperature. The blot was then incubated with a polyclonal antibody directed against oPL (Warren *et al.* 1990b), diluted 1:15 000 in buffer overnight at 4 °C. The blot was then washed three times, 10 min per wash, TBS-T at room temperature. The blot was then incubated for 1 h with anti-rabbit IgG antibody-horseradish peroxidase diluted in blocking buffer. The blot was then washed three times, 10 min per wash, with TBS-T at room temperature, and proteins were visualized by enhanced chemiluminescence (Le Cras *et al.* 1996). To take into account possible differences in gel loading, oPL values were normalized against the cell adhesion protein, Pecam-1 (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following oPL immunodetection, antibodies were removed by incubation in 100 mM 2-mercaptoethanol, 62.5 mM Tris HCl, pH 6.8, 2% (wt/vol) SDS, at 50 °C for 30 min with slight agitation, and then washed with TBS-T twice for 10 min at room temperature. The blot was then blocked in TBS-T, plus 5% (wt/vol) BSA, as described, and then incubated for 1 h with Pecam antibody (1:20 000). The same procedure as described for PL immunodetection followed. The intensity of the bands were determined by densitometry using a scanner and National Institutes of Health Image software.

Figure 1 CBT of TN and HT ewes following entry into environment treatment. Horizontal lines represent mean CBT for each treatment group from 3 dit.

Apparent molecular weight for oPL and Pecam was confirmed by comparison with prestained molecular size markers.

Statistical analysis

CBT of the two treatment groups were modeled to take into account the repeated measures of the data for each ewe. SAS PROC SPECTRA and moving average methodology were used to detect the frequency of the cyclic fluctuations of the equally spaced time series. Following this, a best fit sinusoidal wave equation was determined for each group using circadian rhythm analysis (Zerbe & Jones 1998) in combination with mixed model methodology implemented with SAS PROC MIXED (Littell *et al.* 1996). These equations account for the variability between sheep and the variability between multiple measurements within sheep for each group. The fitting of a first order harmonic equation allowed modeling of the 24 h cyclic nature of the CBT. The model provided estimates of amplitude (peak height above the mean group CBT) and phase (the amount of time between time zero and the first peak of the cosine wave). Ten time periods were defined for the analysis, period 1 was 0–1 dit, period 2, 1–2 dit, period 3, 2–3 dit, period 4, 3–4 dit, period 5, 4–5 dit, period 6, 5–10 dit, period 7, 10–20 dit, period 8, 20–30 dit, period 9, 30–40 dit, and period 10, 40–56 dit.

Hormone data were analyzed using a general linear mixed model, which accounts for the variability within sheep as well as between sheep (Littell *et al.* 1996). For each treatment group, mixed models featuring polynomial group mean response curves were fitted. These were modeled with stochastic parameters in order to account for variability between sheep and between multiple measurements on the same sheep. Polynomial models took the form of a first or second degree, or intercept-only polynomial. The Scheffe method was used to adjust for comparisons of the mean response curves at multiple times as discussed previously (Young *et al.* 1997). Comparisons

were made among the two groups at 1, 10, 20, 30, 40, 50 and 55 dit.

Three TN ewes and one HT ewe were not killed at 90 dpc in order to be available for later gestational studies. As a result, reduced animal numbers were analyzed for placental and fetal parameters using Student's *t*-test. Placental and fetal data analysis was conducted on TN (5) and HT (5). oPL mRNA and protein concentrations were also analyzed using Student's *t*-test, however a shortage of tissue reduced animal numbers to four in each group.

Results

Core body temperature and breaths per minute (bpm)

All animals obtained a resting CBT of 39.2 °C following surgery before being moved to either the TN or HT treatment. TN ewes had a mean CBT throughout the study of 39.19 ± 0.1 °C (Fig. 1). HT ewes had CBT significantly greater than TN ewes within 48 h of entry into the HT environment. From 2 dit onwards, CBT of HT ewes were significantly greater than TN ewes (HT 39.86 ± 0.1 °C vs TN; 39.19 ± 0.1 °C; $P < 0.001$, Fig. 1). Ewes exposed to elevated ambient temperatures (HT), had significantly higher circadian amplitude and altered phase angles when compared with TN ewes. While TN ewes had a flatter amplitude around the mean CBT of 0.091 ± 0.002 °C, HT ewes displayed significantly ($P < 0.0001$) greater fluctuations of 0.181 ± 0.002 °C. The time of peak CBT in the TN ewes, as measured by the calculated phase shift value, occurred at 1800 h, while HT ewes' peaks occurred 3 h later at approximately 2100 h. TN ewes' peak CBT occurred in 100% light, while HT peak CBT occurred approximately 2 h after entry into 0% light and the drop from daytime temperature of 40 °C, to the night-time regime of 35 °C.

Exposure of ewes to elevated ambient temperature resulted in bpm increasing from a pre-exposure rate

Table 1 Arterial hormone concentrations in TN (8) and HT (6), prior to commencement of treatment (37 ± 2 dpc). Values are means $\pm$ pooled standard error of treatment means.

	TN	HT	S.E.M.	P
Progesterone (ng/ml)	6.69	6.67	0.5	>0.1
Placental lactogen (ng/ml)	6.0	7.75	1.95	>0.1
Prolactin (ng/ml)	97.8	125.5	11.21	>0.1

of 50 ± 3 up to 134 ± 9 bpm for the HT group within 3 dit. The HT group appeared to acclimatize with bpm declining to 80 ± 5 at the studies conclusion.

Feed and water intakes, and glucose concentrations

Average feed intake of the HT ewes over the experimental period rose from 9.1 to 10.7 MJ/day at the end of the study period. TN ewe feed intake was that of the gestational age mated HT ewes. Ewes exposed to high ambient temperature had significantly greater water intake than TN ewes (HT; 4.48 ± 0.35 L vs TN; 3.42 ± 0.28 L; $P < 0.05$). Glucose concentrations were not significantly different between groups, though concentrations declined from an average for both treatments of 3.8 ± 0.08 mmol after entry into treatment, settling at 3.5 mmol at approximately 30 dit, corresponding to 70 ± 2 dpc.

Hormone concentrations during hyperthermic exposure

Pre-treatment concentrations of the hormones examined were not significantly different between the allocated groups (Table 1). Stochastic parameter models with second-degree polynomials, fitted for the progesterone, oPL and prolactin data for each of the treatments are provided in the associated figures. Arterial progesterone concentrations became significantly lower in HT ewes between 30 and 40 dit, corresponding to approximately 72 dpc ($P < 0.05$; Fig. 2). HT ewes displayed a constant progesterone concentration throughout the study, whereas TN ewes displayed a pregnancy associated increase. Arterial oPL concentrations became significantly reduced in HT ewes, between 20 and 30 dit, corresponding to approximately 62 dpc ($P < 0.05$; Fig. 3). However, concentrations did increase with advancing gestation, though concentrations remained significantly reduced when compared with TN ewes. Prolactin concentrations immediately rose in the HT ewes to concentrations approximately four times greater than in TN ewes, levels which were significantly different from 5 dit onwards ($P < 0.001$; Fig. 4).

Hyperthermic effects on cotyledonary PL mRNA and protein concentration

Exposure to hyperthermia for 56 days had no effect on cotyledonary oPL mRNA expression, collected at approximately 90 dpc (Fig. 5). oPL mRNA expression,

Figure 2 Gestational changes in arterial progesterone concentrations and regression lines for TN ($\blacktriangle$, $6.958 - (0.014 \times \text{dit}) + 0.002 \times \text{dit}^2$) and HT ($\blacksquare$, $7.025 - (0.011 \times \text{dit}) + 0.00002 \times \text{dit}^2$) ewes. Values are means $\pm$ S.E.M.

normalized for cellular DNA was similar in both groups (Data not shown). The cotyledonary PL protein was also found to be similar in both groups of animals (Fig. 6).

Placental and fetal weights

Placental weights at 93 ± 1 dpc, while not significantly different, were reduced in HT ewes (Table 2). Fetal weights were significantly reduced by 27% ($P < 0.05$; Table 2). CRL were unaffected by heat, though calculated Ponderal index was significantly reduced in the HT fetuses ($P < 0.05$; Table 2). Liver weights were significantly reduced in HT fetuses ($P < 0.05$; Table 3), although when

Figure 3 Gestational changes in arterial placental lactogen concentrations and regression lines for TN ($\blacktriangle$, $4.011 - (0.167 \times \text{dit}) + 0.0127 \times \text{dit}^2$) and HT ($\blacksquare$, $10.090 - (0.646 \times \text{dit}) + 0.0127 \times \text{dit}^2$) ewes. Values are means $\pm$ S.E.M.

Figure 4 Gestational changes in arterial prolactin concentrations and regression lines for TN (▲, 54·402) and HT (■, 139·528 + (3·680 × dit) – 0·04050 × dit²) ewes. Values are means ± S.E.M.

expressed relative to body weight, liver weight was not significantly different. Hyperthermia had no effect on brain and heart weights (Table 3).

Discussion

The results reported here show that following approximately 30 days of heat exposure, during active placental development, progesterone and oPL concentrations

Figure 5 Northern blot analysis of oPL mRNA levels of TN and HT cotyledons. The oPL mRNA was determined to be approximately 1 kb. Quantification of the oPL mRNA levels normalized to the signal from GAPDH mRNA is also shown. The results depict oPL mRNA expression in five TN and four HT ewes. Values are means ± S.E.M.

Figure 6 Western blot of cotyledonary oPL protein content of TN and HT ewes. The oPL antibody detected a single band in the cotyledonary homogenates of 22 kDa. Quantification of the oPL protein signal is also shown. The results depict oPL protein concentration in four TN and four HT ewes. Values are means ± S.E.M.

become significantly depressed. These data suggest that elevated CBT impair placental hormone synthesis and/or metabolism in pregnant ewes. Ovine progesterone concentrations during pregnancy are initially solely a measure of ovarian secretion, but placental secretion becomes the dominant source by approximately 50 dpc, increasing as gestation advances (Bassett *et al.* 1969). We now report that ewes exposed to heat, 20 days earlier than the previous reports (Bell *et al.* 1987), display a reduction in placental hormone concentration around the time of the onset of placental dominance of progesterone secretion. Progesterone concentrations from 35 to 70 dpc (35 dit), during the period of active interdigitation, were similar in TN and HT ewes. However, at the time when

Table 2 Placental and individual placentome weights, placentome numbers, fetal/placental weight ratios and the fetal weights, CRL, Ponderal indexes of TN (5) and HT (5) fetuses following 56 dit. Values are means ± pooled standard error of treatment means

	TN	HT	S.E.M.	P
Placental weight (g)	571·2	363·6	64·3	>0·1
Individual placentome (g)	7·13	5·53	0·91	>0·1
Placentome number	81·0	72·0	4·42	>0·1
Fetal/placenta weight ratios	1·4	1·6	0·2	>0·1
Fetal weight (g)	703·4	514·6	44·3	<0·05
Fetal CRL (cm)	27·0	25·7	0·53	>0·1
Ponderal index	3·57	3·01	0·13	<0·05

Table 3 Fetal organ and relative organ weights of TN (5) and HT (5) fetuses following 56 dit. Values are means $\pm$ pooled standard error of treatment mean

	TN	HT	S.E.M.	P
Organ weight (g)				
Brain	16.11	13.38	0.93	>0.1
Liver	45.16	27.31	4.60	<0.05
Heart	5.18	4.65	0.25	>0.1
Brain/liver	0.37	0.54	0.05	>0.1
Relative organ weight (g/kg fetus)				
Brain	2.29	2.65	0.14	>0.1
Liver	6.35	5.20	0.40	>0.1
Heart	0.73	0.94	0.06	<0.1

concentrations of progesterone would be expected to increase with the decline of ovarian progesterone secretion and increase in placental secretions (50–60 dpc), progesterone concentrations in HT ewes became depressed in comparison to rising TN concentrations.

Circulating progesterone concentrations became significantly lower just prior to term in ewes subjected to heat from 60 dpc, attributed to a reduced placental mass (Bell *et al.* 1987). While placental weight was not significantly different in our study (~ 90 dpc), HT placental weights were 36% less than TN placentae. This suggests that, late in gestation, reduced progesterone concentrations could be a result of a reduced placental mass, as postulated in other studies (Bell *et al.* 1987, Wallace *et al.* 1997). However, the observed reduced progesterone concentrations early in gestation in the present study implies heat adversely effects the development of placental cells responsible for steroid production and metabolism, before factors such as a reduced placental weight might affect circulating hormone concentrations. In contrast, in cattle, progesterone levels increase under HT conditions during the estrus cycle (Abilay *et al.* 1975). Exposure of cattle to elevated ambient temperature appears to promote ovarian synthesis and secretion of steroids during estrus (Abilay *et al.* 1975), and progesterone concentrations remain elevated throughout gestation (Collier *et al.* 1982). This elevation is attributed to stimulation of peripheral thermoreceptors, resulting in the activation of hypothalamic adrenocorticotropin releasing mechanisms (Abilay *et al.* 1975). Differences in the cellular source of progesterone during pregnancy between cattle and sheep may explain differences in responses to elevated temperatures. The corpus luteum is the primary source of progesterone throughout pregnancy in cattle, while in sheep, placental production of progesterone is the dominant source during the later two thirds of pregnancy (Bassett *et al.* 1969). Hyperthermia induced changes in steroid production by the placenta in cattle, could be masked by the dominance of ovarian progesterone secretion, which is stimulated by hyperthermia. Our data shows

that there is a reduced concentration of placental steroids and hormones, following ovarian demise at approximately 50 dpc. This suggests that in sheep, heat may adversely affect placental trophoblast function and or development. Although the exact cause of reduced progesterone concentration in our study is unknown, two possibilities exist. Hyperthermia is affecting either placental steroid synthesis and secretion through interactions upon trophoblast function, or it is altering steroid clearance rate following secretion.

The oPL data may help answer this question. In HT ewes, PL concentrations decline around the same time as progesterone concentrations decline. The binucleated cells (BNC) of the fetal trophoblast are the only cells that produce PL (Kappes *et al.* 1992). PL is considered an important marker of placental integrity and function, with impaired secretion being implicated in altered maternal and fetal metabolism (Freemark & Comer 1989, Handwerger 1991). As gestation advances, PL plasma levels rise (Kelly *et al.* 1974, Oddy & Jenkin 1981, Gluckman & Barry 1988), as a function of total BNC within the placenta (Kappes *et al.* 1992). In our study, heat exposure from 37 dpc, was a critical determinant for circulating oPL concentrations, significantly reduced concentrations suggesting an altered BNC population. Examination of cotyledonary PL mRNA and protein concentration revealed a lack of difference between TN and HT ewes. This suggests that HT treatment does not effect the translation or transcription of oPL, and therefore differences in both maternal PL and progesterone concentrations could be attributable to an impaired trophoblast migration.

Trophoblast cells share the common feature to elaborate and metabolize steroid hormones (Strauss *et al.* 1996). In ruminant placentae, 15–20% of the chorionic epithelia consists of granulated BNC, the percentage of which remains constant over gestation (Wooding 1992). While these cells represent only a fifth of the placental cell population, they are the major source of placental protein and steroid hormones responsible for the establishment and maintenance of pregnancy. Binucleate cells migrate through the apical tight junction of the primary chorionic epithelium, across the microvillar junction of the fetomaternal interface, and fuse into the uterine syncytium (Wooding *et al.* 1986). The syncytium is closely associated with the maternal circulation, and following fusion, the BNC granules are released by exocytosis into the syncytium. The delivery of placental products throughout gestation that this initiates, may be critical to the development of a successful metabolic and/or immunologic dialogue between mother and fetus (Wooding 1992). The failure of human cytotrophoblast cells to differentiate into invasive cells, can result in abnormal placentation and potentially in IUGR and/or preeclampsia (Cross 1996). In human and sheep IUGR, reduced placental hormone and steroid concentrations have been associated with reduced

trophoblast function and trophoblast mass (Westergaard *et al.* 1984, Maruyama *et al.* 1986, Newnham *et al.* 1986). In the sheep, the secretion pattern of protein hormones, such as oPL, is postulated to depend on the continual movement of these cells from the fetal to maternal component (Steven *et al.* 1978, Duello *et al.* 1986, Kappes *et al.* 1992). A failure of normal fetomaternal syncytium development could result in abnormal placental function.

Radiotelemetry has allowed detailed analysis of CBT in both TN and HT ewes during maximal placental growth. An immediate rise in HT ewes' CBT was observed, reaching an asymptote within 2 days of HT treatment. During that period, HT animals maintained their circadian pattern, though with increasing amplitude around their mean CBT. Elevated CBT has been correlated with changes in circulating prolactin concentrations (Wettemann & Tucker 1974, 1976). Hyperthermia increases prolactin concentration in ovariectomized ewes (Schillo *et al.* 1978), similar to rises observed in cattle (Wettemann & Tucker 1974, 1976), mid to late gestation ewes (Bell *et al.* 1989), and those changes reported here. While prolactin is involved in the initiation of lactation (Forsyth 1986), a role during mid pregnancy has not been established in sheep. Circulating concentrations of prolactin do not change during normal gestation until just prior to the onset of lactation (Wettemann *et al.* 1973, Bell *et al.* 1989), and it seems to have no function in human osmoregulation (Baumann *et al.* 1977), suggesting that the observed elevated prolactin is a chronic response to a physiologically stressful situation.

To date, studies concerning the effects of heat stress upon pregnancy have concentrated on the metabolic adaptations by the IUGR fetus to placental failure, in late gestation (Bell *et al.* 1987, 1989, Thureen *et al.* 1992, Ross *et al.* 1996, Anderson *et al.* 1997). Elevated CBT from approximately 35 dpc results in a placental mass that, at term, is significantly reduced and associated with an IUGR fetus (Thureen *et al.* 1992). These fetuses are approximately 40% lighter than a normal fetus and display increased brain/liver ratios and altered fetal biometric ultrasonographic measurements associated with growth retardation (Thureen *et al.* 1992, Galan *et al.* 1999). Reduced birth weights and IUGR are associated with poor maternal nutrition in humans, however, heat induced IUGR in the sheep is considered independent of maternal nutrition (Dreiling *et al.* 1989). In the data reported here, the object of pair feeding the animals was to remove any effects of nutrition on conceptus development and growth. This regime allows developmental or growth impairment to be attributed to the effects of chronic heat exposure. When TN ewes are fed the same feed intake of gestational age matched heat stressed ewes, heat stressed lamb birth-weights are significantly reduced, while TN weights are unaffected (Cartwright & Thwaites 1976, Brown *et al.* 1977). Furthermore, both groups of ewes consumed approximately 10.7 MJ per day, an amount greater than the

estimated 10.5 MJ required metabolizable energy intake of pregnant ewes during the first 105 days of gestation (Subcommittee on Sheep Nutrition 1998). In addition, all ewes displayed normal glycemia over the course of this study, concentrations similar to those previously reported to occur during mid gestation (Bell *et al.* 1989).

Under the environmental regimes used, HT fetal weights were significantly reduced, despite a non-significant reduction in placental weight. The fetus at this stage of gestation is not in a rapid growth stage, and reduced fetal weights suggest that what little nutrients the fetus requires, are not supplied adequately. A direct reduction of available nutrients from the maternal compartment can not be offered as the cause of this fetal weight reduction, since maternal glucose concentrations indicate HT animals are in a state of euglycemia. We postulate that fetal growth failure results from severe placental insufficiency, manifested by abnormal trophoblast development and function. In another report, following exposure to heat between 50 and 75 dpc, fetal weights are observed not to be affected (Vatnick *et al.* 1991). The differences between this report and our data highlight two important suggestions, first that the effect of heat exposure prior and during the maximal growth of the placenta is critical to later placental transfer capacities. Secondly, fetal development and growth occurring between 75 and 90 dpc, can be impaired in a heat stressed placenta. Liver weights were significantly reduced in HT fetuses, similar to changes reported in late gestation IUGR fetuses (Bell *et al.* 1987), which together with a maintained brain weight produced an increased brain/liver weight ratio (Thureen *et al.* 1992). The reduced liver weight in term fetuses has been attributed to a reduced capacity of the heat-stunned placenta to supply nutrients. If this is the case in 90 dpc HT fetuses, then a major placental transport malfunction must be occurring at this relatively early stage in fetal development. Furthermore, fetal CRLs are similar between the two treatment groups, while calculated ponderal index is decreased in HT fetuses. These observations are similar to fetuses subjected to heat following maximal placental development, indicating that the growth of soft tissues is more affected than bone (Bell *et al.* 1989). Of equal importance is the fact that alterations in fetal development and growth are present in 90 dpc fetuses, suggesting that the factors responsible for soft tissue growth are adversely effected during early fetal development by elevated CBT.

Previously it has been suggested that heat induced alterations in plasma progesterone and oPL, associated with IUGR, were consequences rather than causes of placental stunting (Bell *et al.* 1989). However, we report that pregnant ewes exposed to chronic heat stress during maximal placental development display significantly reduced progesterone and oPL concentrations. This occurs prior to a significant placental weight divergence, highlighting a major disruption of early placental function and

or metabolism in the heat stress induced model of placental insufficiency. We postulate that heat stress induced impaired trophoblast development could result in dysfunctional cells that are essential to normal placental development, adversely affecting further placental development and maternal/fetal metabolic interactions. The migration of trophoblast BNC, those cells responsible for steroid and protein synthesis and secretion in the ruminant placenta, plays a major role in the delivery of hormones to the circulatory systems of mother and fetus. Alterations in this system would potentially limit the conceptus' capacity to produce the biochemical signals, such as steroids, hormones and growth factors, necessary for normal placental development and later fetal development and growth.

The relationship between placental and fetal growth implies that placental growth must precede fetal growth, and that potential deficiencies in early placental growth will interfere with subsequent fetal development more severely than heat exposure later in placental development. HT exposure during maximal placental development appears to reduce circulating hormone concentrations, which adversely affects placental and fetal development and growth. As a result of compromised placental development, fetal requirements are impaired earlier in gestation, further impairing fetal development leading to IUGR. This study highlights that IUGR may result primarily from trophoblast cell dysfunction, and secondarily from reduced placental size.

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