

PREGNANCY STAGE AND NUMBER OF FETUSES MAY INFLUENCE MATERNAL PLASMA LEPTIN IN EWES

Margit KULCSÁR¹, Gabriella DANKÓ², H. G. I. MAGDY³, J. REICZIGEL¹, T. FORGACH⁴,
Angella PROHÁČZIK¹, Carole DELAVAL⁵, K. MAGYAR², Y. CHILLIARD⁵, L. SOLT¹
and GY. HUSZENICZA^{1*}

¹Faculty of Veterinary Science, Szent István University, H-1400 Budapest, P.O. Box 2,
Hungary; ²Faculty of Agricultural Sciences, University of Debrecen, Debrecen, Hungary;

³Department of Applied Radiobiology, Nuclear Research Centre, Cairo, Egypt;

⁴Frédéric Joliot-Curie National Research Institute for Radiobiology and Radiohygiene,
Budapest, Hungary; ⁵INRA-Theix, St-Genes Champanelle, France

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Maternal plasma leptin is elevated in ewes during pregnancy. The authors studied whether there was any relation between maternal plasma leptin and insulin concentrations, the number of fetuses and the circulating and faecal levels of gestagens. At the end of the breeding season in January the ovarian activity of Prolific Merino ewes was induced/synchronised with gestagen + eCG treatment. Ewes were inseminated artificially (AI) by laparoscopy. Blood and faecal samples were collected before AI (day 0) and again 41, 81 and 101 days later. The plasma levels of leptin (pL), insulin and progesterone (pP₄), and the faecal P₄ metabolite (P₄-met) content were determined. The day 0 level of pL was significantly higher in pregnant (n = 24) than in non-pregnant ewes (n = 32). By day 41 the pL of pregnant animals had doubled, it showed a further moderate increase on day 81, and decreased slightly thereafter. During pregnancy pP₄ and faecal P₄-met rose continuously and were positively correlated at all stages. The mean levels of pL and pP₄ and the faecal content of P₄-met were lower in ewes bearing single (n = 12) than in those with 2 (n = 6) or 3–5 fetuses (n = 6). Analysis of variance demonstrated significant differences according to the number of fetuses in the pL and pP₄, but not in P₄-met (p = 0.042, 0.044, and 0.051, respectively). Leptin showed positive correlation with insulin before the AI but not during pregnancy. On days 41 and 81 pL showed a slight positive correlation with P₄ and P₄-met, which decreased slightly by day 101. This study shows that although leptinaemia is affected by the number of fetuses and the level of P₄, pregnancy stage is a more important regulator than these additional factors.

Key words: Ewe, leptin, pregnancy, fetus, progesterone

*Corresponding author: Gyula Huszenicza; E-mail: huszenicza.gyula@aotk.szie.hu;
Phone: 0036 (1) 478-4202; Fax: 0036 (1) 478-4230

The recently identified leptin is a cytokine-like protein hormone, which controls the energy homeostasis and body composition, and influences a series of physiological processes (immune response, haematopoiesis, angiogenesis, etc.) (Ingvarsen and Boisclair, 2001; Chilliard et al., 2005; Kulcsár et al., 2005). It is an important regulator of ovarian function, providing a signalling link between nutritional status and reproduction (Zieba et al., 2005). This protein is the product of the *ob* gene and yielded predominantly in white adipocytes (Chilliard et al., 2005), although the contribution of mammary gland in ruminants (Bonnet et al., 2002a and 2002b; Sayed-Ahmed et al., 2004; Bartha et al., 2005) and the placenta in primates and rat (Ashworth et al., 2000) may also have relevance. In adult non-pregnant ruminants the plasma leptin relates positively to the cell size and triglyceride content of adipocytes, and also to the actual feeding level (Delavaud et al., 2000; Ehrhardt et al., 2000; Delavaud et al., 2002). Insulin and cortisol are the main endocrine regulators of *ob* gene expression in adipocytes, and of the circulating level of leptin (Chilliard et al., 2005). However, the additional effect of other factors such as sexual steroids seems to be important (Kulcsár et al., 2005). Plasma leptin level may also vary depending on the stage of pregnancy and lactation (Bonnet et al., 2002a; Vernon et al., 2002). During pregnancy the plasma leptin increases significantly in several species, due to either the enhanced expression of placental leptin mRNA (in primates and rat) or a dramatic elevation in plasma content of soluble leptin receptors, which serve as binding proteins in circulation, and decrease the renal leptin clearance (in mice) (Ashworth et al., 2000). In ruminants the degree of placental leptin gene expression is negligible (Ehrhardt et al., 2001; Vernon et al., 2002), and current knowledge concerning the presence or absence of circulating leptin binding proteins in the blood is uncertain (Chilliard et al., 2005). In spite of these facts, along the gestation elevated plasma leptin levels were reported to occur in ewes (Ehrhardt et al., 2001; Thomas et al., 2001) and nulliparous (but not in lactating primiparous) goats (Bonnet et al., 2005). However, before this study no data were available on whether this pregnancy-associated hyperleptinaemia of small ruminants interacted with the number of fetuses and/or with the gestation-related increase of sexual steroids and insulin.

The purpose of the present investigation was to study whether during early and mid-pregnancy (i) the number of fetuses, (ii) the gestation-associated continuous gestagen load, and (iii) the plasma levels of insulin interacted with circulating leptin in ewes. Gestagen load was quantified by the determination of plasma progesterone (pP₄) and faecal P₄ metabolite (P₄-met) contents.

Materials and methods

Animals, experimental design and sampling

The study was conducted on Prolific Merino ewes ($n = 61$; age: 2 to 5 years; body weight: 33–43 kg) of mild to moderate body condition at the Experimental Farm of the University of Debrecen at the end of the breeding season in January. During the first 72 days the group of animals was kept in a pen and fed grass hay *ad libitum*, whereas after the beginning of the grazing season in April they were moved to a pasture land on alkaline soil, providing abundant grass until lambing in June. Free access to water was available throughout the experiment. In January 2002 the ovarian activity of ewes was induced or synchronised with a 14-day-long gestagen (vaginal sponge of 40 mg Cronolone; ChronogestTM, Intervet International B.V., Angers, France) administration combined with 500 IU eCG (Folligon inj.TM, Intervet International B.V., Angers, France; i.m.) at the time of vaginal sponge removal. Sixty hours later the ewes were inseminated (AI) once with fresh diluted semen, which was deposited with Salamon's method (Evans and Maxwell, 1987) by laparoscopy, after 24-h feed and water deprivation. Pregnancy rate and number of fetuses were calculated based upon the data of lambing in June.

At the beginning of feed deprivation preceding the AI (day 0) and 41, 81 and 101 days thereafter blood samples (10 ml) were taken from the jugular vein into heparinised tubes, cooled and centrifuged within 60 min. Plasma was harvested immediately and stored at -20°C until assayed for progesterone (P_4), insulin and leptin. Simultaneously, faeces (5–10 g) was collected from the rectum into plastic sacs. Faecal samples were frozen (-20°C) and analysed for P_4 -met some weeks later. Body weight was measured simultaneously with the first sampling.

Laboratory procedures

Plasma leptin (pL) concentration was quantified by a modified version of the ruminant-specific, homologous, double-antibody, non-equilibrium ^{125}I -RIA of Delavaud et al. (2000, 2002). The current adaptation of this assay was based on the use of the anti-ovine leptin antibody obtained by Delavaud et al. (2000) in rabbits. Instead of recombinant ovine leptin, however, in this version a commercially available form of recombinant bovine leptin ($_{\text{rb}}$ leptin; DSL-RO 1708-100, Diagnostic Systems Laboratories, Inc., Webster, Texas, USA) was used for radioiodination, as well as for preparing the standards. Furthermore the bound and free ligands were separated using a magnetisable immunosorbent suspension (1.0 mg/ml; containing magnetisable iron oxide microparticles coated with anti-rabbit goat IgG; particle size: 1–5 μm ; minimum binding capacity: 2.5 μg IgG/mg; MIS-4100, Isotope Institute Co. Ltd, Budapest, Hungary), rather than applying a specific anti-rabbit second antibody. The $_{\text{rb}}$ leptin was labelled with ^{125}I by the Chloramine T

method (after Hunter and Greenwood, 1962; with modification). Briefly, 5 µg $_{rb}$ leptin in 10 µl solvent was reacted with 30 MBq ^{125}I (in the form of NaI; Institute of Isotopes Co. Ltd., Budapest, Hungary) and 10 µg Chloramine T (Reanal Ltd., Budapest, Hungary) in boric acid-borate buffer (0.1 mol, pH 8.6). After one minute mixing the reaction was stopped by adding 20 µg sodium metabisulphite. The ^{125}I - $_{rb}$ leptin was separated in a column (20 cm long $\times$ 1 cm diameter) filled with Sephadex G 50-fine (No. 17-0042-01, Pharmacia, Uppsala, Sweden). One-ml fractions were collected, and 1 ml glycerol was added to each fraction containing ^{125}I - $_{rb}$ leptin. The free iodine to ^{125}I - $_{rb}$ leptin ratio was determined by thin layer chromatography on DC-Alufolien Kieselgel 60 (Art. No. 5553, Merck Lab., Darmstadt, Germany). The triplicates of standards were prepared from a stock preparation of $_{rb}$ leptin (1 µg/ml) at the following concentrations: 0.0833, 0.125, 0.25, 0.40, 0.75, 2.00, 2.50, 4.00 and 5.00 ng/tube (in 50 µl). The assay was carried out as follows: 100 µl horse serum was added to all the standards, as well as to the B0 and NSB tubes to obtain a similar protein matrix as in the samples. As samples and quality control tubes ($n = 3$; with 0.058 ± 0.004 , 0.241 ± 0.008 and 0.577 ± 0.023 nmol/l levels as regular control samples with 'low', 'medium' and 'high' leptin content in each assay run) triplicate aliquots of 100 µl plasma were used. Standard, quality control and sample tubes were vortexed with 50 µl of diluted leptin antiserum (in a final dilution of 1:10,000) and then incubated at +4 °C overnight. After that, 100 µl of ^{125}I - $_{rb}$ leptin (12-13,000 cpm, in assay buffer) was added to each tube, and after vortexing the process was continued with a second overnight incubation at +4 °C. Bound and free ligands were then separated by adding 500 µl ice-cold magnetisable immunosorbent suspension. Then the tubes were incubated for 30 min at room temperature. After centrifugation (3600 g, 30 min., +4 °C) all the tubes were put in a magnetic separator (Amersham Lab, Little Chalfont, Buckinghamshire, England), and the supernatant was decanted. The remaining radioactivity on the microparticles was quantified by an automatic gamma counter (Wallac-1470 Wizard, Perkin Elmer, Turku, Finland). The leptin content was calculated by the 1224-310 MultiCalc v2.7 programme (Perkin Elmer, Turku, Finland). Under routine use the sensitivity of this assay (defined as the lower quantity able to generate a diminution of 5% of the B/B0 ratio) was 0.032 nmol/l. The calculated inter- or intra-assay coefficients of variation (CV) were 12.15, 5.61 and 6.13%, or 10.06, 4.57 and 5.28% in the ranges of quality control samples with 'low', 'medium' and 'high' leptin content, respectively.

Plasma insulin content was quantified with a commercial kit (^{125}I -Insulin RIA CT kit, CIS Bio International Ltd., Gif-Sur-Yvette, France) validated for ovine samples (sensitivity: 7.75 pmol/l, intra- and interassay CV: 5.5–8.4 and $\leq 8.8\%$). The *plasma progesterone* (pP_4) levels were determined by a direct ^3H -RIA used for human (Csernus, 1982) and equine (Nagy et al., 1998) samples, which was validated for assaying ovine plasma without any modification (sensitivity: 0.20 nmol/l, intra- and interassay CV: ≤ 4.5 and $\leq 8.9\%$).

For assaying P_4 -met, steroid content was extracted from faeces according to a slight modification of the method described by Palme et al. (1996, 1999). Briefly, 0.50 g of faeces was dispersed in 0.50 ml double-distilled water in thick-walled glass tubes suitable for centrifugation. After adding 4 ml methanol (80%), samples were shaken for 20 min with a multitube vortex. Thereafter 3 ml petroleum ether (bp. 40–70 °C) was added to remove lipids. Then samples were mixed again at high speed for 10 sec by hand-vortex. After centrifugation (3600 g, 30 min, + 4 °C) samples were taken at –70 °C for 25–30 min to separate the three phases of petroleum ether (above), methanol (in the middle) and frozen water with the extracted faeces (below). Then 0.5–1 ml of the methanol phase was pipetted into clean tubes and 1:10, 1:20 or 1:50 working solutions were diluted with PBS buffer (pH 7.4) when low or high P_4 -met levels were expected, respectively (if values were out of the expected range, the sample was re-assayed). P_4 -met concentrations were measured in triplicate 20- μ l aliquots of faecal extracts with a microplate ELISA (Nagy et al., 1998), based on the use of an anti- P_4 monoclonal antibody (5D4) cross-reacting with a wide range of gestagen metabolites (5 β -pregnan-3,20-dion: 100%; 11 α -OH-progesterone: 20%; 5 α -progesterone-3,20-dion: 15.6%; 17 α -OH-progesterone: 3.6%; pregnenolone: 1.8%; 11 β -OH-progesterone: 1.6%) (Siklódi et al., 1995).

In all of endocrine assay systems employed in this study the binding pattern of serially diluted ovine plasma and faecal samples was parallel to that of the standard curves. The recovery rates of added known quantity of hormones were the following: (i) leptin (0.125, 0.250 and 0.500 nmol/sample) and insulin (25, 50 and 100 pmol/sample) to standard ovine plasma samples (n = 3): between 95 and 106%; (ii) P_4 and 5 β -pregnan-3,20-dion (125, 250 and 500 nmol/g of each; both from Steraloids Inc., Newport, USA) to methanolic extract of standard ovine faecal samples (n = 3): between 93 and 102%; (iii) 5 β -pregnan-3,20-dion (125, 250 and 500 nmol/g) to standard ovine faecal samples (n = 3): between 78 and 87%.

Statistics

For presentation of results the group means and their standard errors (SEM) were expressed. The results were subjected to chi-square (χ^2) test (distributions), Student's *t*-test (pair-wise comparison of group means), or a single-trait analysis of variance (ANOVA; comparison of 3 groups in a particular stage or time-related changes within a group). Pregnancy-related changes in the plasma levels of these hormones were evaluated by repeated-measures ANOVA (S-PLUS) using time (day 41, day 81 and day 101) as the within-subject factor, the number of fetuses (1, 2, and ≥ 3) as a between-subject factor and the initial (day 0) measurement as a covariate. Some correlation analyses were also performed (Kleinbaum and Kupper, 1978; Juvancz and Paksy, 1982; Snedecor and Cochran, 1982). Analyses were done using the statistical package S-PLUS 2000 (MathSoft Inc., Seattle, Washington, USA).

Results

Data of 56 ewes were evaluated, whereas the other animals were excluded because of incomplete sampling ($n = 4$) or conception later than the AI in late January ($n = 1$). Twenty-four of the 56 animals (43%) conceived at the AI studied, and delivered one ($n = 12$), two ($n = 6$) or 3 to 5 lambs ($n = 6$) after a regular gestation in June.

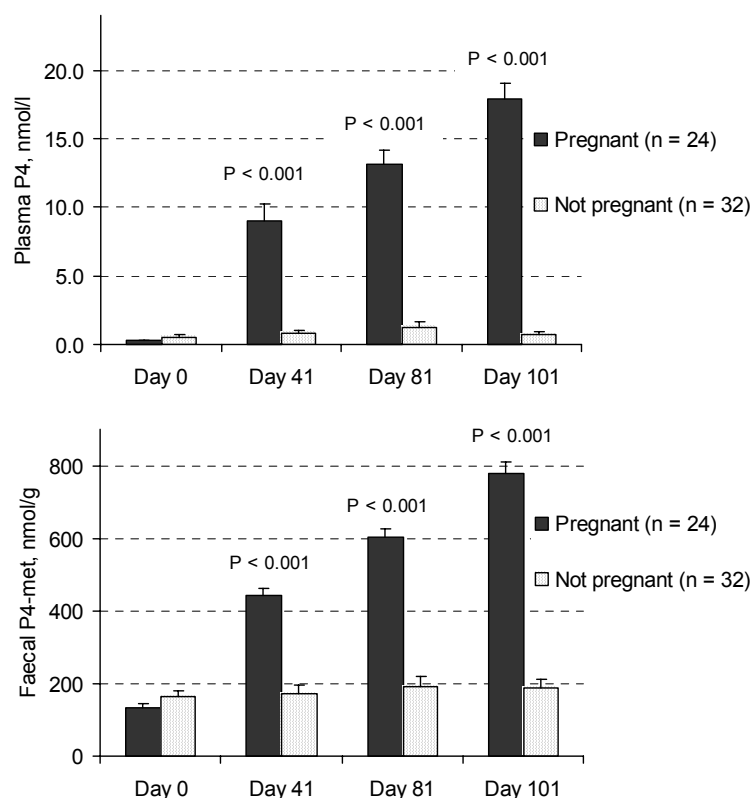


Fig. 1. Plasma progesterone (P₄) and faecal progesterone metabolite (P₄-met) levels of pregnant ewes compared to those of ewes that did not conceive at the AI (day 0) and became acyclic throughout the spring-time sampling period (mean $\pm$ SEM; time-related changes in P₄ and P₄-met of pregnant ewes: $P < 0.001$, with single-trait ANOVA)

All but 4 ewes had low plasma P₄ and faecal P₄-met concentrations (< 1.50 nmol/l and < 250 nmol/g, respectively) at the time of AI, representing the range known as physiological in the follicular phase of cyclic small ruminants (Schwarzenberger et al., 1996; Pereira et al., 2006). None of these 4 individuals with elevated plasma P₄ (1.57 to 4.54 nmol/l) and faecal P₄-met (268 to 424 nmol/g) contents conceived at that time. During pregnancy the P₄ and P₄-met

levels rose continuously, peaking in the last samples taken on day 101 of gestation (Fig. 1). Plasma P_4 and faecal P_4 -met were in significant positive correlation in all stages of the study ($r = 0.526, 0.612, 0.810$ and 0.881 on days 0, 41, 81 and 101, respectively; $P < 0.001$). The P_4 and P_4 -met contents of ewes failing to conceive at the AI remained low (details are not given), proving the lack of cyclic ovarian function in this season.

The initial (day 0) levels of leptin and insulin varied between 0.125 and 0.463 nmol/l and between 64.4 and 96.9 pmol/l, respectively, and showed a moderate positive correlation ($r = +0.455$; $P < 0.001$). This interrelationship persisted throughout the study in non-pregnant ewes only ($n = 32$; $r = +0.438$; $P < 0.001$). No correlation was found, however, between leptin and body weight. The day 0 level of leptin was higher in ewes that became pregnant ($n = 24$) than in those that remained non-pregnant ($n = 34$) (Fig. 2), but the mean body weight in these two groups did not differ (data not shown). The plasma leptin of pregnant ewes had doubled by day 41, it showed a further moderate increase on day 81, and decreased slightly thereafter (Fig. 2). The plasma insulin levels did not show any significant gestation-related changes, and during pregnancy no significant correlation was found between these two hormones (details are not given).

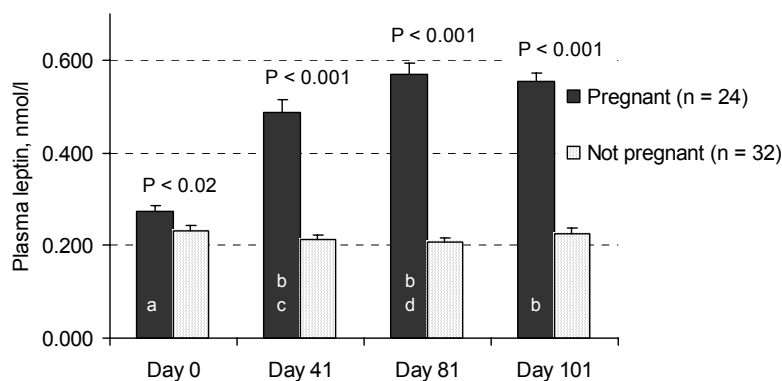


Fig. 2. Plasma leptin levels of pregnant ewes compared to those of ewes that did not conceive at the AI (day 0) and became acyclic throughout the spring-time sampling period (mean $\pm$ SEM; time-related changes of pregnant ewes: bars signed with diverse letters represent significantly different values: $a-bP < 0.001$; $c-dP < 0.05$, with single-trait ANOVA)

The mean levels of P_4 , P_4 -met and leptin were slightly lower in ewes bearing a single fetus than in those with 2 or 3–5 fetuses (Figs 3 and 4). According to the number of fetuses the repeated-measures ANOVA proved significant differences in P_4 and leptin but not in P_4 -met ($P = 0.044, 0.042$ and 0.059 , respectively). On days 41, 81 and 101 the leptin was in slight positive correlation ($P < 0.01$ to 0.05) with P_4 ($r = +0.353, +0.389$ and $+0.329$, respectively) and P_4 -met ($r = +0.297, +0.305$ and $+0.303$, respectively).

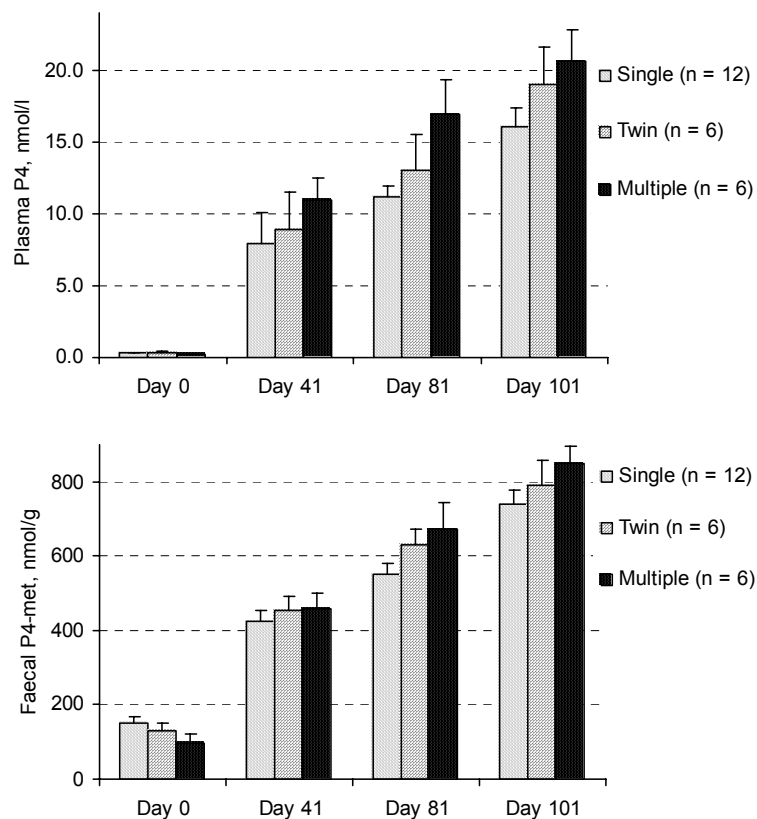


Fig. 3. Plasma progesterone (P₄) and faecal progesterone metabolite (P₄-met) levels of pregnant ewes bearing single vs. twin and multiple (3–5) fetuses (mean ± SEM; pregnancy-related changes in plasma P₄ and P₄-met: P = 0.044 and 0.059, respectively; with repeated-measures ANOVA)

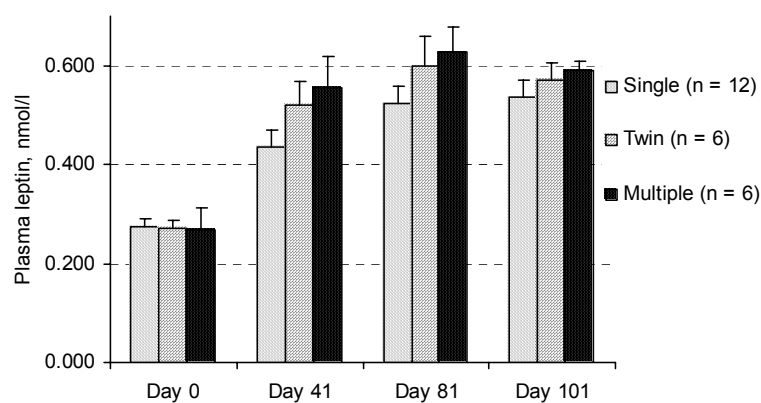


Fig. 4. Plasma leptin levels of pregnant ewes bearing single vs. twin and multiple (3–5) fetuses (mean ± SEM; pregnancy-related changes: P = 0.042; with repeated-measures ANOVA)

Discussion

Pregnancy provides a long-lasting exposure to various hormones for the dam. In ewes the corpus luteum (CL) is the exclusive source of P_4 during the first 50 days. Placental enzymes (Δ^{4-5} isomerase and 3β -ol-dehydrogenase) converting pregnenolone to P_4 become activated on days 50 to 60, and thereafter this hormone comes mainly from the placenta. In general, the greater the amount of placental tissues (as seen in twin or multiple pregnancies), the more P_4 is produced. However, this correlation is not close, and plasma P_4 in ewes bearing single, twin and multiple fetuses may overlap. Placental oestrogen production starts to increase around days 70–80 after conception, but really high levels can be found only in the last 3–4 weeks of gestation (Edqvist and Forsberg, 1997; Senger, 2003). During the ovine pregnancy a distinct alteration is seen also in the function of the adipo-insular axis. As usual in non-lactating ruminants, the concentration of insulin is relatively high (Prior and Christenson, 1978; Harmon, 1992; Petterson et al., 1993) while insulin resistance develops, which is reflected by the decreased ability of adipocytes and skeletal muscle to utilise glucose (Ehrhardt et al., 2001). Also considerable hyperleptinaemia typical of pregnancy occurs in this species (Ehrhardt et al., 2001; Thomas et al., 2001), mainly due to a 2.3-fold increase in leptin mRNA content of adipocytes. Its degree is unrelated to the energy balance of the dam (Ehrhardt et al., 2001). During pregnancy-associated hyperleptinaemia a decrease in hypothalamic leptin sensitivity was confirmed in laboratory rodents (Waddell and Smith, 2003). These alterations in the levels of and sensitivity to insulin and leptin are involved in maternal metabolic adaptation to pregnancy, controlling the partition and expenditure of energy sources and body reserves (Ehrhardt et al., 2001).

According to the experience gained in laboratory rodents and primates (Ashworth et al., 2000; Christou et al., 2003; Henson and Castracane, 2003; Islami and Bischof, 2003; Waddell and Smith, 2003; Yoon et al., 2005; Zieba et al., 2005), this metabolic effect of maternal leptin is completed by the local intrauterine activity of the leptin–leptin receptor system, which may be an important regulator of implantation as well as of placental and fetal growth and development. In human fetuses adiposity is an important factor influencing plasma leptin concentration. Plasma leptin remains static until week 33 of gestation, and then it increases rapidly parallel with the rate of fat deposition. Plasma leptin is already present in the plasma of ovine fetuses by day 40 but, in contrast to humans, it increases at a very slow rate during the fetal life (Ehrhardt et al., 2002). In fetal sheep leptin mRNA abundance in perirenal adipose tissue increases with gestational age toward term (Yuen et al., 1999; Thomas et al., 2001; Forehead et al., 2002; Yuen et al., 2004). These differences may be attributed to the much lower body fat content of fetal sheep, particularly near term. Absence of adipose depots in fetal sheep before day 70 of pregnancy implies that plasma leptin

originates from other tissues (fetal brain and liver) during early and mid-pregnancy. The ontogenic changes in plasma leptin and tissue mRNA levels coincide with the rise in plasma cortisol concentration seen in the fetus near term. On day 138 the fetal (but not the maternal) plasma leptin concentrations are inversely related to uterine blood flow and fetal and placental weight, proving the involvement of this hormone in an adaptive response to intrauterine growth restriction (Buchbinder et al., 2001). These data suggest that the ovine fetus cannot contribute meaningful amounts of leptin to the maternal circulation.

Our samples taken during pregnancy in this study represented the last days of the CL-dependent stage (day 41), and the early and middle period of placenta dependency (days 81 and 101). On days 81 and 101 also the oestrogen concentrations may be elevated but not very high until late pregnancy. Glucocorticoids, which can also stimulate leptin synthesis (Chilliard et al., 2005; Kulcsár et al., 2005), are not increased in the ovine plasma until just before term. Similarly, increase in the plasma level of prolactin and placental lactogen does not occur until late pregnancy (Edqvist and Forsberg, 1997; Senger, 2003). So only the P_4 , P_4 -met and insulin were determined in the current experiment.

In accordance with our expectation the P_4 and P_4 -met levels were continuously elevated throughout pregnancy, and related positively to the number fetuses. The relevance of this tendency was reflected by its interrelation with plasma leptin. The production rate of gestagens relates positively both to plasma P_4 and faecal P_4 -met contents in ruminants (Rabiee et al., 2002). However, plasma P_4 showed closer correlation with leptin than faecal P_4 -met in this study, which was perhaps due to the variability in the dry matter content of faecal samples. It has been clearly demonstrated that leptin acts on the level of the hypothalamus–anterior pituitary axis regulating the secretion of gonadotropins, and thus ovarian and testicular function (Zieba et al., 2005). It is less known, however, that at least in laboratory rodents and primates, after accounting for body fat the second most important determinant of plasma leptin is gender, resulting in more circulating leptin in females than in males of equivalent body fat (Rosenbaum and Liebel, 1999; Rosenbaum et al., 2001). Cyclic women show higher plasma leptin content in the luteal than in the follicular phase (Ludwig et al., 2000; Phipps et al., 2001; Messinis and Domali, 2003). These experiences demonstrate that testosterone suppresses, whereas 17β -oestradiol, P_4 or their combination can stimulate leptin production. However, the plasma levels of 17β -oestradiol and P_4 are more elevated in primates than in ruminants. Our results suggest that the pregnancy-associated long-term increase of gestagens may be one of the factors enhancing leptin production in pregnant ewes.

The plasma insulin levels were relatively high in the day 0 samples and, as compared to the initial values, no further changes were seen throughout the gestation. Insulin and leptin concentrations were correlated only before AI and in non-pregnant animals, but not during pregnancy, which confirms the finding of Ehrhardt

et al. (2001) that in ewes the gestation-challenged increase in leptin production is not related to changes in insulin or whole-body glucose response to insulin.

Body condition correlates well with plasma leptin in ewes ($r = +0.68$; Delavaud et al., 2000). However, lack of significant correlation was observed between the body weight and plasma leptin of dams in this study, obviously due to the age-related variability in the size of our animals. Confirming this idea, the animals that conceived at AI had higher plasma leptin than those failing to conceive, which suggests that ewes in better body condition can become pregnant more easily at the end or out of the breeding season. We think that the body weight was not a sufficiently sensitive parameter to detect this tendency: future studies using more precise parameters of body fat content should be done to improve this hypothesis.

In summary, we think that although the degree of pregnancy-associated hyperleptinaemia is affected by the number of fetuses and the level of P_4 in ewes, pregnancy stage is a more important regulator than these additional factors.

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