Havemeyer Foundation
Monograph Series No. 10

Proceedings of a Workshop on

EMBRYONIC AND FETAL NUTRITION

15th – 18th May 2003
Ravello, Italy

Editors: S. Wilsher and J. F. Wade

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EDITORS' FOREWORD

In the horse, the emphasis on maternal nutrition during pregnancy has tended to focus on the last trimester, when fetal growth is at its maximum. Although the demands for nutrients may not be as high during the earlier stages of pregnancy, organogenesis and the high metabolic and growth rates of the young embryo and fetus are no doubt also nutritionally sensitive.

Although the role of the diffuse, non-invasive, epitheliochorial equine placenta in providing nutrition for the developing foal have been explored, there remains a paucity of data relating to the role of maternal nutrition in both placental development and fetal nourishment *in utero*. It is clear from work in other species that embryonic, fetal and placental development are all governed by maternal diet, both directly by the supply of essential nutrients and indirectly via the governing influences of a myriad of endocrine mechanisms. To add to the complexity of the situation, maternal age, size and bodily condition have also been shown to influence partitioning of nutrients between the gravid uterus and the maternal body.

The lessons to be learnt from research workers studying various species are often of great value. The discussion, speculation and innovative science that often results from the exchange of comparative information is immensely valuable and stimulating. For this reason, the equine research community has much reason to be grateful to the Havemeyer Foundation, and particularly to Mr Gene Pranzo, for their foresight in understanding the importance of a comparative element in workshop meetings. The Havemeyer Workshops have a reputation for spawning international, multi-species collaborations and this workshop was no exception. A number of these had been initiated by the time this monograph went to press; and these will, no doubt, form the basis of discussions at a number of future workshops.

Jan Wade

Sandra Wilsher

HAVEMEYER SCIENTIFIC WORKSHOPS

1981 **First International Workshop on Lymphocyte Alloantigens of the Horse**
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Organiser: Dr D. F. Antczak

1982 **Second International Workshop on Lymphocyte Alloantigens of the Horse**
October - Cornell University, Ithaca, New York, USA
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1984 **First International Symposium on Equine Embryo Transfer**
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July - University of Guelph, Canada
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1989 **Second International Symposium on Equine Embryo Transfer**
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1992 **Workshop on Equine Neonatal Medicine**
January - Naples, Florida
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Third International Symposium on Equine Embryo Transfer
February - Buenos Aires, Argentina
Organisers: Drs D. F. Antczak, W. R. Allen, J. G. Oriol and R. Pashen

1995

Equine Perinatology
July - Cambridge, England
Organiser: Dr P. D. Rossdale

Second International Equine Leucocyte Antigen Workshop
July - Lake Tahoe, California, USA
Organisers: Drs D. F. Antczak, P. Lunn and M. Holmes

First International Workshop on Equine Gene Mapping
October - Lexington, Kentucky, USA
Organisers: Drs D. F. Antczak and E. Bailey

Erection and Ejaculation in the Human Male and Stallion: A Comparative Study
October - Mount Joy, Pennsylvania, USA
Organiser: Dr S. M. McDonnell

Bone Remodelling Workshop
October - Corcord, Massachusetts, USA
Organiser: Dr H. Seeherman

1997

Second International Workshop on Equine Gene Mapping
October - San Diego, California, USA
Organisers: Drs D. F. Antczak and E. Bailey

Maternal Recognition of Pregnancy in the Mare
January - Dominican Republic
Organisers: Drs W. R. Allen and T. A. E. Stout

Uterine Clearance
March - Gainesville, Florida, USA
Organiser: Dr M. M. LeBlanc

Trophoblast Differentiation
September - Edinburgh, Scotland
Organisers: Drs D. F. Antczak and F. Stewart

1998

Third International Genome Workshop
January - San Diego, California, USA
Organisers: Drs D. F. Antczak and E. Bailey

Third International Workshop on Perinatology: Genesis and Post Natal Consequences of Abnormal Intrauterine Developments: Comparative Aspects
February - Sydney, Australia
Organiser: Dr P. D. Rossdale

Horse Genomics and the Genetic Factors Affecting Race Horse Performance
March - Banbury Center, Cold Spring Harbor, New York, USA
Organisers: Drs D. F. Antczak, E. Bailey and J. Witkowski

Allergic Diseases of the Horse
April - Lipica, Slovenia
Organisers: Drs D. F. Antczak, S. Lazary and E. Marti

Equine Placentitis Workshop
October - Lexington, Kentucky, USA
Organisers: Drs D. F. Antczak, W. R. Allen and W. Zent

Septicemia II Workshop
November - Boston, Massachusetts, USA
Organiser: Dr M. R. Paradis

1999 **Equine Genome Project**
January - San Diego, California, USA
Organisers: Drs D. F. Antczak and E. Bailey

Third International Equine Genome Workshop
June - Uppsala, Sweden
Organisers: Drs D. F. Antczak, E. Bailey and K. Sandberg

Fourth International Meeting of OIE and WHO Experts on Control of Equine Influenza
August - Miami, Florida, USA
Organiser: Dr J. Mumford

European Equine Gamete Workshop
September - Lopuszna, Poland
Organisers: Drs W. R. Allen and M. Tischner

Fetomaternal Control of Pregnancy
November - Barbados, West Indies
Organisers: Drs T. Stout and W. R. Allen

2000 **Equine Genome Project**
January - San Diego, California, USA
Organisers: Drs D. F. Antczak and E. Bailey

Uterine Infections in Mares and Women: A Comparative Study
March - Naples, Florida, USA
Organiser: Dr M. M. LeBlanc

5th International Symposium on Equine Embryo Transfer
July - Saari, Finland
Organiser: Dr T. Katila

2001 **USDA International Plant & Animal Genome Conference**
January - San Diego, California

Equine Immunology in 2001
January - Santa Fe, New Mexico
Organiser: Dr D. P. Lunn

Asthma and Allergies II
April - Hungary
Organisers: S. Lazary and E. Marti

From Elephants to Aids
June - Port Douglas, Australia
Organiser: Professor W. R. Allen

International Equine Gene Mapping
July - Brisbane, Australia
Organiser: K. Bell

Second Meeting of the European Gamete Group (EEGG)
September - Loosdrecht, The Netherlands
Organiser: Dr T. A. E. Stout

Foal Septicemia III
October - Tufts University European Center, Talloires, France
Organiser: M. R. Paradis

Infectious Disease Programme for the Equine Industry and Veterinary Practitioners
October - Marilyn duPont Scott Medical Center, Morvan Park, Virginia, USA
Organisers: Drs J. A. Mumford and F. Fregin

From Epididymis to Embryo
October - Fairmont Hotel, New Orleans, USA
Organiser: Dr L. H-A. Morris

2002 **USDA International Plant & Animal Genome Conference**
January - San Diego, California

Comparative Neonatology/Perinatology
January - Palm Springs, California
Organiser: P. Sibbons

Stallion Behavior IV
June - Reykjavik, Iceland
Organisers: S. McDonell and D. Miller

Rhodococcus Equi II
July - Pullman, Washington
Organiser: J. Prescott

Equine Orthopaedic Infection
August - Dublin, Ireland
Organiser: E. Santschi

Inflammatory Airway Disease
September - Boston, USA
Organiser: Dr E. Robinson

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SESSION I:

Chairman:

W. R. Allen

FALLOPIAN TUBE MICROENVIRONMENTS, GAMETES AND EARLY EMBRYONIC DEVELOPMENT

R. H. F. Hunter*, R. Nichol and H. J. Leese

Department of Clinical Studies – Reproduction, Royal Veterinary and Agricultural University, Copenhagen

Because the mammalian egg is endowed with abundant reserves of cytoplasmic substrate, especially lipid droplets, there has been a tendency to view such material as adequate for cleavage requirements until the uterine stage of development. With the notable exception of studies by Brinster (1965), Biggers *et al.* (1967), Hamner (1973) and Rieger (1984), there has seldom been a focus on the metabolic requirements of the embryo during the first 48–72 h; this is despite the fact that the zygote commences development within the lumen of the Fallopian tube, the fluid of which is appreciated to vary both quantitatively and qualitatively with time after ovulation. This paper will argue that changing microenvironments within the Fallopian tubes could have an important influence in programming the embryo for subsequent fetal and placental development, and that this proposal need not be contradicted by results obtained from *in vitro* studies. After a phase of culture, there is invariably a microscopic selection of embryos prior to transplantation into a recipient uterus. Such morphological selection may suggest that *in vitro* procedures are more effective than is the case and that the Fallopian tubes are less critical as a site of development than is now gradually being appreciated, not least from a macromolecular point of view (eg Novak *et al.* 2002).

The nature of tubal luminal fluid was reviewed in depth by Leese (1988) with reference to products of transudation and those of specific secretion. However, that paper treated the fluid as a gross or bulk fluid rather than one composed of regional environments within the Fallopian tube. Indeed, the latter represent a somewhat paradoxical finding, bearing in mind the contractile activity of the myosalpinx and the synchronised beat of cilia, both of which are enhanced at the time of ovulation and shortly thereafter (Hunter 1988). Mixing of luminal fluids would be anticipated, tending to obscure regional differences in composition. Nonetheless, such differences have been demonstrated by acute microsampling in different portions of the ampulla and isthmus (Nichol *et al.* 1992). Stabilisation of regional fluid environments may be achieved in part by the complex arrangement of the epithelial surface (Yaniz *et al.* 2000), in part by the action of viscous glycoproteins (see Leese *et al.* 2001).

In addition to such considerations related to the role of the endosalpinx, it is essential to take note of post ovulatory cell populations within the lumen of the Fallopian tube. Granulosa cells are shed with the oocyte at ovulation, both as cells in suspension in follicular fluid and as cumulus cells usually retaining loose attachment to the zona pellucida. After dissociation from the egg surface, these ovarian somatic cells remain in the close vicinity of the egg and large numbers are synthetically active, although not necessarily mimicking their pre-ovulatory synthetic activity. Even so, their secretions include steroid hormones, peptides and glycosaminoglycans and, together with components of follicular fluid, they generate a particular environment in the immediate vicinity of an embryo. Furthermore, this microenvironment may amplify embryonic signals to the endosalpinx and beyond, leading to modified secretory activity and a positive feedback loop on the embryo (Hunter 2002, 2003). At the most sensitive level, expression of the embryonic genome may be responding to the immediate fluid environment, not only within the tubal lumen but also in the perivitelline space.

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Macromolecules such as oviductin are capable of reaching the vitelline surface and can then enter the cytoplasm - which may suggest functional interactions.

The extent to which individual embryos undergo a crucial phase of genomic sensitisation or programming during passage along the Fallopian tubes requires further exploration. The suggestion here is of a level of fine control due to specific interactions with components of the maternal duct fluid environment that vary with region and time after ovulation. Candidate molecules that might modulate maternal influences would include peptide growth factors and unique glycoprotein secretions. When such putative molecular modulation of the embryonic developmental programme is in abeyance, as in systems of culture prior to embryo transplantation into the uterus, subsequent growth of the placenta and fetus may be less tightly regulated. Indeed, this might provide a partial explanation for the large fetus syndrome in ruminants. The notion that co-culture of embryos with endosalpingeal cells is representative of events in the Fallopian tube is naïve. There are major considerations of timing, not least when using inter-specific systems, and of the secretory integrity of cultured endosalpingeal cells. However, as demonstrated by gamete or embryo transplantation directly into the uterine body, primates offer exceptions to the preceding line of thought. This may be principally because of the nature of the utero-tubal junction and a substantial overlap in constituents of tubal and uterine secretions (Hunter 1998).

If it is accepted that fetal growth within the uterus can have an important influence on post natal development, and that embryonic development within the Fallopian tubes may have a specific influence on placental and fetal growth, then there is one further consideration. It concerns pre-ovulatory nutrition of the oocyte within a Graafian follicle. Leaving nuclear aspects to one side, full development of a primary oocyte represents far more than growth to a mature diameter and volume, and it would be prudent to envisage some variation in the metabolic reserves of individual oocytes released at ovulation. This might contribute to the somewhat tenuous notion of 'oocyte quality'. A well-balanced account of fetal nutrition in the uterus and embryonic nutrition in the Fallopian tubes will ultimately require assessment of oocyte nutrition within the follicular antrum, ie molecular aspects of oocyte maturation.

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REGULATION OF DEVELOPMENT IN THE EMBRYO: IMPACT OF TEMPORARY REMOVAL FROM OR DISRUPTION OF ITS NATURAL ENVIRONMENT ON SUBSEQUENT GROWTH AND VIABILITY

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INTRODUCTION

Factors affecting embryo development *in vivo* include nutrients, maternal physiology, maternal health status and environmental cues. Consequently, alterations to one or more of these may impinge on survival, growth and long-term well-being of mammalian offspring. Likewise, separating an embryo from its native environment, as occurs during *in vitro* embryo culture, can lead to adverse developmental outcomes. In such circumstances, the repercussions may fail to be redressed even after the embryo has been restored to a normal uterine environment.

TEMPORARY REMOVAL FROM NATURAL ENVIRONMENT

A key feature of mammalian embryos during the week following fertilisation is that their blueprints for development are partly pre-ordained and partly self-determined. The former, dictated by maternal mRNA transcripts, informs the initial sequence of events leading to zygotic genome activation; there follows a transition phase when both maternal mRNA and zygotic gene transcripts are active; subsequently, the zygotic genome assumes more complete control. The diploid embryo, thereafter being dependent on transcripts from a unique coalition of genes of maternal and paternal origin, relies on epigenetic events to play a crucial role in determining the expression or silencing of imprinted genes, which express only the maternal or paternal allele. Imprinted genes include some that influence growth, development and placentation, notably *H19, IGF2, IGF2R* and *Mash2*. Consequently, because epigenetic control measures are sensitive to *in vitro* culture conditions, development can be disrupted if, for example, normal DNA methylation patterns are altered. In ruminants (cattle and sheep), placental and fetal anomalies detected following *in vitro* embryo culture have been attributed to this scenario. In some circumstances, legacies of short-term *in vitro* culture have been detected during post natal life, being manifest for example in the enlarged hearts of bulls more than one-year-old (McEvoy *et al.* 1998). More often, however, the consequence of inappropriate culture may be earlier and more lethal aberrations leading to the loss of a conceptus long before a pregnancy has run its course. A recent report from Peterson and Lee (2003) provides some of the most convincing evidence of this eventuality in ruminants and chronicles a dramatic reversal of fortunes in respect of placental pathologies, which had plagued conceptuses derived from *in vitro* culture programmes at their laboratory and elsewhere. The major problem of allantoic malformation – detected in up to 25% of *in vitro* embryo-derived bovine pregnancies examined between Days 22 and 24, as the allantois emerged and inextricably linked with subsequent fetal death approximately one month after the commencement of pregnancy – was resolved when the source of serum albumin, an almost ubiquitous constituent of mammalian embryo culture media, was changed. Although the biological explanation for the radical improvement has not been determined, it is notable in the context of this review that the less harmful albumin variant was, in its protein profile, more representative of albumin fragments that embryos encounter naturally *in utero* (Peterson and Lee 2003).

While severe allantoic malformations have accounted for many of the first trimester losses following transfer of embryos produced *in vitro*, there also has been a legacy of placental

insufficiency among survivors. Bertolini and Anderson (2002) noted differences in placentomes and in cotyledonary surface area in bovine pregnancies subsequent to *in vivo* and *in vitro* embryo transfer. Furthermore, they proposed that high birthweights associated with transfer of embryos produced *in vitro* might be due to disruption of the placental restraint normally imposed on fetal growth in late pregnancy. This may be the case, but is more likely allied to a fetal predisposition to oversize than, as might be inferred, mere passivity on the part of the fetus. Indeed, the large offspring phenomenon associated with ruminant embryos produced *in vitro* is likely to have both fetal and placental components that are intimately linked in terms of their origins and impact. For example, while both placental and cardiovascular developmental anomalies have been reported as discrete occurrences, it is necessary to note that, for their normal well-being, both systems are mutually inter-dependent. A question that arises, therefore, is whether heart enlargement is a phenotypic consequence of having to work extra hard in conditions of compromised placental function. This may be the case since reduced blood flow associated with inferior villous placental development leads to increased blood pressure which, in turn, stimulates cardiomyocyte differentiation (Cross 2001). Moreover, for *in vitro* bovine embryo-derived pregnancies, Bertolini *et al.* (2002) reported that increased fetal heart rate was associated with atypical placentome development from early stages in the first trimester. At a more fundamental level, there is clear evidence, from the murine developmental model, of mechanistic links between the pathways governing cardiovascular and placental development, one of these being *HandI* which is critical in both systems (Cross 2001). Consequently, any disruption of this or some other shared essential factor inevitably would have repercussions for both organ systems.

DISRUPTION OF NATURAL ENVIRONMENT

Disruption of an embryo's natural environment can arise as a result of dietary changes, environmental stresses (eg elevated temperatures), infections or ingestion of toxins. Often the consequence is embryonic loss or early pregnancy failure. Sometimes, however, the effects may not be lethal - at least in the short term. In such instances, the insult to the natural environment may give rise instead to a malnourished fetus, a stillborn or weak neonate or an offspring with limb or other deformities.

The dietary changes most likely to disrupt an animal's natural physiology in a manner detrimental to reproductive performance are those which acutely deprive a pregnant dam of necessary nutrients (as in pregnancy toxaemia), or dramatically alter endocrine status, eg by excessive progesterone degradation in instances of overfeeding. Alternatively, sudden dietary changes can impose a digestive upset or imbalance that, before the liver can adapt, exposes the utero-ovarian environment to toxic levels of ammonia or other harmful by-products of digestion. In the authors' experience, both oocytes and embryos are sensitive to changes in endocrine status, pH and nitrogen balance *in vivo.* Moreover, as outlined by Ashworth and Antipatis (2001), appropriate micronutrient provision is crucial to the development and well-being of concepti, whether in single-bearing or polytocous animals. Inadequate and excessive supplies of vitamin A, for example, have analogous detrimental effects on embryo development (Ashworth and Antipatis 2001).

Of the aforementioned threats, overfeeding is the most likely to induce or aggravate another hazard encountered *in vivo* – namely, heat stress. The damaging impact of high temperatures on embryo survival has been well documented, although it is often difficult to quantify the extent of loss due to elevated environmental temperatures. The loss may even be due to temperature stress prior to ovulation. For instance, data from Rocha *et al.* (1998) indicate that the normality and competence of Holstein *(Bos taurus)* oocytes is compromised by high environmental temperatures. In a subsequent review, Hansen *et al.* (2001) surmised that an inability to synthesise heat shock protein 70 in response to temperature stress could contribute to such oocyte sensitivity.

Frequently, increased body temperatures are associated with additional detrimental complications, such as infections, in which instances it is probably impossible to determine definitively causes of embryonic or later death. Again, other conditions may diminish an animal's heat tolerance and thereby aggravate the threat posed by elevated temperatures to conceptus survival *in vivo.* A case in point is that which

occurs in cattle exhibiting fescue toxicosis. This is caused by a mycotoxin from the fungal endophyte, *Acremonium coenophialum*, and, as well as the associated heat intolerance which has been indicated as a possible cause of increased early embryonic death (McEvoy *et al.* 2001), ingestion of mycotoxins could undermine bovine pregnancies via induction of anorexia and consequent nutritional inadequacies (Zavy 1994). Horses are particularly susceptible to fescue toxicosis (Putnam *et al.* 1991) and outbreaks can quadruple embryo loss (Brendemuehl *et al.* 1994). Non-domestic species held in captivity and domestic animals in tropical or sub-tropical conditions can also be vulnerable to fungal toxins or other dietary contaminants, especially if they are reliant on feeds and forages held in storage for prolonged periods. In the case of non-domestic animals belonging to endangered species, the diet-sensitive reproductive stakes - which are sensitive in terms of level of feeding, suitability of feed and exposure to toxins (McEvoy and Robinson 2003) - may be very high indeed.

In conclusion, as noted in an earlier review in this series (see Robinson *et al.* 2000), there are crucial windows of developmental opportunity and risk throughout pregnancy and beforehand. Therefore, a key factor determining the consequences of *in vivo* perturbations is the time at which the 'insults' arise. Death, the ultimate penalty, can occur either at embryonic or later stages (see Giles *et al.* 1993; Sharp 1994) for relevant equine data). However, the actual repercussions often depend on the stage of development *in utero* (whether pre-implantation, early fetal or later) at which a nutrient deficit or exposure to a toxin or a temperature stress occurs. Some would-be deficiencies or toxic agents are detrimental only if encountered during specific phases of pregnancy, but others must be avoided by the breeding animal throughout development, from before ovulation until after offspring weaning.

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CAN MATERNAL NUTRITION INFLUENCE THE SEX OF OFFSPRING BORN?

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Many invertebrate and some avian species can adjust the sex ratio (usually defined as proportion male) among their progeny in a highly predictable manner depending upon prevailing environmental conditions and the associated relative costs and benefits of producing more offspring of one sex than the other (Hamilton 1967; Charnov 1982; Nunney and Luck 1988; Nager *et al.* 1999; West *et al.* 2000, 2002; West and Sheldon 2002). Sex ratio adjustments appear to contribute to parental fitness by ensuring that parental genes are transmitted most efficiently to future generations at the least cost. Although there are numerous reports of sex ratio variation in mammals in relation to factors such as food availability and competition for resources (see Clutton-Brock and Iason 1986), and evidence that some of the changes might be adaptive and in accordance with evolutionary theory, this area remains controversial (Clutton-Brock and Iason 1986).

Perhaps the best-known examples of major changes in sex ratio in mammals have been reported for various species of deer, either in the wild or under some form of confinement (Clutton-Brock and Iason 1986; Wauters *et al.* 1995; Flint *et al.* 1997; Kruuk *et al.* 1999). Trivers and Willard (1973) in their sex allocation theory predicted that in such wild polygynous species, where a small proportion of males sire most of the young yet invest little in their care, females in the best body condition would be anticipated to produce more sons than daughters, because such male offspring would be more likely to join the class of elite breeder males when they reached adulthood. Conversely, mothers in poorer body condition would be expected to invest more in female young, as their sons would have a relatively lower chance of reproductive success than their daughters. Although relatively straightforward in concept, this theory has been difficult to prove, with sex ratios sometimes deviating from the direction predicted (Clutton-Brock and Iason 1986; Kruuk *et al.* 1999). As far as the author is aware, there are no data available for horses either in the wild or under domestication.

Surprisingly, few studies aimed at examining whether nutrition of the mother can affect sex ratio have been carried out under laboratory conditions. Rodents, like most mammals, tend to produce roughly equivalent numbers of males and females, although litters with marked imbalances in sex ratio can occur spontaneously. When rodents are food-restricted, however (Wright *et al.* 1988; Meikle and Thornton 1995), or provided diets sub-optimal in essential fatty acids (Rivers and Crawford 1974), they tend to produce small, female biased litters, most probably reflecting the

TABLE 1: Relative energy content (Kcal%) of major nutrients in mouse diets

Diet	D12450B* (LF)	D12492* (VHF)	Purina 5015† (CLC)
Protein	20	20	18
Carbohydrates			
Starch	31	0	51
Maltodextrin	4	13	NS
Sucrose	35	7	1
Total carbohydrates	70	20	56
Fats			
Soybean oil	6	6	NS
Lard	4	54	NS
Total fat	10	60	26

*Defined Research Diet (Research Diets, Inc., New Jersey, USA) with equivalent amounts of casein, cellulose, minerals, vitamin mixes. D12450B diet had a caloric density of 3.8 kcal/g, D12492, 5.2 kcal/g.
†Purina Complete Life Cycle (CLC) 5015 diet, 4.4 kcal/g. (Purina Inc., Missouri, USA).
NS – Not specified.

TABLE 2: Weight at conception, litter size, gestation length, fraction male pups, and number of male-biased litters over 4 successive pregnancies in mice maintained on the LF and VHF diets

Treatment	Litter	Conception weight (g)	Litter size	Pregnancy gestation length (days)	Fraction male pups	Number of Male-biased litters
LF	1 (n=15)	20.8 (±1.4)	9.4 (±1.7)	20.0 (±1.4)	0.48	3
	2 (n=14)	26.7 (±2.2)	10.8 (±2.9)	19.8 (±1.4)	0.45*	4
	3 (n=15)	29.4 (±5.0)	9.1 (±2.3)	19.3 (±1.5)	0.35†	1
	4 (n=10)	30.8 (±2.3)	9.1 (±4.8)	20.0 (±1.4)	0.38*	0
VHF	1 (n=16)	23.1 (±2.2)	9.5 (±2.0)	19.6 (±2.1)	0.51	10
	2 (n=15)	30.6 (±4.3)	10.7 (±2.8)	18.8 (±1.9)	0.66†	12
	3 (n=14)	35.7 (±5.9)	9.9 (±2.3)	20.0 (±1.2)	0.65†	12
	4 (n=9)	38.0 (±5.8)	8.6 (±4.3)	19.9 (±1.5)	0.71†	7

Females were housed in pairs. When they were aged approximately 10, 20, 28 and 40 weeks of age, they were introduced to a stud male. After vaginal plugs were noted, the females were removed. Females were housed individually from the end of Week 2 of pregnancy until pups were weaned. Cannibalism, death of 3 females and failure of some females to conceive account for the reduced litter numbers over the course of the study.
Values for maternal weight at conception, litter size, and pregnancy length are means, with standard deviations provided in parentheses to indicate extent of variability. Weights of mothers on VHF and LF diets deviated significantly at second conception and thereafter.
,†Sex ratio deviated significantly from 0.5; $P<0.05$; $P<0.01$†.

greater vulnerability of male fetuses to stress. In the experiments that follow, the authors have chosen to examine the effects of 2 complete diets (Table 1), which differ markedly in their sources of dietary energy, on the sex of offspring born to female NIH Swiss mice. Diet 1 was low in saturated fat (LF), with the majority of calories provided as sugars and complex carbohydrate. The second was very high in saturated fat (VHF), with most energy provided as lard (Table 1). The aim was to determine whether these diets could influence the sex ratio of pups born.

Mice appeared to tolerate both diets well and showed no obvious ill effects. They gained weight and demonstrated normal fertility. Table 2 summarises the data for 108 pregnancies and 1,048 pups born over 4 parities. The weights of the mothers on the 2 diets did not differ significantly between Day 30, when the mice were first placed on the diets, and the time they were first bred ($P>0.1$), but the VHF group was significantly heavier ($P<0.05$) by the beginning of the second parity, and weights continued to deviate as the mice aged (Table 2). By Parity 4, the females on the VHF diet were about 20% heavier ($P<0.001$) than females on the LF diet (Table 2), although there was considerable variation within groups. Litter sizes and lengths of pregnancy in the 2 groups were similar and did not change with parity, indicating that the diets did not suppress fertility in one group relative to the other. Pups born to mice on the 2 diets had similar weights at Day 2 post partum (data not shown). These values were similar to those observed with NIH Swiss mice fed Purina 5015 (data not shown). Together, the data suggest that reproductive performance had not been compromised on either experimental diet, nor was there any indication that the dams on the LF and VHF diets differed in their abilities to feed and care for their pups.

In contrast to the lack of difference in litter size and gestation length, mothers on the LF diet tended to produce female-biased litters and VHF mothers male-biased litters (Table 2). This trend was noticeable at first parity but became more exaggerated at litters 2, 3 and 4. The overall sex ratio of total pups born within the 2 dietary groups over 4 parities differed markedly ($P<0.0001$), with the LF group producing a preponderance of female pups and the VHF group more males. Although the sex ratio of pups born to LF mothers was not significantly different in first litters, it became skewed towards daughters at Litters 2, 3 and 4 (Table 2). Conversely, the sex ratio of pups born after Litter 1 to mothers on the VHF diet became highly male-biased. In contrast, comparably aged mice on the Purina 5015 diet gave birth to almost equal numbers of male and female pups at first and second parity (sex ratios 0.52 and 0.48, respectively).

Several hypotheses have been proposed to explain skewing of sex ratios in mammals where the male is always the heterogametic sex (Clutton-Brock and Iason 1986). These theories fall into 2

classes, those that operate prior to conception and those that favour one sex over the other after fertilisation has occurred. It is feasible, for example, that conditions within the reproductive tract, such as vaginal pH or the viscosity of cervical mucus favour Y-sperm over X-sperm or vice versa in terms of movement to the egg or in fertilisation potential. Conversely, there could be selective loss of conceptuses either before or after implantation. These mechanisms for sex selection in mice are currently being tested in this laboratory.

Whether calorie intake and diet can be used to influence the sex of offspring born in the horse is not known. The species, like the deer, is generally recognised as polygonous so that analogous reproductive strategies might be expected. However, it should be recognised that if the process of sex selection involves post fertilisation processes, eg failure to implant the embryo or failure to maintain a pregnancy through flawed signalling to the mother, the outcome would be loss of the pregnancy and a need to re-breed the mare.

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SESSION II:

Chairman:

G. R. Foxcroft

PATTERNS OF TROPHOBLAST INVASION IN PRIMATES AND RODENTS: AN EXAMPLE OF CONVERGENT EVOLUTION

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INTRODUCTION

Although primates and rodents share a haemochorial type of placentation, there are marked differences in the positioning of the extra-embryonic membranes including the yolk sac, the histological structure of the placenta (villous and labyrinthine, respectively) and the route of entry of maternal blood into the placenta. In man and other Old World primates, maternal blood enters the placenta as fountain-like spurts from spiral artery openings in the basal plate. In rodents, maternal blood is carried through the whole thickness of the placenta via trophoblast-enclosed arterial channels, which are functional extensions of the spiral arteries of the basal (mesometrial) decidua (Wooding and Flint 1994). On the fetal side of the placenta, the maternal arterial channels divide into several superficial branches which lead into the small sinusoids of the labyrinth (Adamson *et al.* 2002).

Haemochorial placentation is always associated with decidualisation of the endometrium, characterised not only by decidual swelling of endometrial stromal cells but also by the appearance of numerous uterine natural killer (uNK) cells. A peculiar feature of several rodent species (including rats and mice) is a marked development of the mesometrial triangle area between circular and longitudinal myometrium overlying the mesometrial decidua. Development of this region leads to a progressive dispersion of the circular myometrial layer at the placental insertion site. The mesometrial triangle area, formerly called 'metrial gland', can be considered as an extra decidualised compartment showing extensive coiling of spiral arteries and containing numerous uNK cells. In primates there is no development of an equivalent extra-decidual structure within or beyond the myometrium. Here the uNK cells are abundantly present in the decidua basalis only, and are absent from the myometrium which is exceptionally thick compared with all the other mammalian groups.

In spite of these differences, events concerning trophoblastic invasion show remarkable similarities in some rodent and primate species.

TROPHOBLAST INVASION PATTERNS

In man and other primates, placental 'villous' trophoblast has to be distinguished from 'extra-villous' trophoblast populations, which arise as cell columns at the tips of anchoring villi and invade the maternal placental bed. In rodents, a similar distinction can be made between the placental labyrinth and 'extra-labyrinthine' trophospongium from which subpopulations of trophoblast invade the mesometrial decidua. There are 2 possible pathways of invasion: an interstitial pathway comprising the decidual stroma, which may be extended into the myometrium (primates) or mesometrial triangle (rodents); and a vascular (arterial) pathway, which may involve decidual and myometrial (primates) or mesometrial triangle (rodents) segments of spiral arteries. Interstitial invasion occurs abundantly in the decidua in man, rat and mouse, but is restricted to superficial decidual layers near the basal plate in baboons (Table 1). Deeper interstitial invasion in myometrium or mesometrial triangle occurs in man and the rat. In all species mentioned on Table 1, decidual spiral arteries undergo invasion, but the mouse is exceptional because the invasion follows a perivascular (outside the endothelial lining) rather than an endovascular (intraluminal) route (Fig 1). Endovascular invasion is extended into the myometrium or the mesometrial triangle in man

TABLE I: Occurrence and succession of interstitial and arterial invasion pathways of trophoblast in primate and rodent species

Species	Interstitial invasion		Arterial invasion	
	Decidua*	Myometrium (Myo)* Mesometrial triangle (MT)*	Decidua*	Myometrium (Myo)* Mesometrial triangle (MT)*
Primates				
Human	+ [1]	+ (Myo) [1]	+ [2]	+ (Myo) [2]
Baboon	(+)†	- (Myo)	+	- (Myo)
Rodents				
Rat	+ [2]	+ (MT) [2]	+ [1]	+ (MT) [1]
Mouse	+ [2]	- (MT)	+ ‡[1]	- (MT)

*Numbers 1 and 2 indicate whether this invasive pathway is first or second; †Only very few interstitial trophoblastic cells near basal plate; ‡Arterial invasion follows a perivascular pathway in the mouse, in contrast to the endovascular pathway in the other species on this table.

and the rat, respectively (Legrand 1974; Pijnenborg *et al.* 1983), but not in the baboon and the mouse (Pijnenborg *et al.* 1996; Adamson *et al.* 2002).

TROPHOBLAST INVASION AND ARTERIAL CHANGES

The typical changes associated with spiral artery invasion in man have been described amply. Endovascular trophoblast penetrates the endothelium and is embedded intramurally within an amorphous fibrinoid matrix replacing the vascular smooth muscle. Following this invasion phase the maternal endothelium is restored. As a result these vessels have lost their muscular coat, implying that they can no longer respond to vasoactive influences. This 'physiological change' is therefore thought to adapt the uterine vasculature to an increasing maternal blood supply to the placenta. In man, interstitial invasion precedes the endovascular, and there is evidence that the interstitial trophoblast induces early disorganisation of the vascular smooth muscle, thus preparing myometrial spiral arteries for subsequent endovascular invasion (Pijnenborg *et al.* 1983). Similar physiological changes have been described in decidual spiral arteries of the pregnant baboon, but in this case interstitial trophoblast is virtually absent and can therefore play no role in early arterial remodelling. At the time of trophoblast invasion, numerous uNK cells are present in the decidua, and notably in the baboon the spiral arteries are cuffed by these cells. The uNK cells are increasingly considered as important regulators for trophoblast invasion, but the exact mechanism is not yet clear (Moffett-King 2002).

In the rat the histological changes of the spiral arteries show remarkable similarities to those in man. The endothelium is replaced temporarily by endovascular trophoblast, which secretes a fibrinoid layer, and the underlying muscle layer undergoes substantial fragmentation. Near the end of pregnancy the endothelium is restored while the trophoblast has acquired an intramural position (unpublished results). In contrast to the situation in man, endovascular invasion in the rat precedes interstitial invasion. In both compartments numerous uNK cells are clustered around the spiral arteries, but their possible interaction with invading trophoblast has not yet been studied closely in this species. In the mouse the interaction of the perivascular trophoblast invasion with vessel wall components is not yet completely clear; in particular the disappearance of the muscle layer does not seem to be related to trophoblast (Adamson *et al.* 2002). As in the rat, this vascular invasion precedes interstitial invasion but, in the mouse, neither invasion extends beyond the circular myometrial layer. Also, in the latter species uNK cells are numerous, especially in the mesometrial triangle where they take up a more interstitial position than in the rat. A reciprocal relationship between distribution of uNK cells and interstitial trophoblast invasion in both rat and mouse is suggestive for a controlling function of invasion, but this needs further clarification.

ANIMAL MODELS FOR DEFECTIVE TROPHOBLAST INVASION

Trophoblast invasion patterns and interaction with maternal vessels show remarkable analogies

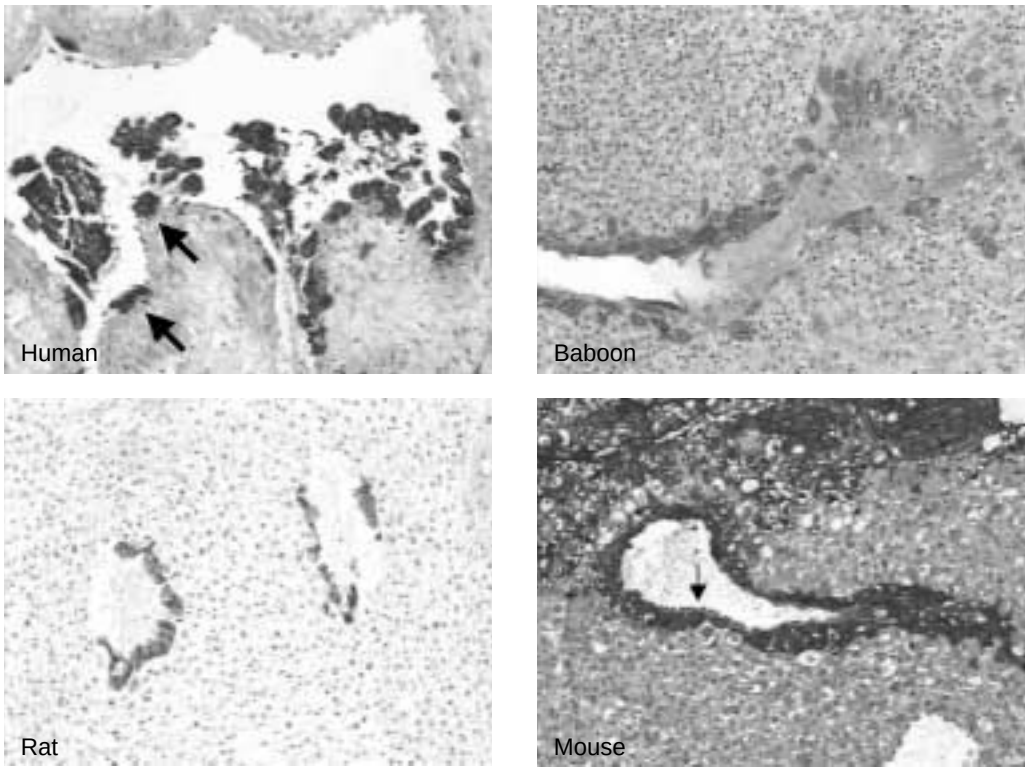

Fig 1: Trophoblast invasion of spiral arteries in the decidua (human, baboon, mouse) and mesometrial triangle (rat). Trophoblast penetration of the endothelial layer is shown for man (arrows). In the mouse, vascular invasion follows a perivascular pathway, and the endothelial layer remains intact (arrow).

between different species of the 2 diverse mammalian groups. Therefore this is an intriguing example of convergent evolution, implying that the similar trophoblast-related vascular changes have the same essential function in the 2 disparate groups. Carter (2001) pointed out the convergence in evolution of trophoblast invasion in different mammalian groups with haemochorial placentation, and the present work shows that this convergence also applies to some of the histological details of trophoblast-maternal cell interaction.

Human pregnancy can be complicated by gestational hypertension or pre-eclampsia. Although the real causes of this disease are still unclear, there is agreement that abnormal placentation plays a pivotal role. Notably trophoblast invasion and associated vascular changes in spiral arteries are deficient in such women, as the normal vascular adaptation is restricted to the decidua and does not extend into the myometrium (Brosens *et al.* 1972). Such vascular defects are associated with reduced placental perfusion, which may lead to further physiological disburbances (Roberts and Lain 2002). Further research in this field may depend on experimental work on well-chosen animal models, and therefore selected species should undergo deep invasion, comparable to that in women. For such studies, the rat is therefore preferable to the mouse. Models of pre-eclamptic rats are being developed (eg Faas *et al.* 1994) but trophoblast invasion patterns in such animals have not yet been examined. While deep invasion seems to be necessary for normal placentation and fetal development in women, it is not clear how other species such as the baboon can cope with a more superficial vascular adaptation. There must be subtle physiological differences in the regulation of maternal blood supply to the placenta in the different species, but these have not yet been clarified. Comparative studies remain essential for better insights into the adaptive value of various physiological processes, including placental function.

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DEVELOPMENTAL BIOLOGY AND ROLE OF ENDOMETRIAL GLANDS IN UTERINE FUNCTION

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Conceptus (embryo/fetus and associated extra-embryonic membranes) growth and development is dependent initially on the uterine endometrium, then on both endometrium and placenta once implantation and placentation are completed. All uteri contain endometrial glands that transport or synthesise and secrete a complex array of proteins and related substances termed histotroph, consisting of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins and other substances. Histotrophic nutrition is primarily derived from the secretions of endometrial glands that bathe the conceptus and are absorbed by placental areolae. Areolae are unique placental structures in ruminants and pigs that develop over the mouth of each endometrial gland opening as specialised areas for absorption and transport of histotroph. Evidence from primate and subprimate species during the last century supports an unequivocal role for secretions of endometrial glands as primary regulators of conceptus survival, development, production of pregnancy recognition signals, implantation and placentation (reviewed in Gray *et al*. 2001a). Following placentation, most micronutrients are derived from the maternal uterine blood supply, but the requirement for uterine histotroph remains particularly critical in domestic animals with an epitheliochorial (pig) or synepitheliochorial (sheep, cattle, goat) placenta.

Recent studies of the uterine gland knockout (UGKO) ewe model revealed an essential role for endometrial glands and their secretions in normal oestrous cycles and in peri-implantation conceptus survival and growth (Gray *et al*. 2001b, 2002). Continuous administration of a synthetic, non-metabolisable progestin to neonatal ewes from birth to post natal day (PND) 56 permanently ablated differentiation of the glandular epithelium (GE) from luminal epithelium (LE) in the endometrium and produced an UGKO phenotype without altering development of other Müllerian duct-derived female reproductive tract structures or the hypothalamic-pituitary-ovarian axis. Adult UGKO ewes are unable to establish pregnancy, and transfer of normal hatched blastocysts into the uteri of timed recipient UGKO ewes failed to ameliorate this defect (Gray *et al*. 2001b, 2002). Morphologically normal blastocysts are present in uterine flushes of bred UGKO ewes on Days 6 or 9 post mating. In contrast, Day 14 uterine flushes of bred UGKO ewes contained either no conceptus or a severely growth-retarded tubular conceptus. Integrin expression on endometrial LE of UGKO ewes was not different from normal ewes (Gray *et al.* 2002). Furthermore, expression of receptors for oestrogen (ERα), progesterone (PR) and oxytocin (OTR), as well as several LE-specific genes were not different between endometrium of UGKO and normal ewes (Gray *et al.* 2000a, 2002). Uterine flushes of UGKO ewes were analysed for the presence of osteopontin (OPN) and glycosylated cell adhesion molecule one (GlyCAM-1) proteins, which are expressed by GE of the ovine uterus and implicated in regulation of conceptus implantation (Gray *et al.* 2002). Uterine flushes of Day 14 bred UGKO ewes contained lower amounts of OPN and GlyCAM-1 compared to Day 14 pregnant ewes. Genomics and proteomics are being used to identify specific components of histotroph that are absent or diminished in the UGKO ewe (Spencer *et al.* 1999a). Collectively, studies of the UGKO ewe model unequivocally support a role for uterine glands and, by default, their secretions in peri-implantation conceptus growth and survival in ruminants.

Given that the success of endometrial gland development determines, in part, the embryo-

trophic and functional capacity of the adult uterus, it is fundamentally important to determine the hormonal, cellular and molecular mechanisms regulating endometrial gland morphogenesis. Post natal uterine development in sheep involves differentiation and development of endometrial glands or adenogenesis, development of endometrial folds, organisation of intercaruncular endometrial stroma, and, to a lesser extent, growth of endometrial caruncular areas and myometrium (Taylor *et al.* 2000). Endometrial adenogenesis in the neonatal ewe involves budding and tubulogenesis of the GE from the LE, followed by coiling and branching morphogenesis of the glands as they develop from the LE to the myometrium between birth and post natal day (PND) 56. Although the ovine uterine wall is histoarchitecturally mature by PND 56, final maturation and growth may not occur until after puberty and, perhaps, even after the first or second pregnancy (Stewart *et al.* 2000). In both sheep and pigs, endometrial glands undergo extensive hyperplasia and hypertrophy during pregnancy, presumably in response to placental hormones to meet increasing demands of the growing fetus for uterine histotroph.

Prolactin (PRL), a member of the helix bundle peptide hormone/cytokine superfamily, regulates growth and differentiation of a number of epitheliomesenchymal organs. In the endometrium of adult sheep, humans and primates, the PRLR gene is expressed exclusively by GE and, in ewes, PRLR expression during pregnancy correlates with hyperplasia and hypertrophy of endometrial glands (Stewart *et al.* 2000). There is a primary role for pituitary PRL acting on PRLR in GE in the regulatory system controlling endometrial gland branching morphogenesis in the neonatal ovine uterus. In neonatal ewes, circulating levels of PRL are relatively high on PND 1, reach a maximum on PND 14, and then decline slightly to PND 56 (Taylor *et al.* 2000; Carpenter *et al.* 2003a). Expression of mRNAs for both short and long PRLR is restricted to nascent GE buds on PND 7 and proliferating and differentiating GE from PNDs 14 to 56 (Taylor *et al.* 2000). Hyperprolactinemia, induced in neonatal ewes by treatment with recombinant ovine PRL from birth to PND 56, resulted *in uteri* with over 60% more endometrial glands (Carpenter *et al.* 2003a). On the other hand, hypoprolactinemia, induced in neonatal ewes by treatment with bromocryptine, a PRL secretion inhibitor, from birth to PND 56, reduced endometrial glands by 35% (Carpenter *et al.* 2003a). Thus, PRL acting via PRLR in GE is a pivotal factor regulating endometrial gland development in neonatal ewes. Similarly in adult ewes, intra-uterine administration of placental lactogen (PL), a member of the PRL/growth hormone (GH) family that binds and activates the PRLR, stimulated proliferation of endometrial glands, particularly the coiled and branched glands found in the stratum spongiosum, and increased production of OPN and uterine milk proteins (UTMP) (Spencer *et al.* 1999b). Sequential production of hormones from the placenta, including interferon tau (IFNτ), PL and placental GH, form a servomechanism regulating endometrial gland morphogenesis and differentiated function during pregnancy in the ewe.

In rodents, pigs and sheep, expression of ERα is abundant in the endometrial GE and stroma of the neonatal uterus. In fact, endometrial adenogenesis in the pig is ovary-independent and requires activation of ERα in a ligand-independent manner (Tarleton *et al.* 1999). Neonatal ewes treated with EM-800, a pure and potent ERα antagonist and anti-oestrogen, from birth to PND 56 did not affect uterine weight and horn length (Carpenter *et al.* 2003b). On PND 14, uteri from EM-800 ewes appeared histologically similar to control ewes, except for a slight reduction in coiled endometrial glands. However, on PND 56, the intercaruncular endometrium of EM-800 ewes had 44% fewer ductal gland invaginations and 22% fewer endometrial glands that were less coiled and branched. Treatment of ewes from birth to PND 56 with CGS 20267, an aromatase inhibitor, did not affect uterine development or endometrial adenogenesis on either PND 14 or PND 56. Thus, post natal ovine uterine development is oestrogen-independent and partially ERα-dependent. Although ERα does not regulate the initial stages of endometrial adenogenesis, it does influence coiling and branching morphogenetic differentiation of endometrial glands in the neonatal ewe.

Previous studies indicated that endometrial adenogenesis was ovary-independent between birth and PND 14, but the ovary did regulate uterine growth after PND 14 in ewes. Ewes were ovariectomised on PND 7 and uterine wet weight was reduced by almost 50% on PND 56 with no effects on circulating levels of oestradiol-17ß (Carpenter *et al.* 2003c). Surprisingly, the uterus

of ovariectomised ewes had fewer coiled and branched endometrial glands on PND 56 as compared to sham control ewes. Thus, the ovary and an ovarian-derived factor(s) influences the coiling and branching morphogenesis of endometrial glands in the neonatal ewe. The ovarian factor(s) would presumably be secreted from the abundant growing and antral follicles in the ovary, but is not oestrogen. Candidate ovarian factors include follistatin, activins or inhibin (Carpenter *et al.* 2003c) as well as insulin-like growth factor two (IGF-II). Additionally, expression of growth factors in the endometrial stroma, including IGF-I and IGF-II, hepatocyte growth factor, and fibroblast growth factors-7 (FGF-7) and FGF-10 that have receptors specifically expressed in the endometrial epithelium are also implicated as regulators of endometrial adenogenesis in the neonatal ewe (Taylor *et al.* 2001).

Collectively, available evidence indicates that unexplained high rates of peri-implantation embryonic loss in domestic animals and humans may reflect defects in endometrial gland morphogenesis or differentiated function due to genetic errors, epigenetic influences of endocrine disruptors, and pathological lesions. Knowledge of the developmental biology and functional roles of endometrial glands is necessary for developing therapeutic strategies to reduce pregnancy losses in domestic animals as well as humans.

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ENDOMETRIAL SECRETIONS AND DEVELOPMENT OF THE HUMAN FETOPLACENTAL UNIT DURING EARLY PREGNANCY

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It has long been recognised that endometrial secretions play an important role in supporting mammalian fetal development. According to Amoroso (1952) the presence of nutritive secretions within the uterus of ruminants during pregnancy was described by William Harvey in a letter in 1655. Because of their fatty content these secretions often have a milky appearance, and the term 'uterine milk' was first applied to them by W. Needham in 1667. These secretions are often voluminous in early pregnancy, and in all species sustain the conceptus until implantation occurs. Their role subsequent to establishment of the placenta has classically been considered to be more variable, however, and has been related to the form of placentation observed (Amoroso 1952). Thus, in those species with an epitheliochorial placenta, the secretions may continue to provide an important pathway for nutrients throughout gestation. Specialised areas of the chorion, the areolae, form opposite the mouths of the uterine glands, and the columnar trophoblast cells actively phagocytose the secretions. In this way histiotrophic and haemotrophic pathways for exchange operate side by side. Uterine secretions and histiotrophe still play an important role in mammals with endotheliochorial placentae, such as the carnivores, but are thought to be of little importance beyond the earliest stages of development in species with haemochorial placentae, including man.

In man, the uterine secretions have been considered particularly insignificant post implantation for 2 main reasons. Firstly, the interstitial form of implantation displayed by the human blastocyst removes it from the uterine lumen, and hence access to the secretions, by Day 9–10 post fertilisation. Secondly, it has generally been assumed that the maternal intra-placental circulation is established shortly after implantation (Moore and Persaud 1993), leading to the precocious onset of haemotrophic exchange. Recent data indicate that this is not the case, however, and although maternal erythrocytes may be observed within the lacunar precursors of the intervillous space, their presence does not equate with an effective circulation. There is now considerable evidence from a variety of techniques supporting the concept that the maternal intra-placental arterial circulation is not fully established until 10–12 weeks of pregnancy (Hustin *et al.* 1988; Jauniaux *et al.* 2000). Prior to this time the intervillous space is filled with a clear fluid that may represent a plasma transudate originating in the superficial endometrium, or a plasma filtrate percolating between the invading endovascular trophoblast cells that plug the tips of the spiral arteries (Burton *et al.* 1999).

More recently it has been observed that the uterine glands also contribute to this fluid during the first trimester (Burton *et al.* 2002). Secretions from glands in the decidua basalis are delivered directly into the intervillous space through openings in the basal plate of the developing placenta. These secretions disperse between the villi, and it has been shown immunocytochemically that two proteins synthesised in the glands but not in the placenta, MUC-1 and glycodelin, are phagocytosed by the syncytiotrophoblast. In the earliest placenta-*in situ* specimen available to the authors, estimated to be of 43 days gestational age, the endometrium beneath the implantation site is 5.3 mm thick. The glands are highly active and their lumens represent nearly 50% of the tissue volume. Their epithelium is columnar, but very irregular in form with some cells possessing long apical protrusions. Large

accumulations of glycogen are present within the apical portions of the cells, and secretory vesicles are conspicuous. The secretions within the lumens are carbohydrate rich, contain free glycogen rosettes and numerous lipid droplets. By 8 weeks the thickness of the decidua basalis is reduced to 2.3 mm, but the glands remain active and still open into the intervillous space. The glandular epithelial cells are more cuboidal in shape but still contain secretory vesicles. Although the authors have no direct proof at present, it seems extremely likely that uptake and subsequent breakdown of the maternal secretion may serve as an important source of energy and substrates for the early fetus. In addition, the glandular epithelial cells are heavily immunoreactive for alpha tocopherol transport protein and for lactoferrin during the first trimester, suggesting that they may be involved in the transport of vitamin E and other specific substances.

The endometrial secretions may play other roles in fetoplacental development besides nutrition, however, as the glands are known to be an important source of growth factors. The epithelial cells are immunoreactive for vascular endothelial growth factor (VEGF), transforming growth factor ($TGF\beta_3$), and leukaemia inhibitory factor (LIF). These factors are known to modulate trophoblast proliferation, differentiation and migration *in vitro* (Lala *et al.* 1998). The glandular secretions may be pivotal in regulating placental development, both in terms of elaboration of the villous tree and the extravillous invasion of the endometrium. For example, failure of extravillous trophoblast to fully invade during the first few weeks after implantation is associated with incomplete plugging of the spiral arteries, and early and disorganised onset of the maternal intra-placental circulation (Jauniaux *et al.* 2003). This results in excessive oxidative stress within the placental tissues that undoubtedly contributes to the loss of these pregnancies.

The picture that emerges for man, therefore, is that during the first trimester the intervillous space is perfused by a clear fluid derived from uterine secretions, possibly supplemented by a transudate percolating through the superficial layers of the decidua. Towards the end of the first trimester the maternal arterial intra-placental circulation is established, initially in the periphery of the placenta and subsequently extending towards the centre. During the first trimester the fetus thus develops in a low oxygen environment (Jauniaux *et al.* 2000). This corresponds to the period of organogenesis when the body plan is being established and the main organ systems are differentiating. This is a time of rapid cell division, and there is increasing evidence that many of the events taking place may be perturbed by excessive concentrations of oxygen (Nicol *et al.* 2000; Burton *et al.* 2003). Free radicals, generated through aerobic metabolism, can cause oxidative damage to DNA, or disrupt signalling pathways. Although antioxidant defences have evolved to scavenge these radicals, they cannot provide complete protection as most radical reactions are diffusion limited (Halliwell and Gutteridge 1999). Limiting the production of radicals by maintaining a low oxygen environment during this period may therefore represent a protective adaptation.

Comparisons across species support this view. Morphologically, attachment of the equine conceptus does not occur until 6–7 weeks, by which time organogenesis is complete. Similarly, in the ruminant attachment does not begin until the second month and may not be complete until the end of the third month. In the mouse and rat the chorioallantoic labyrinth does not develop until halfway though gestation, by which time most of the main systems have differentiated. Measurements of the oxygen tension surrounding these embryos are not available, but metabolism in many mammalian species is largely anaerobic during the period of organogenesis (New 1978). The few measurements that have been performed in the chick yielded values of approximately 10 mmHg in the tissues at Day 4 of incubation (Meuer and Baumann 1987). Our measurements indicate an oxygen tension of <20 mmHg in the human fetoplacental unit during the first trimester, and confirm that metabolism is largely anaerobic (Jauniaux *et al.* 2000, 2001).

In conclusion, it would appear that in those mammals studied to date organogenesis takes place in a low oxygen environment, possibly as an adaptation to limit the risk of free radical induced teratogenesis. When viewed in this light the interstitial form of implantation displayed by the human as classically described appears to represent a paradox. Whilst the extensive and intimate apposition of the 2 circulations that it provides for undoubtedly facilitates the exchange of oxygen and nutrients necessary to support rapid fetal growth during the second and third trimesters, it may place the developing embryo at risk of radical mediated damage during early

pregnancy. Plugging the spiral arteries by extravillous trophoblast and supplying nutrients via the uterine glands during the first trimester may resolve this conflict. Adopting such an approach brings the human situation into line with that in many domestic species. Although the uterine secretions are not necessarily visible externally, there is now strong reason to believe that they may a significant role in fetoplacental development post implantation.

Acknowledgements

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EQUINE BLASTOCYST CAPSULE FORMATION *IN VIVO* AND *IN VITRO*

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INTRODUCTION

A characteristic feature of horse embryo development is the formation of an acellular glycoprotein 'capsule' between the trophectoderm and zona pellucida (ZP) on Days 6–7 after ovulation, soon after the embryo has entered the uterus and coincident with blastulation. Shortly after the capsule has formed, the ZP is shed leaving the rapidly expanding blastocyst surrounded by a tight fitting capsule that increases equally rapidly in dry weight until approximately Day 18 of gestation; thereafter, the capsule is attenuated steadily until it disappears altogether at around Day 23 (Oriol *et al.* 1993). Although the exact functions of the capsule are unknown, it is essential to conceptus survival *in vivo* (Stout *et al.* 1997) probably because it provides vital physical protection during the maternal recognition of pregnancy period when the delicate conceptus is propelled throughout the uterine lumen by powerful myometrial contractions. In addition, the capsule has been proposed to play roles in maternal-conceptus communication (Herrler *et al.* 2000), nutrient uptake (Crossett *et al.* 1998), ZP loss (Stout *et al.* 1997) and in the protection of the conceptus against micoorganisms and maternal immunological recognition (see Betteridge 1989 for review). Growth of the capsule appears to be primarily a function of trophoblast-secreted glycoproteins, at least after Day 11 of gestation (Oriol *et al.* 1993). However, a uterine contribution has been advanced to explain why embryos that blastulate *in vitro* fail to produce a visible capsule (see Betteridge 1989), and it is possible that the uterine component is represented by the endometrial lipocalin, P19, which associates with the capsule in great quantities (Stewart *et al.* 1995). The aims of the current study were to determine whether initial capsule formation is from the same trophectodermal glycoproteins that predominate during later growth, and to investigate why the capsule fails to form *in vitro.*

MATERIALS AND METHODS

Horse embryos were produced *in vitro* (IVP) by injecting frozen-thawed spermatozoa into the cytoplasm of oocytes matured *in vitro* for 24 h. The injected oocytes were cultured for 2 days in a modified synthetic oviductal fluid (SOF) medium, at the end of which zygotes that had cleaved were selected for further culture. The selected 2–4 cell embryos were cultured for a further 5 days in one of 2 systems; 1) 20 µl droplets of modified SOF medium, under oil at 38.5°C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2; or 2) the ligated oviduct of progesterone-supplemented ewes. At the end of the 5 day culture, the embryos were harvested and either fixed (Day 7 IVP embryos) or cultured for a further 3 days in a 1:1 mixture of DMEM and M199 supplemented with 5% FCS and 5% serum replacement (Day 10 IVP embryos). Control *in vivo* produced embryos were flushed from the uterus of inseminated mares 6–9 days after ovulation.

All embryos were fixed for 24 h in 4% paraformaldehyde and then stored in PBS at 4°C. Before staining, the embryos were permeabilised with 0.1% Triton X-100. Capsular glycoproteins were then labelled using a monoclonal antibody raised in mice against Day 13.5–15.5 equine capsule (OC-1: Oriol *et al.* 1993) and a goat anti-mouse second antibody coupled to the fluorochrome Alexa Fluor 488 (Molecular Probes; A-11029). The embryos were concurrently stained

with AlexaFluor 568 Phalloidin (Molecular Probes; A-12380) and 4,6-diamino-2-phenyl-indole (DAPI) to enable visualisation of the microfilaments and cell nuclei, respectively. A small number of *in vitro* and *in vivo* matured oocytes were stained similarly to check for cross-reactivity of OC-1 with ZP, and a few embryos were incubated with labelled second antibody without prior exposure to OC-1, to rule out the possibility of non-specific staining. Finally, the pattern of OC-1 expression was examined using a multiphoton laser-scanning microscope.

Results

There were no significant differences in embryo development or capsular glycoprotein expression between IVP embryos cultured in SOF medium or in the oviduct of a sheep. In general, Day 7 IVP embryos were smaller, had fewer cells and were more compact than equivalently aged *in vivo* produced embryos (Tremoleda 2003). Day 6 *in vivo* embryos had a confluent capsule sandwiched between the ZP and trophectoderm, with no infiltration of capsular glycoproteins into the substance of the ZP. Older *in vivo* embryos had hatched completely from their ZP and were surrounded by a capsule with the classical bilaminar appearance described by Oriol *et al.* (1993). By contrast, Day 7 IVP embryos showed only scattered patches of OC-1 staining over the apical surface of trophectoderm cells, together with accumulations of stained material within the perivitteline space. After 10 days of culture, IVP embryos had partially hatched through the hole created during ICSI. Where the trophectoderm had herniated from the ZP, capsular glycoproteins were present in abundant patches scattered over the surface of the trophectoderm cells, but had not coalesced into a confluent layer. The ZP of these Day 10 IVP embryos was lined by a relatively thick layer of capsular material with extensive infiltration of OC-1 labelled glycoproteins into the substance of the ZP, via the transzonal channels. Removal of the ZP by micromanipulation demonstrated convincingly that this 'pseudo' capsule was adhered to the ZP.

Conclusions

In this study, it was demonstrated that the initial layer of capsule formed between the trophectoderm and ZP of a Day 6 horse embryo contains abundant OC-1 reactive glycoproteins; previously, OC-1 expression had not been examined for conceptuses recovered before Day 11 after ovulation (Oriol *et al.* 1993). As the trophectoderm cells of IVP embryos also expressed OC-1, it appears that the initial layer of capsule is formed from OC-1 reactive glycoproteins secreted by trophectoderm cells, independently of a maternal (endometrial) input. However, *in vitro*, the secreted glycoproteins failed to coalesce into a confluent layer, presumably either because they failed to reach a critical concentration or because of the absence of some vital aspect of the uterine environment. In the former respect, the low cell numbers in Day 7 IVP embryos and the presence of the ICSI-derived hole in the ZP may have hindered the accumulation of glycoproteins. However, this is unlikely to have been significant given that an intact ZP is not an absolute requirement for capsule formation; zona-free demi-blastocysts develop a capsule after transfer to the uterus of recipient mares (McKinnon *et al.* 1989). More probably, the critical role of the uterus in capsule formation is to provide an appropriate microenvironment for crosslinking and hydration of the mucin-like glycoproteins. In the absence of this microenvironment, capsular glycoproteins adhered to, and infiltrated into, the ZP; a feature not seen in *in vivo* embryos. This infiltration of the ZP with capsular glycoproteins may help to explain the aberrant mechanism of ZP loss seen *in vitro*, ie herniation through a small hole in the ZP rather than rapid, complete ZP loss as occurs *in vivo*. It is probable that infiltration with capsular glycoproteins alters the response of the ZP to both stretching and enzymatic digestion.

As a result of the current findings, it is suggested that IVP horse embryos should be transferred into the uterus of recipient mares by no later than the late morula to early blastocyst stage to ensure that survival is not compromised by aberrant capsule formation. Because Day 7 IVP embryos can develop into normal pregnancies after transfer (Li *et al.* 2001), it must be assumed that subsequent capsule coalesence can and does occur.

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SESSION III:

Chairman:

R. M. Roberts

NUTRITION AND UTERINE CROWDING AS REGULATORS OF CONCEPTUS SURVIVAL AND PRE-NATAL DEVELOPMENT IN THE PIG

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GILT AND SOW NUTRITION

Indirect and latent effects of maternal nutrition and metabolic state on embryonic survival have been reported in both cyclic gilts and lactating and weaned first parity sows. In gilts, feed restriction during the late (Days 8–16) compared to the early (Days 1–7) luteal phase of the cycle has deleterious effects on embryonic survival, without affecting ovulation rate (Almeida *et al.* 2000; Table 1).

An extension of this study included the HR and RH groups shown in Table 1 and an HR + I group that received daily injections of long-acting insulin during the later period of feed restriction, together with a supplement of energy in the form of corn oil to prevent acute hypoglycaemia. A sub-population of these gilts (n=9 per treatment) were cannulated and used to study endocrine responses on Days 15 and 16 of the cycle and through the peri-oestrous period. Time of ovulation was determined by transcutaneous ultrasonography and gilts were subjected to surgery between 12 and 20 h after ovulation for the recovery of fertilised embryos, oviductal flushings, oviductal tissue and luteal tissue. Culture of 1–2 cell embryos *in vitro* resulted in approximately 85% development to the morula stage and 40% to the blastocyst stage by 144 h of culture, with no treatment effects on early development (Novak *et al.* 2002), suggesting that in contrast to nutritional effects reported in sheep, either previous treatment does not affect the developmental competence of the embryo, or that inherent differences in developmental potential were not evident using the *in vitro* methods used in these experiments. No endocrine differences were observed on Days 15 and 16 of the cycle. However, the magnitude of the pro-oestrus rise in plasma oestradiol and the pre-ovulatory LH surge, and the rate of increase in plasma progesterone were all lower in HR than in RH gilts. Treatment with insulin during feed restriction on Days 8 to 16 reversed the negative effect of restriction (Almeida *et al.* 2001). The *in vitro* culture of luteal tissue indicated that previous treatment affects luteal function, with luteal cells from HR gilts being less responsive to LH stimulation than cells from RH gilts. Additionally, insulin enhanced luteal function, as measured by progesterone

TABLE 1: Reproductive characteristics at Day 28 of gestation and plasma progesterone concentrations at 48 and 72 h after onset of oestrus in gilts fed at 2.8 x Maintenance (approximately 85% of to-appetite intake) throughout the oestrous cycle (HH), or restricted to 2.5 x M from Day 1 to 7 (RH) or Day 8 to 16 (HR)

Treatment	N	Ovulation rate	Embryo survival (%)	Plasma progesterone (ng/ml)	
				48 h after ovulation	72 h after ovulation
HH	22	17.1 ± 0.6	83.6 ± 4.3^{a}	1.44 ± 0.16^{x}	4.92 ± 0.44^{x}
HR	19	18.5 ± 0.6	68.3 ± 4.8^{b}	0.82 ± 0.18^{y}	3.64 ± 0.49^{y}
RH	21	17.7 ± 0.6	81.7 ± 4.5^{a}	1.24 ± 0.18^{x}	4.98 ± 0.47^{x}

^{a,b} LS means within a column with different superscripts differ P<0.05 (arcsin transformed data)
^{x,y} LS means within a column with different superscripts differ P<0.05 (log transformed data)

TABLE 2: Data from a population of commercial, dam-line, sows of different parities in which reproductive characteristics were measured at 3 stages of gestation (S. Town, unpublished data, 2003)

Author: Please cite in text

Parity	N	Mean ovulation rate	Percentage survival rates at different gestation days (no. of surviving conceptuses)		
			Day 30	Day 55	Day 90
1 and 2	146	20.1	N/A	61 (12.1)	60 (12.1)
3 and 4	124	23.5	65 (15.3)	57 (13.4)	55 (12.9)
5 and 5+	157	24.6	62 (15.3)	43 (10.6)	42 (10.3)

release *in vitro* and the increased mRNA expression of key regulators of luteal steroidogenesis (Mao *et al.* 2001). As *in vitro* production of progesterone was strongly correlated to plasma progesterone concentrations at the time that luteal tissue was recovered, latent effects on luteal function may be one of the mechanisms by which previous nutrition affects progesterone status in early pregnancy. Finally, evidence was obtained for treatment effects on the secretion of oviductal proteins, and specifically POSP 1–3 (Novak *et al.* 2003). As local countercurrent transfer of ovarian steroids to the oviductal vasculature regulates protein secretion into oviduct fluid (Novak *et al.* 2002), the more rapid switch from oestrogen to progesterone dominance in RH and HR + I gilts may create an oviductal environment that is advantageous to the developing embryo. These changes may also act to alter the priming of uterine secretory function and the essential uterotrophic nutrition of the conceptus in the pig. Direct evidence for such effects awaits comprehensive proteomic analysis of uterine secretions in early gestation.

Nutritional studies in lactating and weaned first parity sows indicate a number of similar effects on embryonic survival, independent of effects on ovulation rate (Zak *et al.* 1997a). Futhermore, increased catabolism in late lactation results in decreased follicular maturity at weaning (Clowes *et al.* 2003) and limits follicular development and oocyte maturation in the first wave of pre-ovulatory follicles developing after weaning (Zak *et al.* 1997b; Yang *et al.* 2002). Based on studies in the cyclic gilt, it assumed that the relative immaturity of pre-ovulatory follicles after weaning is linked to lower plasma progesterone concentrations reported in early pregnancy in catabolic sows.

Author 2000 in ref list

Results of these experiments provide support for the concept of 'nutritional' and 'metabolic' imprinting of embryonic development. Additionally, high feed intake in the immediate post ovulatory period is reported to decrease circulating steroid concentrations through increased metabolic clearance rates. The authors of this abstract are currently exploring the possibility that epigenetic effects due to genomic imprinting may also mediate effects of previous nutrition on oocyte maturation and early embryonic and placental development.

Effects of uterine crowding

Direct selection for litter size in some prolific dam-lines appears to have led to disproportional selection for ovulation rate compared to uterine capacity. Although embryonic survival to Day 30 of gestation is considered generally to be the major component of pre-natal loss in swine, data collected recently from a number of contemporary commercial genotypes suggest that the distribution of pre-natal loss may vary widely among genotypes. Even within genotypes, parity, health status and litter of origin may create major differences in the extent and timing of pre-natal loss. Generally, embryonic survival is the major determinant of litter size in gilts and in first parity sows. In healthy multiparous sows, high ovulation rates are often associated with a number of conceptuses at Day 30 that exceeds uterine capacity. In this situation, post implantation loss of conceptuses becomes a major determinant of litter size and in individual sows, more than 50% of the conceptuses may be lost in the post implantation period.

In gilts with high ovulation rates and low pre-implantation embryo loss, uterine crowding around Day 25 to 35 of gestation has been associated with a negative impact on average placental size (Almeida *et al.* 2000). Even in parous sows with only 60% embryonic survival to

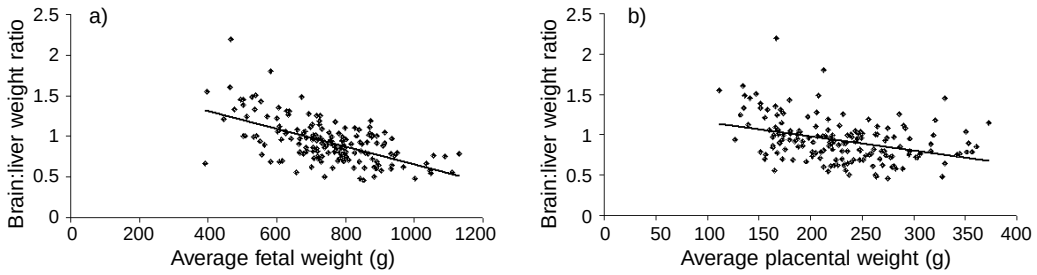

Fig 1: Correlations between mean brain:liver weight ratio and: a) average fetal weight at Days 85–90 of gestation (r^2=-0.35, P<0.0001); and b) average placental weight at Days 85–90 of gestation (r^2=-0.14, P<0.0001).

Day 25 of gestation, a highly significant positive correlation (r = +0.50; P<0.001) between numbers of viable conceptuses on Day 25 and ovulation rate, was associated with negative correlations between placental weight and the numbers of viable conceptuses recovered from a gravid uterus at any stage of gestation (Vonnahme *et al.* 2000). Although no association was observed between embryonic or fetal weight and the number of viable conceptuses *in utero* in either of these studies, the authors of this abstract have nevertheless become interested in possible effects on the proportional development of the embryo and fetus.

Author 2000 in ref list

Using a number of experimental approaches, Town (unpublished observations) has observed effects analogous to intra-uterine growth retardation (IUGR) in other species. In one study (Fig 1), positive associations between the brain:liver weight ratio and fetal number *in utero* at Day 90 of gestation were apparent in the sow, in the absence of any effect on birthweight. Thus, in more extreme situations of uterine crowding created by an imbalance between high ovulation rates in multiparous sows and uterine capacity, increased variability in post natal growth performance could arise from effects analogous to IUGR in other species. Effects on myogenesis are therefore being evaluated as a commercially important trait that may be influenced by changing patterns of pre-natal loss in contemporary pig genotypes.

Conclusions

In swine, inappropriate maternal nutrition, or the catabolism of body tissues frequently seen as the result of low voluntary feed intake during the first lactation relative to the demands of milk production, can produce latent detrimental effects on early embryonic survival. Multiple mechanisms mediate such outcomes, including 'nutritional/metabolic imprinting' of follicular development and oocyte maturation. In turn, this affects early luteal function and the integrity of the oviductal environment. Knock-on effects on early embryonic development have yet to be demonstrated in the pig, but seem likely. Similarly, it is anticipated that nutritionally driven differences in the steroid priming of the uterus will be likely to affect uterine secretory function and the uterotrophic nutrition of the conceptus. Nutritional effects on genomic imprinting are also possible.

Embryonic and fetal development may also be deleteriously affected in swine through naturally occurring intra-uterine crowding. In early gestation this is associated with negative effects on placental development and may represent a nutritional limitation to the embryo at critical stages of organogenesis. Although the absolute size of the fetus at term may not be different, reprogramming of myogenesis as a consequence of 'head-sparing' effects may compromise post natal growth, analogous to the lifetime effects on organ function associated with IUGR in other species. This has important implications for the efficiency of commercial grow-finish operations.

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FACTORS INFLUENCING PLACENTAL GROWTH AND EFFICIENCY IN THE PIG

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On approximately Day 11 of gestation, free floating 1 cm diameter pre-implantation pig embryos will begin to secrete vast quantities of oestradiol-17ß, which serves as the signal for maternal recognition of pregnancy. Embryonic oestradiol-17ß secretion is believed to be part of a feed forward growth loop in which embryonic oestradiol-17ß stimulates uterine secretion of IGF-I, which in turn stimulates embryonic growth and further oestradiol-17ß production. Shortly after the dramatic burst of oestrogen production the embryo will be transformed from a 1 cm sphere to a 1 m long thread in approximately 24 h, a process referred to as elongation. Immediately after elongation, approximately 30–40% of the embryos in a litter will be lost. This loss appears to be a result of relative asynchrony within the litter and is a relatively constant percentage of the embryos, regardless of how many are present. Between Day 30 of gestation and full term, additional embryos will be lost primarily as a result of the limits of uterine capacity. The prolific Chinese Meishan pig has 3 to 5 more pigs per litter than occidental breeds (Yorkshire, Large White, Landrace, etc) in spite of having a similar ovulation rate and uterine size, at least in gilts of the same reproductive age. Meishan embryos exhibit a reduced growth rate, specifically of the trophectodermal layer, as early as Day 5 to 6 of gestation. This reduced trophectodermal mitotic rate continues until the embryo has reached a large spherical shape immediately prior to elongation. Meishan pre-implantation embryos also produce less estrogen and consequently stimulate less endometrial IGF-I production. Following elongation, the Meishan embryo is shorter than similar stage Yorkshire embryos, probably as a result of the reduced trophectoderm mitotic rate. Up to Day 90 of gestation the Meishan conceptus will exhibit a reduced size compared to occidental controls. It was hypothesised that the reduced growth rate of the Meishan embryo led to a reduced placental size later in gestation as a result of the reduced pre-implantation secretion of oestradiol-17ß and concomitant reduced endometrial secretion of IGF-I which resulted in the reduced growth rate of the elongating embryo and therefore the placenta throughout gestation. To test this hypothesis, Meishan gilts were given exogenous oestradiol-17ß around the time of elongation and placental size at full term was increased by 40%. The reduced size of the Meishan conceptus during gestation had led to the hypothesis that the increase in litter size observed was simply the result of more smaller conceptuses in a similar amount of uterine space. Reciprocal embryo transfer between Meishan and Yorkshire gilts on Day 2.5 of gestation results in smaller pre-implantation embryos 9 days later when they are gestated in a Meishan uterus, compared to those gestated in a Yorkshire uterus. However, within a given uterine environment (either Yorkshire or Meishan) Meishan embryos are smaller than similar stage Yorkshire embryos. When both Meishan and Yorkshire conceptuses are co-transferred to a Yorkshire recipient gilt, the piglets are born at the same weight despite a marked difference in placental weight, with the Meishan placenta ~70% the weight of the Yorkshire placenta, and markedly more vascular. The compensatory growth of the Meishan fetus during the last 25 days of gestation indicated that the Meishan placenta was not only smaller throughout gestation, but had the capability to become markedly more efficient in the extraction of nutrients, a concept that has been termed placental efficiency. Within litter variation in placental efficiency was significant

(approximately 3-fold), supporting the concept that there was phenotypic variation available to respond to selection pressure. More importantly from a conceptual basis was the fact that placental efficiency was negatively associated with placental but not fetal weight. This indicated that large and small fetuses can develop on efficient or inefficient placentae, but that large placentae are less efficient than small placentae and, therefore, the difference in litter size between the Meishan and our occidental breeds may be the result of thousands of years of indirect selection for small, efficient placentae. If this was indeed the case, and if variation in placental efficiency existed in the Yorkshire breed, and was heritable, it should be possible to directly select animals based on the efficiency of their placenta (as measured by the fetal weight to placental weight ratio) and potentially increase litter size. To test the hypothesis that placental size, and therefore efficiency, is a component of the increased litter size in the Meishan, individuals were identified within our Yorkshire herd that grew either on a large inefficient or a small efficient placenta and bred males and females within these 2 groups. Not only was the efficiency of the placenta heritable, but the group that had developed on small efficient placentae exhibited an increased litter size. More recent observations indicated that even as early as Day 25 of gestation, prior to any reduction in conceptus numbers resulting from uterine crowding, placental efficiency is negatively correlated with placental weight, not associated with fetal weight, and positively correlated with litter size. Furthermore, placental efficiency was found to be positively correlated with both placental and endometrial vascular density. The association between the efficiency and vascularity of the placenta, and associated endometrium, suggested that more efficient placentae might exhibit a greater expression of compounds involved in angiogenesis, specifically vascular endothelial growth factor (VEGF) and its receptors, VEGF-R1 (Flt-1) and VEGF-R2 (Flk-1/KDR). In general, there is a progressive increase in the density of blood vessels in the placenta throughout gestation. Similar to the increase in blood vessel density in the placenta during the course of gestation, placental expression of both VEGF and VEGF concentration in fetal blood increases steadily from approximately Day 25 of gestation to full term. Within a given day of gestation, placental efficiency and placental expression of VEGF are correlated. In addition to exhibiting a greater placental efficiency throughout gestation, Meishan conceptuses have greater concentrations of circulating VEGF in fetal blood. In the population of animals directly selected for placental efficiency, both placental efficiency and placental expression of VEGF were elevated in the group selected for high placental efficiency compared to those selected for low placental efficiency. Although there does not appear to be a difference in the expression of VEGF-R2 in individuals selected for high versus low placental efficiency, the animals selected for high placental efficiency have greater expression of VEGF-R1 than the low selected group. This body of work describing the tremendous variation within litter mate conceptuses in their ability to adapt to the particular uterine environment has led to the suggestion that the capacity of the uterus is not adequately described by uterine size or numbers of conceptuses gestated (as though they were all equivalent), but instead should be described as the total placental mass that can be carried to term and therefore the number of conceptuses surviving is a result of both uterine size and average placental efficiency. In addition to the contribution of placental efficiency to litter size regulation, recent evidence has emerged demonstrating that pigs selected for post natal survival exhibit an increased placental efficiency. These data have been interpreted as suggesting that not only is a small efficient placenta conducive to increasing litter size, but also to increasing the provision of nutrients at critical times during gestation, thus resulting in more vigorous pigs at birth. Therefore, a variety of events impact upon conceptus survival and growth during gestation in the pig. First, the amount of oestradiol-17ß produced by the pre-implantation embryo influences the size of the placenta which, in combination with the conceptus' potential to increase its placental efficiency, will influence whether it survives to term. The efficiency of that placenta appears to be influenced by the density of blood vessels in the placenta, probably a direct result of the level of expression of VEGF and its receptors in the placenta and adjacent endometrium. And finally, the combination of late pre-implantation growth, placental size and efficiency of the placenta (via regulation of angiogenic factor signaling systems) all contribute to the number of pigs born and their likelihood of survival.

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DEVELOPMENT OF THE EQUINE CHORIONIC GIRDLE: A ROLE FOR ALLANTOIC MESENCHYME?

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Sadly Francesca Stewart died in December 2000

INTRODUCTION

Many features of equine embryonic development and placentation are unique to the genus. One of these is the development of a discrete annular region of specialised invasive trophoblast called the chorionic girdle. This narrow band of cells develops on the surface of the equine conceptus, between Days 25 and 35 after ovulation, in a narrow region of the chorion at the point where the regressing yolk sac and enlarging allantoic membranes abut one another (van Niekerk and Allen 1975). Between Days 34 and 36 the girdle adheres to the overlying epithelium of the endometrium and the now mature, binucleate cells begin to invade and migrate through the maternal tissue. Once in the endometrial stroma they enlarge quickly, round up and become tightly bunched together to form a series of ulcer-like endometrial protruberances known as endometrial 'cups'. These structures secrete large quantities of equine chorionic gonadotrophin (ecG) which, in turn, stimulates the formation of secondary corpora lutea (CL) in the maternal ovaries, thus ensuring sufficient progesterone and progestagens are produced to maintain pregnancy. By mid-gestation, however, the secondary CL and endometrial cups degenerate and disappear (Allen and Moore 1972) and the non-invasive allantochorion, that gives rise to the diffuse epitheliochorial placenta, takes on the role of progesterone production.

It has long puzzled developmental biologists why the equine chorionic girdle should develop in such a precise manner. Consequently, the mechanisms which stimulate an invasive phenotype for this portion of horse trophoblast are of considerable interest, and yet the precise timing and stimuli for the discrete hyperplasia are not entirely clear. It is most likely, however, that local, fetally derived mitogenic signals are responsible and the allantoic mesenchyme is the most probable source of these (Stewart 1996). It is also known that the proliferation occurs in the region where avascular chorion is in close contact with adjacent vascularised allantoic mesenchyme (Stewart *et al.* 1995) that has been shown to express a number of cytokines and growth factors, including insulin-like growth factor-2 (IGF-2; Lennard *et al.* 1995) and hepatocyte growth factor-scatter factor (HGF-SF; Stewart *et al.* 1995). HGF-SF, known to have mitogenic, motogenic and morphogenic effects on a variety of cells, is probably the more important of the 2; moreover its receptor, the proto-oncogene c-met, is expressed strongly by equine trophoblast. These findings, and the fact that both HGF-SF and HGF-SF receptor knock-out mice fail at around mid-gestation due to a placental defect involving the lack of labyrinthine trophoblast (Bladt *et al.* 1995; Smith *et al.* 1995; Uehara *et al.* 1995), led Stewart *et al.* to conclude that HGF-SF secreted by the allantoic mesenchyme is the most likely stimulant for chorionic girdle formation.

Author: Please cite in ref list

Stewart *et al.* 1995?

To investigate this question further, the present study aimed to examine the precise relationship between the chorion and the allantoic mesenchyme during chorionic girdle formation using immunohistochemistry. Monoclonal antibodies against cytokeratin and vimentin were employed to identify trophoblast and mesenchymal tissues accurately. In addition, a monoclonal antibody against the cell proliferation marker Ki67 (MIB1) was used to assess the extent of cell division within the different membranes.

MATERIALS AND METHODS

Tissues

Eight horse conceptuses were recovered non-surgically by uterine lavage between Days 29 and

34 after ovulation. A length of chorionic girdle was dissected free and the edges were trimmed to leave a small band of chorion and allantochorion, respectively, on either side. A piece of this was fixed in Bouin's fluid and the remainder was frozen in OCT embedding medium (BDH Ltd, Dorset, UK) in isopentane over liquid nitrogen and stored at -70°C. Cryostat sections (6 µm) were fixed in 4% paraformaldehyde for 5 min, rinsed in PBS and dehydrated in an ethanol series (50, 70 and 95%), before being stored in 95% ethanol at 4°C in readiness for immunohistochemical staining.

Immunohistochemistry

Sections were rehydrated and incubated with the appropriate dilution of the primary antibody overnight at 4°C, or for 60 min at room temperature. The antisera used were specific for cytokeratin (Dako Diagnostics, Cambridgeshire, UK), vimentin (clone V9; Boehringer Ingelheim Ltd, Berkshire, UK) and Ki67 (MIB-1; Dianova, Hamburg, Germany). All of the antisera were diluted in Tris-buffered saline containing 3% bovine serum albumin (TBS-BSA). The sections were then washed repeatedly in TBS-0.01% Tween (T)-20 before the second antibody (goat anti-mouse IgG; Dako Diagnostics) was applied for 60 min at room temperature. Antigen-antibody complexes were then visualised by labelling with the Vectastain Avidin and Biotinylated horseradish peroxidase macro-molecular Complexes (ABC) and DAB peroxidase kits (both from Vector Laboratories, Cambridge, UK) before final counterstaining with Harris's haematoxylin.

RESULTS

As expected, the cytokeratin antibody labelled all the trophoblast cells of the chorionic girdle, chorion and allantochorion regions of the membranes, while the vimentin antibody bound to the mesothelial cells (Mt) underlying the trophoblast in all three regions. The mesothelial layer underlying the chorion was uniformly one cell thick, whereas that underlying the chorionic girdle was much thicker and was multilayered. Furthermore, the mesothelium underlying the girdle was thickest at the allantochorionic border and became progressively thinner as it approached the chorion. In the mature (Day 34) girdles, this gradation from allantochorion to chorion mirrored the pattern of trophoblast proliferation since the girdle itself was thickest at its allantochorionic edge and tapered off markedly towards the chorion. Also, the mesenchyme underlying the girdle contained fetal capillaries, as it does when beneath the allantochorion, but not when beneath the avascular chorion. These capillaries were invariably close to the allantoic border of the girdle and their presence demonstrated convincingly that the mesenchyme underlying the chorionic girdle is derived from the allantois.

Staining with the antibody for the proliferation marker, Ki67, demonstrated that as many as 90% of the trophoblast cells in the developing chorionic girdle were actively dividing, compared to <10% of cells in the adjacent chorion.

DISCUSSION

These immunohistochemical staining experiments demonstrated that the mesothelial layer underlying the chorionic girdle originates from the vascularised allantois rather than from avascular chorion. It may be concluded, therefore, that allantoic mesenchyme not only fuses with the chorion to form the rapidly expanding allantochorion, but also migrates up to 4 mm in the opposite direction to underlie the existing chorion and so provide the stimulus for the development of the chorionic girdle. The extremely high rate of proliferation in the trophoblast cells overlying this region of reverse migration of allantoic mesenchyme, and the possible correlation between mesenchymal thickness and breadth and thickness of the chorionic girdle, provide strong circumstantial evidence that a mitogen secreted by the allantoic mesenchyme initiates the differentiation of the invasive trophoblast in the mare.

Invasive equine chorionic girdle cells are comparable to the primary syncytiotrophoblast in primate pregnancy, which is also invasive and secretes human chorionic gonadotrophin (hCG). The allantochorionic placenta is common to all eutherian mammals, but its precise anatomy and function differs greatly between species. Despite this diversity, however, the cellular composition of the allantochorion seems to provide a unifying theme. It is proposed that, as a general rule, an allantochorion originating from ectodermal, mesodermal and endodermal derivatives gives rise to non-invasive trophoblast, whereas the allantochorion composed only of ectodermal and

mesodermal components, with no underlying endoderm, gives rise to trophoblast with an invasive phenotype.

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STRUCTURAL AND HAEMATOLOGICAL ASPECTS OF THE EQUINE PLACENTA IN MID-PREGNANCY

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INTRODUCTION

The placenta is an organ with a limited life span, and is considered to be the only channel for transport of nutrients from the mother to the conceptus. The demands of this structure increase exponentially with the progress of pregnancy, and are accompanied by a wide variety of structural modifications (Wooding and Flint 1994). In the horse, the placenta is diffuse, microcotyledonary and epitheliochorial (Steven 1982), and it is suggested that the fetomaternal blood flow interrelationship is multivillous (Leiser and Kaufmann 1994).

One of the most important placental structural modifications involves the fetal and maternal vasculature, the volume of which depends particularly on the size of the microcotyledon. The latter increases gradually with the progress of pregnancy (Samuel *et al.* 1974) and reaches 1–2 mm average diameter at term (Wooding *et al.* 2000). Despite the importance of the fetomaternal blood vascular systems, there is a lack of information concerning the microvascular architecture of the horse placenta. Therefore, the present study was undertaken to clarify the architecture of the maternal and fetal blood vessels in the microplacentomes of the mare.

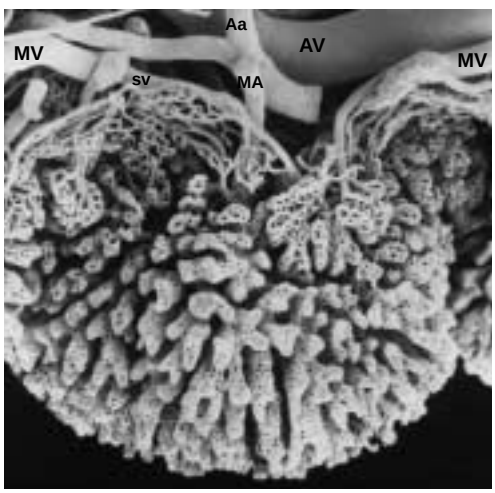

Fig 1: Vascular cast of a fetal microcotyledon showing the supplying microcotyledonary artery (MA) that originated from the allantochorionic artery (Aa) and 2 relatively small microcotyledonary veins (MV) that formed from more than 2 stem veins (SV) to join the large allantochorionic vein (Av). Note the tightly meshed capillary network of terminal villi. x 480.

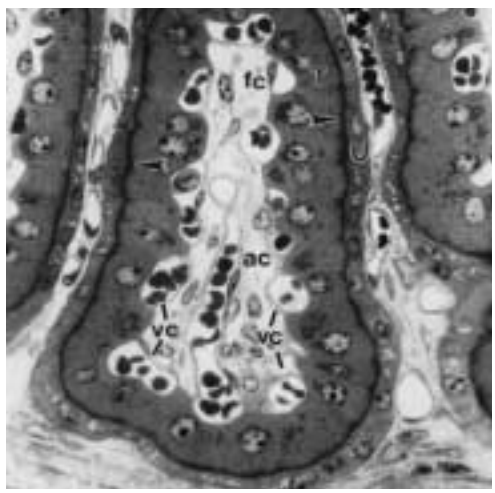

Fig 2: Light micrograph of a terminal villus showing a single arterial capillary limb (ac) extending to the top of the terminal villus and 4 venous capillary limbs (vc) appearing in the axial periphery. The fetal capillaries (fc) partly indent the trophoblast cells (T). The uterine epithelium (U) is thin and connected to the trophoblast cells through interdigitating microvilli (arrowhead). x 475.

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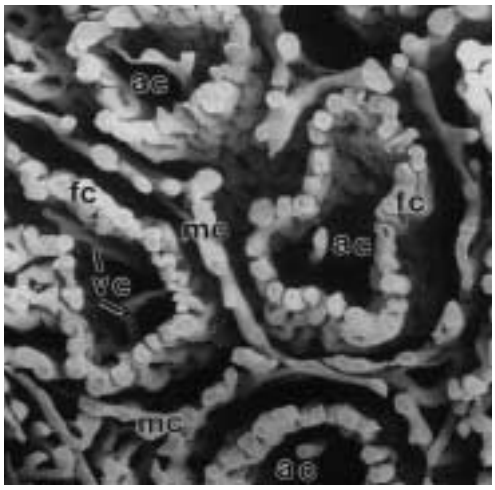

Fig 3: Frozen-cut combined fetomaternal vascular cast showing dense fetal capillaries (fc) which are completely surrounded by relatively loose, maternal ones (mc). Note, the arterial capillary limbs (ac) and the venous capillary limbs (vc) on the vasculature of the fetal terminal villi. x 1100.

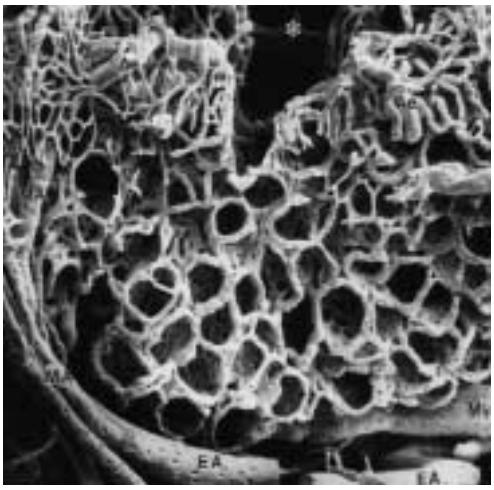

Fig 4: Maternal vascular cast showing microcaruncular artery (MA) arising from endometrial arteries (EA) and divided into arterioles (Ae) near to the top of the microcaruncle. The arterioles are subsequently divided into capillaries to constitute the vascular skeleton of the orifice or primary crypt (*), the side and base of microcarucle. Large microcaruncular vein (Mv) originating from the base of microcaruncle. Note the honeycomb-like arrangement of capillaries on the side and base of the microcaruncle. x 700.

MATERIALS AND METHODS

Pregnant uteri were removed post mortem from 6 Thoroughbred mares between 180 and 197 days of gestation and pieces of endometrium with placenta attached were fixed for light microscopy. In addition vascular casts, formed by injection of liquid plastic (0.5 g catalyst, 20 ml Mercox and 5 ml Methylmethacrylate) through the umbilical and uterine arteries, were also examined.

RESULTS

The microcotyledons were globular in shape and were completely enclosed in the corresponding microcaruncles. Each constituted a microplacentome with a diameter of 240–660 µm. Each microcotyledon consisted of 4–5 stem villi, with each villous divided into 6–8 intermediate villi. The latter were subdivided further into 4–5 terminal villi, which penetrated deeply into the endometrial tissue to anchor the microcotyledon in its corresponding maternal crypt.

Vascularity of the microcotyledon was achieved by a single microcotyledonary artery, which followed a short straight course before dividing into 4–5 stem arteries. Drainage was via 2–3 microcotyledonary veins (Figs 1, 5). The stem arteries subdivided into 6–8 smaller arterial branches according to the number of intermediate villi and, located peripheral to the artery, were the smaller stem veins. More than one stem vein was seen to constitute each microcotyledonary vein, which then joined the allantochorionic veins (Fig 1). On each intermediate villus, one fine artery or arteriole (15–25 µm) could be observed giving rise to 4–5 arterioles to vascularise the terminal villi. The venules originated at different levels from the capillary complex and were located peripheral to the artery. Tightly arranged capillaries, with each terminal villous capillary ending in 3–5 capillary loops, formed the vasculature of the terminal villi. The latter consisted of a centrally located arterial capillary limb (12 µm) and was drained by 1–4 venous capillary limbs (15 µm). The fetal capillaries were of smaller diameters (8 µm) and were round-to-oval in cross section (Figs 2, 3).

The blood supply to the microcaruncle was usually via 2 straight arteries that ran in a materno-fetal direction to the top, at which point each artery ramified into 2–4 arterioles (40 µm). These arterioles ran a short course and/or directly ramified into capillaries which constituted the capillary networks of the orifice, at the side and base of the microcaruncle (Fig 4). Maternal capillaries were relatively dense and irregular at

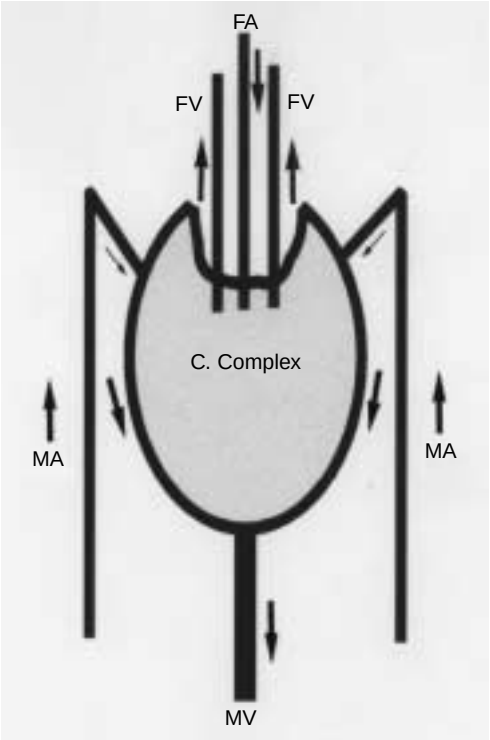

Fig 5: Schematic drawing of the mare microplacentome with the supplying arterial and venous vessels on both fetal and maternal side. Fetal microcotyledonary artery (FA), fetal microcotyledonary vein (FV), maternal microcaruncular artery (MA), maternal microcaruncular vein (MV), and capillary complex (C. complex). The arrows show the direction of blood flow in the fetal and maternal vessels.

the orifice but, at the side and base of the microcarucle, they were organised in a honeycomb-like fashion. These maternal capillaries appeared rounded to oval in cross section and larger (9 µm average diameter) than the fetal ones. However, they became flattened and enlarged (12 µm), before converging into the venules. The venous drainage of the microcaruncle occurred via a large number of venous capillary limbs and venules which ranged from 15–60 µm in diameter. These venules originated from the capillary complex at the base of the microcaruncle before converging to form a large microcaruncular vein (Figs 4, 5).

Discussion

The study of equine placentae at mid-gestation was limited by the materials available. The advantage of focusing on this period was that the placenta was not overly complicated in structure, despite the fact that the microcotyledons were fully formed and the vascular system is already established by Day 150 of gestation (Samuel *et al.* 1975).

The vascular architecture of the horse microcotyledon comprises a long straight artery and the capillary network invests long villi that are arranged in a partly fan-like fashion, drained by a single vein (Steven 1968). However, the present study has demonstrated that each fetal microcotyledon is vascularised through a single, centrally located artery and drained by 2 veins situated peripherally. The current study is in accordance with Steven and Samuel (1975) because the maternal circulation to the microcaruncles is in the form of long, straight microcaruncular arteries. These arteries break over the rim and give rise to a dense vascular network on the walls of the maternal crypts, then drain to a single microcaruncular vein as in the camel (Abd-Elnaeim 1998). The vascular supply of the maternal placenta is similar in the 2 species, but the horse represents the most complex situation, because of the ramifications of the microcaruncle into primary, secondary and tertiary crypts. In the horse, the venous outflow takes place through several venous capillary limbs that join to form venules which converge into a large microcaruncular vein at the base of microcaruncule.

Most transplacental exchange takes place at the capillary bed (Benirschke and Kaufmann 1995). In order to favour this area, the supplying vessels have to run as straight as possible. The present study supports this notion, with the stem vessels and their ramifications almost completely following the whole length of the microcotyledons, with a central and straight course for the arterial vessels and a winding peripheral one for the venous vessels. On the maternal side, also, the microcaruncular arteries follow a straight fetomaternal course to the top of microcaruncles before ramifying into arterioles and capillaries. This type of vessel ramification provides the shortest distance from the supplying vessels to the capillaries of the terminal villi on the fetal side and from the arterioles to the capillaries of the terminal crypts on the maternal side, as in the bovine (Leiser *et al.* 1997; Pfarrer *et al.* 2001).

The area of exchange is represented by the thin walled vessels which are located at the periphery of the microcotyledon and are close to the maternal microcaruncular tissue. The capillary

convolutions of the terminal villi are still simple at this stage but we expect an increase during the second half of gestation. There have been several attempts to classify the variety of placentae according to the geometrical arrangement of fetal and maternal vessels. However, in the present study, evidence from histological examination of semithin sections and microvascular casts indicates that the fetal and maternal blood flow is complicated and occurs mostly in opposite directions suggesting that the fetomaternal blood flow interrelationship is countercurrent in nature.

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SESSION IV:

Chairman:

T. A. E. Stout

ACUTE NUTRIENT RESTRICTION DURING MID-PREGNANCY IN THE EWE: RESPONSES AND CONSEQUENCES

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INTRODUCTION

Perturbations in fetal growth may have adverse consequences for the health and survival of the offspring, both in the post natal period and in adult life. The intra-uterine environment, which is modulated by nutrients, growth factors and hormones, determines the pattern of fetal growth. The nutritional status of the mother has the potential to alter all of these factors and so may have a significant influence on both placental and fetal growth. Nutritionally mediated alterations in the concentration of systemic factors may play a role in the modulation of fetal and placental growth through their regulation of metabolism, nutrient partitioning, uterine blood flow and nutrient transport kinetics. The insulin-like growth factor (IGF) axis is thought to play a fundamental role in the development of the fetus and placenta. Many of the components of the IGF axis have been localised to the ovine placenta and several have been shown to be nutritionally sensitive (Osgerby *et al.* 2002). Nutritional modulation of the expression of these factors may provide a link between maternal nutrition and placental growth (Wathes *et al.* 1998). Although the effects of chronic under nutrition during pregnancy are discussed frequently, the effects of a period of acute under nutrition remain unknown. Therefore, this study assessed the effect of a period of acute under nutrition during mid-gestation on parameters of fetal and placental growth. Although the absolute requirements for nutrients are relatively low at this time, the metabolic rate of the fetus and the proportion of its weight represented by rapidly growing tissues are at their highest (Robinson and Symonds 1995). This time point also enabled the implementation of a relatively long period of maternal re-feeding, to examine whether the effects observed were permanent or reversible. Maternal and fetal systemic factors and the expression of the placental IGF axis were examined as potential mediators of any effects observed.

MATERIALS AND METHODS

All procedures were performed under the UK Animals (Scientific Procedures) Act 1986. Pregnant Welsh Mountain ewes of body condition score 2.0–2.5 were fed a pelleted diet providing 100% of their maintenance requirements from 4 weeks prior to conception. At Day 83 of gestation, ewes were allocated to control (C) or nutrient restricted (NR) groups. The concentrate ration of NR ewes was reduced from Day 83 to 85 and withdrawn between Days 85 and 90. The ewes remained on a bed of wheat straw to maintain rumen function and some feeling of satiety, whilst providing minimum nutritional value. At Day 90, half the ewes (NR: n=7, C: n=8) were humanely slaughtered. The remainder (NR: n=9, C: n=9) were fed their maintenance diet until slaughter at Day 135 (term ~147). Maternal blood samples were collected fortnightly during pregnancy and analysed for glucose, insulin, IGF-I and non-esterified fatty acids (NEFAs). At slaughter, fetal blood samples were collected, physical measurements recorded and organs dissected out and weighed. All placentomes were dissected from the uterus, weighed and assigned a type according to their morphology (Vatnick *et al.* 1991). Placentome tissue was snap frozen for analysis of the expression of the IGF axis by *in situ* hybridisation. At each time-point data were compared using the Student T test. Distribution of placentome types was compared between the

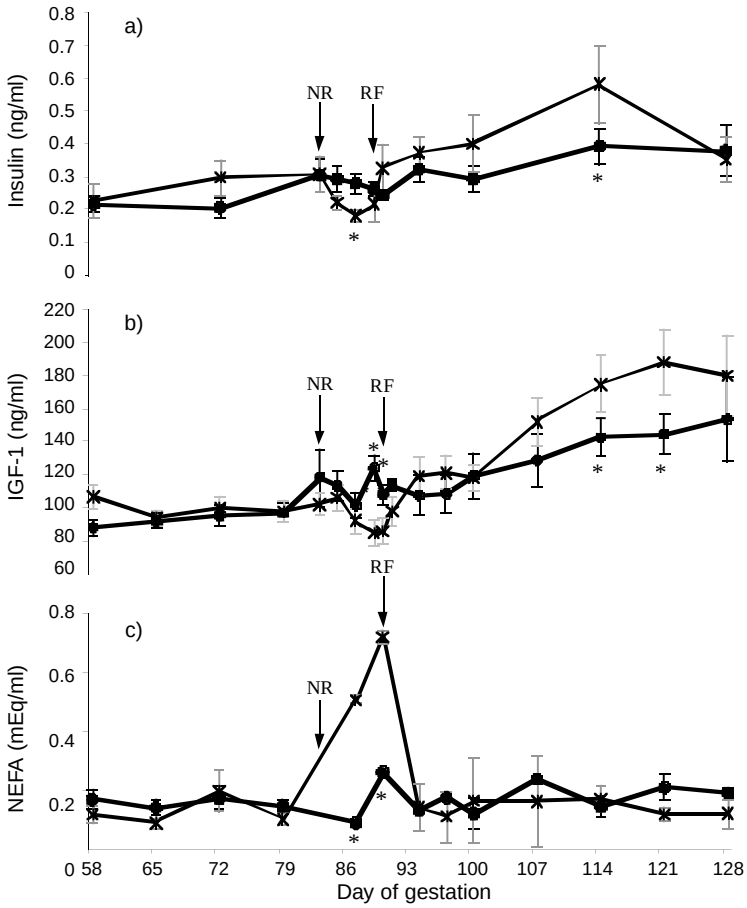

Fig 1: Maternal plasma: a) insulin; b) IGF-I; and c) NEFA concentrations in control (———) and nutrient restricted (– – –) ewes throughout gestation and during nutrient restriction (NR) and re-feeding (RF) (corresponds to P<0.05).*

groups using Chi Squared. Maternal metabolic profiles were assessed by Mixed Model Repeated Measures ANOVA.

RESULTS

Maternal insulin and IGF-I concentrations were significantly lower in NR ewes during the restricted period (Fig 1). Surprisingly, maternal glucose was unaffected. Following re-feeding, the increase in insulin and IGF-I over time appeared greater in the NR ewes and this difference became significant on Day 114 (insulin and IGF-I) and Day 121 (insulin). The concentration of maternal NEFAs was significantly increased in NR ewes during the period of nutrient restriction. No difference in fetal insulin or IGF-I was observed at either Day 90 or Day 135.

Fetal weight was unaffected by the nutrient restriction at both time-points (Table 1). However, at Day 90 there was a tendency towards decreased weight of the fetal lung in the NR ewes, both as actual weight and as a percentage of fetal body weight. This was associated with significantly decreased thoracic girth and uterine fluid volume. At Day 135, the difference in actual lung weights between the groups was significant. Placental weight was significantly lower in NR ewes at Day 90 but not Day 135 (Table 1). At Day 135, a significant shift in the distribution of placentome type had occurred towards the everted type (Table 1). Placentome fetal mesoderm expression of IGF-II was unaffected by maternal diet (Table 2). However, expression of IGFBP-2 in the placentome capsule and of IGFBP-3 in the placentome capsule and maternal stroma was significantly reduced in NR ewes at Day 90 and 135, respectively (P<0.05).

TABLE 1: Placental and fetal measurements at Day 90 and 135 of gestation

		Day 90			Day 135		
		Control n = 8	NR n = 7	P a>b	Control n = 9	NR n = 9	P a>b
Total placentome weight (g)		767 ± 53[a]	598 ± 31[b]	0.05	492 ± 25	486 ± 26	ns
Placentome Type	Type A	69.0 ± 8.4	63.5 ± 9.6	ns	58.3 ± 8.9	29.8 ± 9.5	0.05
	Type B	15.8 ± 7.8	23.3 ± 7.2		25.9 ± 6.6	48.3 ± 8.3	
Distribution (%)	Type C	0	0.8 ± 0.6		2.6 ± 1.9	8.5 ± 4.3	
	Type D	0.5 ± 0.5	0.4 ± 0.4		1.1 ± 1.0	2.7 ± 2.0	
Fluid volume (ml)		724 ± 50[a]	530 ± 67[b]	0.05	1386 ± 124	1413 ± 93	ns
Fetal weight (g)		556 ± 20	538 ± 24	ns	4458 ± 122	4297 ± 162	ns
Thoracic girth (cm)		17.4 ± 0.3[a]	16.5 ± 0.3[b]	0.05	35.0 ± 0.5	35.0 ± 0.6	ns
Lung weight (g)		27.1 ± 1.6[a]	22.9 ± 0.9[b]	0.06	116.4 ± 6.0[a]	100.0 ± 3.2[b]	0.05
Lung (% of body weight)		4.87 ± 0.25[a]	4.28 ± 0.19[b]	0.1	2.70 ± 0.14	2.43 ± 0.13	ns

DISCUSSION

The significant reduction in lung weight at Day 135 may have adverse consequences for the respiratory function of the neonate. In man, lung hypoplasia is often associated with respiratory insufficiency at birth and is present in 14–20% of neonatal autopsies (Sherer *et al.* 1990). In lambs, persistent impairments in respiratory function during early post natal life have been observed in response to placental insufficiency (Joyce *et al.* 2001) and amniotic drainage (Jakubowska *et al.* 1993). The growth of the lungs is dependent largely on expansion by lung fluid, secreted by the pulmonary epithelium. The physical environment, including changes in luminal epithelium pressure, affects secretory activity (Harding and Hooper 1996). In sheep, a reduction in amniotic fluid volume imposes exaggerated trunk flexion on the fetus, narrowing the thoracic cavity and displacing the diaphragm upwards. This results in decreased volume and increased pressure of the thoracic cavity, which may limit lung expansion (Harding and Higgins 1991). In man, reduced amniotic volume is one of the most common factors associated with fetal lung hypoplasia (Sherer *et al.* 1990). The association of reduced amniotic volume and thoracic girth with decreased lung weight in fetuses from NR ewes suggests that lung growth was restricted by this mechanism in response to the nutrient restriction (Fig 2). The mechanisms behind the regulation of amniotic volume are complex. However, placental insufficiency has been shown to decrease amniotic volume with no associated decrease in fetal urine production and it has been proposed that the oligohydramnios observed is caused by an excess of intra-membranous fluid transfer (Gagnon *et al.* 2002).

The decreased maternal insulin and IGF-I, and increased maternal NEFA concentrations indicate reduced anabolic drive and mobilisation of lipid stores in the maternal compartment, which may act to protect glucose for placental transfer to the fetus. Indeed, despite the relatively severe level of nutrient restriction, both fetal insulin and IGF-I were unaffected, suggesting that the fetuses remained normoglycaemic. The shift towards the everted placentome and the reduction in amniotic volume in the NR ewes may be additional compensatory responses contributing towards the maintenance of fetal growth. Although no difference in placental weight was observed at Day 135, the distribution of placentome types had shifted significantly towards the everted type. A similar change in placentome morphology has been observed in response to hypoxia, carunclectomy and chronic maternal under nutrition (Clarke *et al.* 1998; Penninga and Longo 1998; Osgerby *et al.* 2002) and so seems to be a general response to any environment in which the nutrient supply to the fetus is disturbed. Although it seems certain that IGF-II is fundamental to

TABLE 2: Effect of nutrient restriction on placental expression of IGF-II, IGFBP-2 and IGFBP-3 at Day 90 and 135 of gestation

Probe	Localisation	Day 90	Day 135
IGF-II	Fetal mesoderm	⇔	⇔
IGFBP-2	Capsule	⇔	⇓
IGFBP-3	Capsule	⇓	⇔
	Caruncular stroma	⇓	⇔

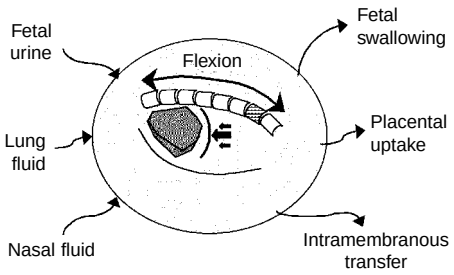

Fig 2: Increased fetal trunk flexion during oligohyramnios may limit the expansion of the fetal lungs by reducing thoracic volume and pressure (Harding et al. *1991). Routes by which amniotic fluid is controlled are shown. It is suggested that an increase in intramembranous transfer may be the cause of oligohydramnios during placental insufficiency (Gagnon* et al. *2002).*

placental growth, the results of this study and others (Osgerby *et al.* 2002) indicate that it is not nutritionally sensitive and therefore does not provide a direct link between maternal diet and placental growth. However, the significant reduction in placental expression of IGFBP-2 and IGFBP-3 may have influenced placental development by altering the mitogenic activity of IGF-II.

In summary, reduced anabolic drive and mobilised lipid stores in the maternal compartment seem successful in maintaining fetal nutrient status and body weight during the mid-gestation nutrient restriction. Despite this, possibly as a side effect of a compensatory increase in intra-membranous transfer, fetal lung growth was compromised and this may have adverse consequences for neonatal respiratory function. In addition, this study clearly demonstrates the nutritional sensitivity of placental IGFBP-2 and IGFBP-3 expression and associated alterations in placentome morphology.

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THE EFFECTS OF PRE-NATAL NUTRITION ON CARDIOVASCULAR FUNCTION IN OFFSPRING – SOME INSIGHTS FROM COMPARATIVE BIOLOGY

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The concept of the fetal origins of adult disease proposes that a fetus develops appropriately to cope with the environment into which it predicts, on the basis of cues from the mother and/or the placenta, that it will be born. If such cues indicate that environmental conditions are harsh, eg that nutrition is poor or ambient oxygen tension is low, then the offspring develops phenotypic characteristics which may enable it to survive better in such an environment. This was the essence of the 'thrifty phenotype' hypothesis of Hales and Barker (2001). This proposition, directed towards human disease, was largely based on observations in the rat, but both the requisite pre- and post natal components of the phenomenon have been confirmed more recently in sheep. The concept has also been extended and the range of characteristics of such a phenotype now includes not only insulin resistance but altered exercise and feeding behaviour (including appetite and food preference (Vickers *et al.* 2000), reduced skeletal muscle mass (also giving insulin resistance), central fat deposition, reduced vascularity in some tissues and alterations in autonomic control involving the hypothalamic pituitary adrenal axis and the sympathetic nervous system. The phenotype is highly appropriate to any mammal with restricted food supply and to the hunter-gatherer lifestyle, in which the individual will favour fat ingestion and, in man, store it when available as central fat. This is analogous to the camel's hump (Wolff *et al.* 2001), and indeed this hump and human omental fat have remarkable similarities as both represent the major labile fat storage depot for episodic feeders. Studies of the related camelid, the llama, have revealed a plethora of adaptive responses which develop during fetal life to defend the offspring, in this case from hypoxia (Riquelme *et al.* 2002).

Recent attention has focused on the way in which the post natal environment contributes to the actual risk of disease in later life, especially if it provides conditions which were not predicted by the offspring pre-natally and which challenge its homeostatic mechanisms. This may occur if the environment changes rapidly, as has happened during the lifespan of the last generation because nutrition is plentiful and of high calorific value. The fetus is then maladapted to its new environment, and poor diet and lifestyle serve to amplify the risk of later disease. Demographic changes in the third world, and the epidemic of obesity in young people in developed societies, will lead inexorably to a prevalence of chronic disease of nightmarish proportions.

The effects may in addition be apparent for more than one generation. If a woman experienced an adverse environment when she herself was *in utero,* this can impair her reproductive capacity and such transgenerational constraints make fetal maladaptation all the more likely. Thus adaptations to undernutrition and other adverse environmental influences at critical periods of early development can lead to cumulative and persisting changes in the body's structure, physiology and metabolism, increasing the risk of cardiovascular disease and type 2 diabetes in society.

Rodent models have provided many valuable insights into the mechanisms underlying the programming of metabolic and vascular dysfunction in the offspring of dams which have received an unbalanced diet such as the low protein diet (LPD; Brawley *et al.* 2003c), global undernutrition (Ozaki *et al.* 2001), a high fat diet (Khan *et al.* 2003) or exogenous glucocorticoids (Seckl *et al.* 1999) during pregnancy. These models have advanced our understanding rapidly.

Because it is possible to produce clear and reproducible adaptive effects in the offspring of a species in which development differs in many respects from the human (viz. number of offspring, maturity of cardiovascular, renal and neural development at birth, length of gestation etc) this work supports the concept that such adaptive responses are a widespread biological phenomenon: they are not just a feature which results from a unique combination of human development, diet or lifestyle and our increasing longevity as a species.

In the rat, a LPD in pregnancy produces hypertension and vascular dysfunction in small arteries in the adult offspring (Langley and Jackson 1994; Brawley *et al.* 2003b). Endothelial dysfunction is accompanied by changes in NO release. The defect may result from impaired maternal cardiovascular adaptation to pregnancy such as perfusion of the reproductive tract (Ahokas *et al.* 1983; Itoh *et al.* 2002), which partly determines nutrient delivery to the fetus. However, it is noteworthy that offspring are not uniformally smaller at birth (Hanson and Hoet 1999) and this reinforces the fundamental point that predictive adaptive responses pre-natally are not necessarily coupled to changes in overall body growth: certainly, a severe challenge will induce adaptations which include reduced growth; but this may occur in parallel with, rather than as a cause of, other adaptive responses. The point is of more than semantic importance, for recently epidemiologists have been much exercised by the size of the link between reduced birth weight and later manifestations of disease such as elevated blood pressure. Experimental observations from a range of animal species make it clear that such debate is relatively pointless: birth size is a poor proxy for fetal growth and it gives little information on pre-natal adaptive responses.

Attention has focused recently on the role of glycine as a conditionally essential amino acid during pregnancy. This is because whilst the mother, placenta and fetus are able to synthesise glycine from serine to a variable degree, the fetal requirements for glycine, at least in late gestation in the human fetus, exceed those of other amino acids (Widdowson 1979). Glycine supplementation of the LPD prevents vascular effects in the offspring (Jackson *et al.* 2002). Glycine is a major source of methyl groups during development, and this metabolic pathway requires folate and vitamin B12 as cofactors. Folate supplementation of the LPD has similar effects to glycine supplementation (Brawley *et al.* 2003a). A likely mechanism underlying the glycine/folate effects is via deficiency of methyl groups for both DNA synthesis and its methylation (Rees *et al.* 2000; Petrie *et al.* 2002). By altering the methylation patterns of key genes involved in growth, vasculo- and angiogenesis, some of which are known to be imprinted, the balance between organ growth and blood supply will be perturbed. Altered patterns of blood flow result in a plethora of secondary effects, eg endothelial dysfunction and vascular remodelling which increase risk of atherogenesis and hypertension. The effects of the LPD may occur much earlier in development than the fetal stage; giving it to rats in just the pre-implantation phase produces changes in the allocation of blastocyst cells to the inner cell mass and trophectoderm lineages, and the offspring become hypertensive as adults (Kwong *et al.* 2000). In mice, embryo transfer involving a short period of *in vitro* culture also results in hypertensive offspring (Watkins *et al.* 2002). Such epigenetic effects may be passed to future generations, and indeed female offspring of LPD dams have vascular dysfunction when they in turn are pregnant (Torrens *et al.* 2002a) and so do their (second generation, F2) offspring (Torrens *et al.* 2002b).

Thus, studies in a range of animal species are giving mechanistic insights into the processes by which pre-natal factors influence the risk of chronic disease in later life in people. They raise many questions, eg about the interpretation of family linkage analysis in establishing the aetiology of cardiovascular disease; but by shifting our attention away from such predominantly genetic bases for disease, they will also suggest possible interventions to alter the pattern of disease in human populations.

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COMPARATIVE ASPECTS OF FETAL METABOLISM

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During late gestation, the fetal nutrient requirement varies amongst species (Fowden 1997). In large domestic animals in which the fetus can be studied in the anaesthetised state *in utero*, fetal rates of carbohydrate and amino acid metabolism differ 2- and 10-fold, respectively across the species (Table 1). Compared with non-ruminant herbivores, ruminant species tend to have lower rates of fetal glucose uptake and higher rates of fetal urea production, an index of amino acid catabolism (Table 1). In contrast, fetal rates of O_2 consumption appear to be more uniform across the species (Table 1). Of the O_2 consumed by the fetus, less is used to oxidise glucose carbon in fetal sheep (25–35%) than in fetal pigs or horses (35–45%). Fetal sheep are, therefore, less dependent on glucose and more dependent on amino acids for oxidative metabolism than the fetal horse or pig (Fowden 1997).

The reasons for the species differences in fetal metabolism are multifactional and involve differences in maternal nutrient availability, placental morphology and function, and in the demand for specific nutrients by the fetus itself. In ruminants, maternal glucose availability is low compared to non-ruminant herbivores as glucose is not enterally derived but synthesised from volatile fatty acids by gluconeogenesis in the liver and kidney. Hence, the glucose concentration gradient across the placenta, which drives transplacental glucose transfer, is lower in ruminant than in non-ruminant herbivores (Silver *et al.* 1973). This may account, in part, for the lower rates of umbilical glucose uptake in sheep and cows than in pigs and horses (Table 1).

The diffuse placenta of the non-ruminant herbivores also appears to be more efficient at nutrient transfer by diffusion than the cotyledonary placenta of the ruminants (Silver *et al.* 1973). By term, more grams of fetus are produced per gram of placenta in horses and pigs (15–20g/g) than in sheep (10–12g/g). The gradient between fetal and maternal arterial concentrations of glucose and O_2 are also steeper in horse and pigs than in sheep and cows (Silver *et al.* 1973). In part, this may be due to the longer diffusion distance across ovine than equine or porcine placentae but may also reflect species differences in placental vascular architecture (Steven 1995). In the equine placenta, fetal and maternal blood vessels have a counter-current arrangement, which is more efficient at diffusion than the cross-current

TABLE 1: Mean ranges of the rates of umbilical uptake of glucose, lactate, oxygen and acetate and of urea production by fetuses of different species during late gestation (≥85% gestation)

Species	Umbilical uptake µmol/min/kg				Fetal urea production µmol/min/kg
	Glucose	Lactate	Oxygen	Acetate	
Sheep	25–35	15–25	290–310	25	5-10
Cow	25–35	45–55	290–310	15	4-5
Horse	35–45	10–15	290–320	-	3-4
Pig	35–45	30–40	320–350	-	2-3

Data from Silver *et al.* (1973); Fowden and Silver (1995); Fowden (1997); Fowden *et al.* (2002)

TABLE 2: Mean rates (µmol/min/kg) of uteroplacental consumption of glucose and oxygen and of uteroplacental lactate production in different species during late gestation (≥85% gestation). Uteroplacental tissue weights = combined weight of uterus, placenta and membrane

Species	Glucose consumption	Oxygen consumption	Lactate production
Sheep	120	500	70
Cow	120	500	70
Horse	250	700	100
Pig	330	650	170

Data from Fowden (1997); Fowden *et al.* (2002)

TABLE 3: Factors influencing the fetal nutrient requirement during late gestation in different species

	Sheep	Pig	Horse
Growth rate g/kg fetal wet wt/day	36.0	32.8	9.2
Brain weight % total body wt	1.5	3.3	3.5
Glucose requirement for glycogen deposition g/day/kg	0.50	1.40	0.20
Incidence of FBM (% time)	32.0	41.0	30.0

FBM = Fetal breathing movements. Data from Fowden (1997); Fowden *et al.* (2001)

vascular arrangement found in ovine placentomes (Silver *et al.* 1973; Steven 1995). There may also be species differences in the abundance and localisation of the placental glucose transporters that are essential for transplacental glucose transfer (Wooding 2003). In contrast, diffuse epitheliochorial placentae may not be as active as cotyledonary placentae in the active transfer of amino acids; the fetal to maternal ratio of alpha amino nitrogen is close to unity in horses and pigs but is 1.4–1.9 in sheep and cows (Silver *et al.* 1994). Certainly, measurements of the concentration differences in specific amino acids across the umbilical circulation of the horse suggest that amino acids are not taken up in excess of the requirements for fetal tissue accretion in this species as occurs in the sheep (Fowden 1997; Fowden *et al.* 2002). This may limit the availability of amino acid carbon for fetal oxidation in the horse and, hence, explain the low rate of urea production in this species (Table 1).

Nutrient utilisation by the uteroplacental tissues also varies with species (Table 2). Weight specific rates of glucose and oxygen consumption are lower in cotyledonary than diffuse placentae (Table 2). The rate of uteroplacental lactate production also varies amongst species but does not appear to be related to gross placental morphology (Table 2). No significant species differences are observed in the distribution of uterine glucose and oxygen uptake between the uteroplacental and fetal tissues. Of the uterine uptakes, approximately 50% of glucose and oxygen are delivered to the fetus in both sheep and horses during late gestation (Fowden 1997). Hence, the higher rates of fetal glucose uptake in non-ruminant than ruminant herbivores are not the consequence of either lower rates of uteroplacental glucose consumption or preferential distribution of uterine glucose uptake to the fetus (Fowden *et al.* 2002).

The fetal demand for nutrients also varies between species and with gestational age (Fowden

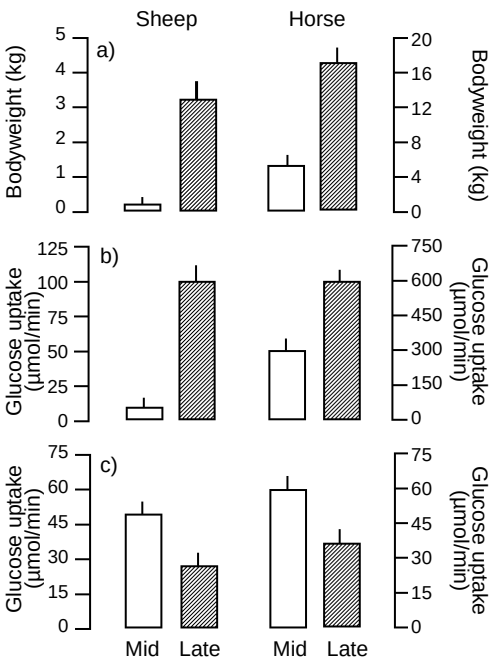

Fig 1: Mean (± se) values of: a) body weight; b) absolute; and c) weight specific rates of umbilical glucose uptake in fetal sheep and horse at mid (□) and late (▨) gestation. Data from Fowden (1997); Fowden et al. *(2002).*

1997). The fetal growth rate is higher in sheep and pigs than in the horse although the absolute weight gain during late gestation will be greater in the horse (Table 3). Fetal sheep and pigs also have a greater glucose requirement for glycogen deposition during late gestation than fetal horses (Table 3). The brain, which is an obligatory glucose consumer, accounts for a larger percentage of body weight in pigs and horses than sheep (Table 3). Energy expenditure on activities, such as breathing movements, also varies between species (Table 3). Consequently, as the fetus grows and nears term, its absolute demand for glucose rises (Fig 1), which places an increasing drain on the maternal glucose pool (Fowden and Silver 1995). This drain is minimised by reducing fetal muscular activity near term and by lowering the weight specific rates of fetal glucose uptake by 30–50% between mid and late gestation (Fig 1). In some species, the increasing fetal demand for nutrients is also met by increasing placental size and, thus, the area for nutrient exchange (Macdonald *et al.* 2000). In others, the weight specific rates of uteroplacental nutrient consumption are reduced (Fowden 1997; Fowden *et al.* 2001). A greater proportion of the uterine uptake of glucose and O_2 is, therefore, distributed to the fetal tissues during late gestation (Fowden 1997; Fowden *et al*. 2002). In addition, distribution of uteroplacental lactate production switches from supply predominantly into the uterine circulation at mid gestation to preferential delivery into the umbilical circulation close to term in both sheep and horses (Fowden 1997; Fowden *et al.* 2002).

The species differences in fetal glucose availability have important consequences for survival both *in utero* and at birth. Compared to fetal horses, fetal sheep have a good glucogenic capacity during late gestation (Table 3) and can activate glucogenesis in response to impending delivery and adverse intra-uterine conditions earlier in gestation (Fowden *et al.* 2001). Fetal horses have more difficulty in maintaining their circulating glucose levels in these circumstances and are more vulnerable to nutritional and other challenges than fetal sheep as a consequence (Fowden *et al.* 2001). Certainly, hypoglycaemia is a common clinical feature of prematurity in foals. The high rate of glucose consumption by the equine uteroplacental tissue will also increase the susceptibility to pre-term delivery during undernutrition as a rapid fall in glucose availability triggers uteroplacental prostaglandin (PG) production (Fowden *et al.* 1994, 2001).

In part, the species differences in the ability to store and utilise glucose reflect differences in the fetal endocrine environment. Hormones, such as insulin, glucagon and catecholamines have all been shown to affect fetal glucose utilisation and production (Fowden *et al.* 1998). Cortisol, in particular, has an important role in enhancing the fetal glucogenic capacity during late gestation (Fowden *et al.* 1998). It also alters the partitioning of uterine glucose uptake in favour of the uteroplacental tissue in sheep (Ward 2003), which may limit uteroplacental PG production until the fetus is sufficiently mature to survive the transition to extrauterine life (Fowden *et al.* 1994). Because fetal cortisol levels rise later in gestation in the horse than in other species (Fowden *et al.* 1998), there is a relatively narrow window for metabolic maturation of the fetal horse, which may explain the tendency for both prematurity and neonatal hypoglycaemia in this compared to other species.

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PLACENTAL LIMITATION OF FETAL NUTRIENT SUPPLY: THE OVERNOURISHED ADOLESCENT PARADIGM

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INTRODUCTION

The adolescent pregnancy rate in the UK is the highest in Western Europe (30 births per 1,000 women aged 15–19 years). This is of concern because adolescent mothers have an increased risk of delivering premature and low birthweight babies compared with those >20 years of age. Additionally, both pre-term delivery and fetal growth restriction are associated with a greater risk of neonatal mortality and morbibity. The risk of adverse pregnancy outcome in the adolescent has been variously attributed to poor socioeconomic status, gynaecological immaturity or the growth and nutritional status of the mother at the time of conception. Gynaecological immaturity undoubtedly pre-disposes adolescent girls to poor pregnancy outcome in that the rates of spontaneous miscarriage and of very pre-term birth are highest in girls aged 13–15 years. However, maternal growth and nutritional status during pregnancy also appear to play a potentially modifiable role. Almost 50% of adolescents continue to grow while pregnant and this growth is associated with larger gestational weight gains, increased fat stores and greater post partum weight retention. Paradoxically, in spite of these maternal changes, which typically are associated with increased fetal size, the offspring are significantly smaller compared with non-growing pregnant adolescents and mature women (Scholl *et al.* 1994). This reduction in fetal growth has been attributed to competition for nutrients between the maternal body and her gravid uterus. It is axiomatic that the placenta is a major player in this paradoxical partitioning process and it was against this background that the ovine adolescent model was developed.

ADOLESCENT SHEEP PARADIGM

The experimental paradigm uses a single sire and embryo transfer techniques to establish singleton pregnancies on Day 4 of an induced oestrus cycle in peripubertal adolescent sheep (aged 7–10 months). This removes the potentially confounding influence of partial embryo loss and maximises the homogeneity of the resulting fetuses. Immediately after embryo transfer, recipient dams are offered a high or moderate quantity of a complete diet (10.2 MJ metabolisable energy and 140 g crude protein per kg dry matter) to promote rapid or low maternal growth, respectively. Thus, maternal liveweight gain during the first 100 days of the 145 day gestation ranges from 200–350 g/day in high intake compared with 50–85 g/day in moderate intake groups. Thereafter, the feed intake of the moderate intake group is adjusted weekly to maintain body condition score and to meet the increasing nutrient demands of the pregnant uterus in late pregnancy.

CHARACTERISTICS OF THE OVINE ADOLESCENT PARADIGM

Table 1 summarises pregnancy outcome data obtained following the application of these nutritional treatments throughout gestation in 8 individual studies. These studies were all initiated during the mid-breeding season using the same recipient genotype and a single sire. Within studies, the adolescents were of equivalent age, liveweight and body condition score (adiposity) at the time of embryo transfer, thus removing the confounding effect of differences in gynaecological age and pre-conception nutrition. Overnourishing adolescent dams by feeding a high

TABLE 1: Pregnancy outcome in adolescent sheep delivering live young. Adolescent ewes were offered either a high or moderate nutrient intake throughout their entire pregnancy. Values are mean ± sem (see Wallace *et al.* 2001) for original references plus J.M. Wallace, J.S., Milne and R.P. Aitken, unpublished data)

	Maternal intake		Significance
	High (n=89)	Moderate (n=75)	
Gestation length (days)	142.1 ± 0.29	145.1 ± 0.28	***
Lamb birthweight (g)	3664 ± 119	5044 ± 89	***
(range)	(1850 – 6940)	(2950 – 7050)	
Fetal placental weight (g)	322 ± 10.4	483 ± 12.7	***
(range)	(134 – 612)	(245 – 796)	
Number of fetal cotyledons	80.2 ± 1.57	92.4 ± 1.79	***
Total fetal cotyledon weight (g)	73.6 ± 2.76	135.7 ± 4.66	***
(range)	(23 – 147.4)	(61 – 262)	
Fetal: placental weight	11.7 ± 0.22	10.7 ± 0.20	**

intake throughout their entire pregnancy results in a major restriction in fetal placental mass (33%) which leads to a significant decrease in lamb birthweight relative to that for normally-growing adolescents (27%). Within both high and moderate intake groups, total placental mass and fetal weight were highly correlated. The higher fetal: placental weight ratio in the high intake dams reflects that placental growth was more perturbed than fetal growth in this group.

High maternal dietary intakes are also associated with an increased incidence of non-infectious spontaneous abortion or stillbirth in late gestation. Low or absent secretion of pregnancy-specific protein B by the binucleate cells of the placenta implies that this is preceded by severe placental insufficiency during mid-gestation (Wallace *et al.* 1997a). For ewes delivering live young, high maternal dietary intakes are also associated with a modest but highly significant reduction in gestation length. The precise endocrine changes underlying premature parturition in the overnourished adolescent have not been examined but may be initiated by nutritionally-induced alterations in placental hormone secretion (primarily progesterone). Alternatively, limitations in placental nutrient transfer resulting in fetal hypoxia and hypoglycaemia during late gestation (see below) may accelerate the maturation of the fetal hypothalamic-pituitary-adrenal axis which is central to the initiation of parturition.

Alterations in the competition for nutrients are also evident within the mammary gland in that overfeeding is associated with a major reduction in the initial yield, nutrient composition and IgG content of colostrum accumulated pre-natally (Wallace *et al.* 2001). Thus, in the absence of human intervention, many of these premature and low birthweight lambs would not survive the rigours of the neonatal period. However, with meticulous neonatal care procedures, the majority do progress to adulthood.

ENDOCRINE REGULATORS OF NUTRIENT PARTITIONING

Nutritionally-sensitive hormones of either maternal or placental origin may orchestrate nutrient partitioning at the expense of placental growth in this paradigm. In rapidly growing adolescent dams, maternal insulin, and IGF-1 concentrations are high and promote a sustained anabolic drive to maternal tissue deposition, primarily of adipose tissue (Wallace *et al.* 1997b). The elevated maternal leptin concentrations from the end of the first third of pregnancy are thought largely to reflect this increasing adiposity (Wallace *et al.* 2001). Intriguingly the adolescent animals appear to be leptin resistant in that the high intakes, once established, are maintained throughout pregnancy. Conversely, maternal concentrations of GH, progesterone and placental lactogen are low in the rapidly growing dams (Wallace *et al.* 1997a,b, 2001). Progesterone supplementation to restore maternal concentrations to control values during early pregnancy (Day 5–55) enhances fetal but not placental growth in the rapidly growing dams. This effect of progesterone is attributed to a direct effect on the inner cell mass as delaying the period of supplementation to Day 11 of pregnancy in the rapidly growing dams has no beneficial effect on lamb birthweight at term (Wallace 2000). In

TABLE 2: Summary of the key features of the overnourished adolescent paradigm compared with human intra-uterine growth restriction see Regnault *et al.* (2002) for original human references

	Ovine IUGR	Human IUGR
Placental weight	↓	↓
Fetal weight	↓	↓
Gestation length	↓	↓ →
Brain:liver weight	↑	↑
Fetal oxygenation	↓	↓
Fetal glycemia	↓	↓
Fetal lactate	↑	↑
Fetal anabolic hormones	↓	↓
Uterine blood flow	↓	?
Umbilical blood flow	↓	↓
Umbilical oxygen uptake	↓	↓
Umbilical glucose uptake	↓	?
Umbilical amino acid uptake	↓	↓

contrast, GH administration throughout the period of rapid placental proliferation reduces maternal adipose deposition and enhances uteroplacental mass as assessed at Day 81 of gestation (Wallace *et al.* 2003a). Currently we are examining whether these effects are due wholly to alterations in maternal metabolism or if they also reflect a direct effect of GH or IGF at the level of the uteroplacenta.

PLACENTAL VASCULAR BED DEVELOPMENT, BLOOD FLOWS AND NUTRIENT UPTAKES

Preliminary studies reveal that the placentae of rapidly growing dams exhibit significantly less proliferation in the fetal trophectoderm compared with moderately growing controls at Day 81 of gestation, the apex of placental growth (Lea *et al.* 2003). Similarly, quantitative real-time RT-PCR determination of placental angiogenic growth factor mRNA expression has revealed that the expression of a range of genes, including vascular endothelial growth factor (VEGF) is markedly attenuated in the placentae of rapidly growing dams at this mid-gestation timepoint (Redmer *et al.* 2003). These changes occur before differences in placental wet weight are apparent but they clearly impact on the subsequent growth and vascularity of the placenta.

By late pregnancy, placentome mass in the rapidly growing versus the control dams is reduced by ~45%. While no major change in the allometric coefficients of all the major fetal organs has been detected at this stage of pregnancy, there is evidence of brain sparing in that the brain:liver weight ratio is significantly higher in the growth restricted compared with the normally growing fetuses. The growth restricted fetuses are relatively hypoxic and hypoglycemic, and have low anabolic hormone concentrations, while lactate and urea levels are high (Wallace *et al.* 2000, 2002a). Chronically catheterised animals have been used to measure uteroplacental blood flows, transplacental nutrient uptakes and metabolism in these pregnancies at ~Day 130 of gestation. The growth restricted pregnancies are associated with major reductions in absolute uterine and umbilical blood flows leading to attenuated fetal oxygen, glucose and amino-acid uptakes (Wallace *et al.* 2002a, 2003b). Uteroplacental metabolism is reduced absolutely in proportion to the reduction in placental mass but is normal when expressed on a placental weight specific basis. In addition, glucose clamp procedures at 3 maternal-fetal glucose concentration gradients reveal that absolute placental glucose transport capacity is markedly reduced in the rapidly growing dams but is normal when expressed on a weight specific placental basis (Wallace *et al.* 2002b). Similarly, placental GLUT 1 and GLUT 3 mRNA expression is independent of maternal growth and nutritional status. Thus it is the small size of the placenta *per se* rather than alterations in its nutrient metabolism or transfer capacity which is the major limitation to fetal growth in the rapidly growing adolescent.

Inadequate growth and vascularisation of the placenta is clearly the primary cause of fetal growth restriction in the rapidly growing adolescent sheep. Future studies will continue to unravel the underlying nutritionally-mediated mechanisms and how they impact on transplacental nutrient exchange, fetal growth and metabolism, and post natal well-being. Information obtained from this highly controlled ovine paradigm is clearly relevant to the clinical management of human adolescent pregnancies. In addition, our paradigm provides a robust model of placental growth restriction which replicates many of the key features of human intra-uterine growth restriction *per se* (Table 2).

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COMPARATIVE ASPECTS OF PLACENTAL GLUCOSE TRANSPORTERS: IS THE LOCALISATION OF THE EQUINE ISOFORMS UNIQUE?

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Glucose is one of the major nutrients for all mammalian fetuses and the mother's blood the only primary source. Diffusion across the membrane barriers in the placenta is slow but can be speeded up to 10,000 times by the presence in the membranes of glucose transporter proteins (Barrett *et al.* 1999). These move the glucose by facilitated diffusion requiring no energy but a concentration gradient higher in the maternal than the fetal circulation; invariably the case in a normal pregnancy. So far they are the only type of glucose transporter found in the placenta.

These glucose transporters are members of an enormous superfamily of transporter molecules, comprising a significant part of the total genome, all of which show a similar molecular structure with 12 membrane spanning helices which, together, form a central pore through the membrane (Zuniga *et al.* 2001).

There are several different isoforms which transport glucose, fructose and other polyols (Joost and Thorens 2001). The expression of the various isoforms is under a variety of hormonal, nutritional and developmental controls. So far, 12 isoforms have been identified, with characteristic distributions in various tissues, but currently only glucose transporters 1 (GT1) and 3 (GT3) have been found in the placenta. Northern and Western blotting are among the methods used to demonstrate their presence but immuno-cytochemistry is necessary to show exactly where the different isoforms are located on the membrane barriers separating maternal and fetal blood. The author uses gold label immunocytochemistry with silver intensification on K4M acrylic sections to locate the transporters, allowing an inferred localisation on the light microscope to be verified conclusively on electron microscope sections from the same block of tissue.

All mammalian placentae so far investigated show GT1 and the simplest arrangement is to have this isoform on both maternal and fetal surfaces of the single layered trophoblast as in the haemochorial human and primate system (reviewed in Illsley 2000). There has been considerable controversy over the presence of GT3 in the human placenta. The current consensus (Illsley 2000) seems to be that GT3 is only expressed on the endothelium of the larger arteries of the villi, at some distance from the syncytiotrophoblast exchange site.

In the endothelial carnivore placenta a similar distribution of GT1 (and an absence of GT3) with GT1 on the apical and basal membrane surfaces of the single layered syncytiotrophoblast is found. There is also a major GT1 presence on the fetal capillary endothelium which is closely adjacent to the basal syncytiotrophoblast membrane; but none on the maternal capillaries.

Other orders express both GT1 and GT3 on the placental barrier layers as in the triple layered rodent placenta. The outermost layer (Layer I) expresses neither, but has large discontinuities so that maternal blood bathes the surface of Layer II which carries both GT1 and GT3. This layer is linked by numerous gap junctions through which the glucose can easily pass from Layer II to Layer III and then to the basal plasma membrane of Layer III, which expresses only GT1. Glucose can therefore cross the placental layers either using GT1 alone, or GT3 then GT1 (Shin *et al.* 1997). Because GT3 works more efficiently at lower glucose concentrations than GT1, best use of the physiological range of glucose concentrations found in maternal blood is facilitated.

The horse also has GT1 and GT3, but on different layers of its multi-layered epitheliochorial placenta; GT1 on basolateral

surfaces of the trophoblast and uterine epithelia, but only GT3 on the interdigitated apical surfaces of those 2 epithelia which form the microvillar junction (Wooding *et al.* 2000). GT1 is also highly expressed on the fetal, but not the maternal, placental capillaries, similar to the carnivore. This pattern of GT1 and GT3 distribution is found from the earliest development of the microcotyledons and persists to term (Wooding *et al.* 2000). The microvillar junction consists of apposed maternal and fetal membranes. Where these have been artifactually separated during processing, it is clear that both express GT3, but there is more on the fetal than the maternal membrane.

Author: 2001 in ref list

In the multi-layered synepitheliochorial ovine placenta, Das *et al.* (2000) have shown the presence of both GT1and GT3. The author used a LM/EM technique to establish in bovine and ovine placentomes that there is a similar distribution to the horse, with GT1 basolateral on the trophoblast and uterine epithelium or derivative, but only GT3 on the microvillar junction between the maternal and fetal layers. There is no indication of localisation of either on the 2 capillary beds. Where the microvillar junction is separated, there seems to be an even distribution of GT3 on either side in the bovine, whereas the ovine definitely shows far more on the fetal side, as in the horse.

In the multi-layered epitheliochorial pig and camelid placentae, GT1 is expressed basolaterally on both trophoblast and uterine epithelium as in the horse and ruminants, with no localisation to the microvillar junction. The GT3 antibodies are much more species specific than the GT1, and none of the GT3 antibodies used give any localisation to pig or camel placenta. However, the 45 oligomer *in situ* hybridisation probe for GT3, which works well on ruminant placentomes and horse microcotyledons, also reacts strongly with pig trophoblast. It therefore seems possible that GT3 is present on the microvillar junction but the specific antibody to demonstrate this is not available.

In such multi-layered placentae, each glucose molecule therefore has to use GT1 and GT3 in sequence, and the overall rate will always be of the slowest transporter, not the ideal system for maximising maternal to fetal transfer with varying maternal glucose availability.

The results detailed above suggest a possible correlation between multi-layered placentae and use of the sequential GT1 to GT3 arrangement. However, Allen *et al.* (2002) have recently been investigating the unique endotheliochorial placenta of the elephant, and immuno-cytochemical results show a GT1 and GT3 expression from implantation to term. In the elephant, the trophoblast is a single cellular layer throughout gestation (unlike the syncytium in the similarly endotheliochorial carnivores) with GT1 present basolaterally and GT3 exclusively on the apical surface facing the maternal capillaries. However, it is similar to the endotheliochorial carnivores in showing a high level of GT1 on the fetal but not the maternal capillary endothelium.

A possible explanation for the sequential GT1/GT3 system in the placenta is that it may increase glucose transit time and enable the placental tissues to retain enough for local use, to support the high level of placental metabolism including synthesis and functioning of the many other transporters necessary for a successful pregnancy.

For sufficient glucose transport the amount of transporter protein is obviously as important as the location. In man, analysis shows that the apical syncytio trophoblast membrane has much more than the basal throughout pregnancy but the total levels vary little during normal pregnancy (Illsley 2000). In rodents and sheep on the other hand, the GT3 isoform increases as pregnancy progresses whereas the levels of GT1 decrease before term (Zhou and Bondy 1993; Currie *et al.* 1997; Ehrhardt and Bell 1997; Shin *et al.* 1997). Hahn *et al.* (1999) have shown that in human full term trophoblast cells in culture, and in rodent placenta *in vivo*, the levels of GT1and GT3 can be decreased by glucocorticoid administration and Das *et al.* (1999) showed that levels of ovine GT1 and 3 *in vivo* can be affected by circulating glucose and insulin concentrations.

Author: 2000 in ref list

From these and other studies, it seems that control of transporter proteins is undoubtedly multifactorial but there is no evidence so far to suggest that changes in the localisation of GT1 and 3 occur during pregnancy. The variety of patterns in the different orders and suborders is specific and established very early in placental development. Later in gestation, it seems to be a question of fine tuning the various systems to maintain optimal glucose transport to both placenta and fetus. The strange glucose transporter localisation in the horse is therefore not unique but it does highlight once again the remarkable variety of placental stucture and function.

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Author: 2000 in text

SESSION VI:

Chairman:

R. Pijnenborg

TOTAL FEEDING FAILURES IN THE EQUINE FETUS

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The products of the myriads of exocrine secretory glands that are uniformly and densely distributed throughout the endometrial stroma of the mare's uterus ('uterine milk') are vital for the sustenance of the unimplanted embryo during the first 40 days of gestation (Amoroso 1952; van Niekerk and Allen 1975). Furthermore, they remain an important histotrophic component of the total nutrition of the fetus throughout the remainder of gestation (Steven and Samuel 1975). A stable microvillus attachment of the trophoblast cells of the allantochorion to the lumenal epithelial cells of the endometrium is established at Day 40-42 after ovulation. During the next 100 days of gestation, the allantochorion extends steadily to occupy the entire uterine lumen while an increasingly branched and complex interdigitation between the allantochorion and endometrium gives rise, by mid-gestation, to the mature microcotyledons (Fig 1) that cover the entire allantochorion. These provide the essential haemotrophic nutritional, gaseous and waste product exchanges between fetus and mother throughout the remainder of gestation (Samuel *et al.* 1974; Macdonald *et al.* 2000).

The lumenal and glandular epithelia in the mare secrete a range of other important pregnancy-associated molecules which are clearly vital for embryonic, fetal and placental growth. These include: uteroferrin, required for the transport of iron to the fetus (McDowell *et al.* 1982); uterocalin, the 19 KDa carrier protein which accumulates in large quantities on the surface of, and becomes incorporated into the structure of, the unique equine blastocyst capsule between Days 7 and 23 after ovulation (Stewart *et al.* 1995), and is concerned with the transport of essential vitamins and minerals to the fetus (Stewart *et al.* 2000); and the mitogenic growth factor, epidermal growth factor (EGF), the production of which by only the apical portions of the endometrial glands is hugely upregulated

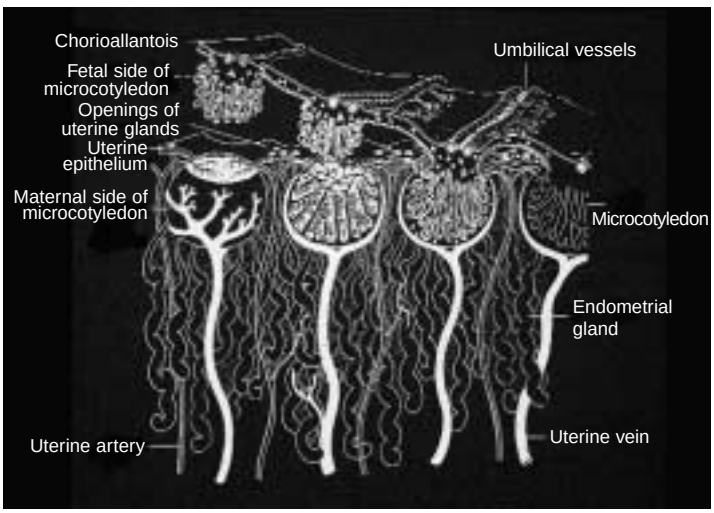

Fig 1: Microscopic architecture of the microcotyledonary placental interface in the mare (from Steven and Samuel 1975).

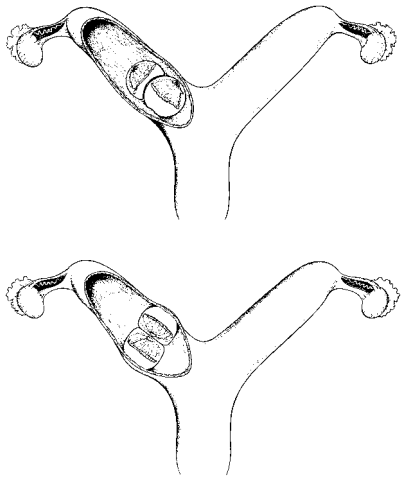

Fig 2: Abutment of the absorptive bilaminar choriovitelline membrane of one unicornuate twin conceptus to its co-twin conceptus instead of the endometrium (from Allen 2001).

between Days 35 and 40 of gestation (Stewart *et al.* 1994; Gerstenberg *et al.* 1999).

The onset of secretion of all 3 of these proteins is stimulated by progesterone alone and it is puzzling that uterocalin production declines markedly after Day 23, despite the continued dominance of progesterone beyond this time. Conversely, the marked upregulation in EGF secretion that occurs around Day 35–40 in the pregnant mare can only be mimicked in the anoestrous or ovariectomised mare by the continuous administration of exogenous progesterone for as long as 35 days (Gerstenberg *et al.* 1999).

Restriction of either the production or uptake of 'uterine milk' in early pregnancy, or the development of an inadequate total area of functional microcotyledons on the surface of the allantochorion in later gestation, will have deleterious effects on embryonic and/or fetal growth which, if sufficiently severe, may cause fetal death and abortion. Common examples of such nutritional restriction include:

i) spontaneous embryonic death and resorption of one of unicornuate twin conceptuses due to the abutment of the absorptive choriovitelline portion of the conceptus membranes to those of its co-twin, rather than to the milk-producing endometrium (Fig 2);

ii) abortion of twin conceptuses around 7–9 months of gestation due to competition between the 2 allantochorions to interdigitate with the limited area of endometrium available, leading to starvation and death of the more disadvantaged of the 2 fetuses (Jeffcott and Whitwell 1973);

iii) age-related degenerative changes in the mare's endometrium which result in the laying down of fibrous tissue around groups of endometrial glands to form functionless 'gland nests'. These cause stasis of lymph drainage leading to development of lymph-filled endometrial cysts and stromal lacunae (Ricketts 1975; Kenney and Doig 1986). This patchy degeneration in the endometrium is reflected in the opposing allantochorion where fewer and less well developed microcotyledons, together with inadequate numbers of healthy and productive endometrial glands, cause a

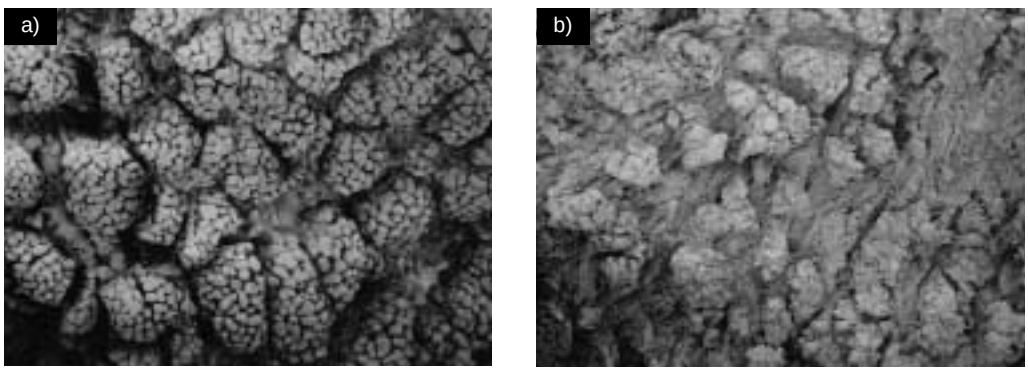

Fig 3: Scanning electron micrograph of the maternal surface of the allantochorion at 120 days of gestation in: a) a young fertile mare with a healthy endometrium; and b) an aged mare suffering severe degenerative endometrial changes. Note the poor development and reduced numbers of microcotyledons (from Bracher et al. *1996).*

serious reduction in the level of nutrition available to the fetus (Fig 3; Bracher *et al.* 1996). Depending upon the severity of these degenerative changes, either fetal death and abortion will occur or the foal will be born runted many days or weeks after full term.

One other form of nutritional inadequacy that results in early fetal death is observed in the donkey-in-horse model of extraspecific equine gestation created by embryo transfer (Allen 1982). In around 70% of such pregnancies, the xenogeneic donkey fetus appears to develop normally to around Day 60–65 after ovulation but, over the next 15–25 days, becomes increasingly thin and cachetic in appearance before it finally dies and is aborted between Days 80 and 95 (Allen *et al.* 1987). Histological examination of the fetomaternal interface reveals partial or complete failure of the donkey allantochorion to interdigitate with the surrogate horse endometrium after Day 40, which may, in turn, be related to the complete failure of the donkey chorionic girdle to invade the surrogate horse endometrium around Day 36–38 to form endometrial cups. Despite dense accumulations of lymphocytes and other immune cells in the endometrium opposed to the poorly attached xenogeneic allantochorion, and a definite indication of immune memory in mares carrying a transferred donkey conceptus in repeated pregnancies (Allen *et al.* 1987), experiments have indicated that unidentifiable genetic differences may be more significant than adverse immunological responses at the maternofetal interface in the aetiology of this model of equine pregnancy failure (Allen and Antczak 2000).

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THE EFFECTS OF MATERNAL NUTRITION ON PLACENTAL AND FETAL DEVELOPMENT IN MAIDEN THOROUGHBRED MARES

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Using stereological techniques, Wilsher and Allen (2003) demonstrated that the age and parity of Thoroughbred mares have a profound effect on the development of microcotyledons on the surface of their placenta. A reduction in the surface area, or plexiform nature, of the microcotyledons was apparent, not only in older mares with age-related degenerative changes in the opposing endometrium, but also in young primigravid mares. The resulting curtailment of fetomaternal contact in these maiden mares resulted in lower birthweights compared to younger multiparous mares. Experiments described by Wallace *et al.* (2001) indicated that maternal overnutrition compared to maintenance nutrition in the pregnant adolescent sheep resulted in nutrient partitioning in favour of the ewe at the expense of the growing fetus which was facilitated by a major reduction in placental mass. The present study investigated whether the common practice on commercial Thoroughbred stud farms of dramatically changing the body condition score of 3-year-old fillies from that of a fit, lean racehorse to a well-rounded broodmare might mirror the adolescent sheep model. Namely, the combination of immaturity and overnutrition would underlie the observed reductions in microcotyledon surface density and foal birthweight in primigravid versus young multiparous mares.

Twenty 3- and 4-year-old Thoroughbred fillies, recently retired from racing, were maintained at a moderate body condition score until they were impregnated by a single Thoroughbred stallion. When pregnancy was diagnosed ultrasonographically at 14 days post ovulation, the mares were assigned randomly to be overfed or maintenance fed for the remainder of gestation. Weekly jugular blood samples were collected for measurement of progestagen, chorionic gonadotrophin (eCG), insulin, leptin and IGF-I profiles. Placentae were recovered at spontaneous third stage labour and measured grossly and stereologically. Foal birthweights were recorded and Ponderal indices calculated. During the course of the experiment both groups of fillies became infected with *Streptococcus equi* resulting in the disease known as 'strangles'. The infection caused dramatic weight loss in both groups, in effect imposing a nutritional insult at varying times between 90 and 150 days of gestation.

At the time of writing, 8 maintenance fed and 11 overfed fillies have foaled. Most hormone assays are still being undertaken, but significant increases in eCG production have been revealed already in the maintenance fed animals ($P<0.001$).

During gestation, weight increase differed significantly between the 2 groups, when expressed as either liveweight gain (97.1 ± 6.4 vs 135.5 ± 4.1 kg, $P<0.001$) or as a percentage increase of bodyweight from the time of conception (20.4 ± 1.3 vs. $27.2 \pm 0.7\%$, $P<0.001$). Mean gestation length was longer in the maintenance group (342 ± 2.9 vs. 333 ± 2.6 days). Placental area, weight and volume did not differ significantly between the groups. However, placental weight was correlated with foal birthweight ($r=0.7$, $P=0.001$). Mean foal birthweight did not vary significantly between the maintenance and overfed groups (44.3 ± 1.4 vs 45.8 ± 1.1 kg), nor did body composition of the foals, judged on Ponderal indices. Weight loss, inflicted by the *Strep. equi* infection, was correlated to the weight and volume of the placenta with increased weight loss resulting in a reduced placental mass and volume ($r=0.43$, $P=0.039$; $r=0.54$, $P=0.01$, respectively). Conversely, placental efficiency (kg

foal birthweight per kg of allantochorion) was enhanced by reductions in weight.

Thus, although it appears that 3- and 4-year-old Thoroughbred fillies may not exhibit the same degree of biological immaturity as the adolescent sheep studied by Wallace *et al.* (2001), and hence failed to demonstrate equivalent partitioning of nutrients, the results have been confounded by the nutritional insult caused by the *Strep. equi* infection. However, this study has shown the effects of a nutritional insult around 90 to 150 days of gestation, coinciding with the rapid proliferative growth of the placenta. Namely, enhanced placental efficiency coupled with decreases in placental mass and volume.

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THE EFFECTS OF INTRA-UTERINE DISTURBANCES ON EQUINE FETAL GROWTH AND ORGAN DEVELOPMENT

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INTRODUCTION

The horse is a precocious species which reaches an advanced state of development by full term gestation (320–365 days). Healthy foals born at term show physical and functional maturity of their skeleto-muscular and visceral organ systems and rapidly adapt to extra-uterine life. In contrast, foals born prematurely (before 320 days), show clinical signs of immaturity, for example, skeletal underdevelopment, hypoglycaemia, respiratory distress syndrome, adrenocortical hypofunction, renal dysfunction and neonatal maladjustment syndrome (Rossdale *et al.* 1984). Furthermore, these foals are often described as having a low body weight although they may be, in fact, appropriately sized for their gestational age. There are many clinical conditions which can disrupt fetal growth and development during gestation. The most common causes are infectious agents or vascular compromises of the placenta or umbilical cord which tend to result in fetal abortion during the second half of pregnancy when fetal growth and nutritional/oxygen demands on the placenta are increasing (Platt 1978; Whitwell 1987; Giles *et al.* 1993). In the Thoroughbred (TB) breeding industry, chronic undernutrition of the mare is rarely encountered, although acute starvation, for example from disease, increases the risk of fetal expulsion due to uterine prostaglandin release (Silver and Fowden 1982). Fetal undernutrition has not been well studied in horses except in twin pregnancies which are a naturally occurring model of intra-uterine growth retardation (IUGR). They compete for placental blood supply and, invariably, are aborted when the placenta can no longer sustain them at around 7–9 months of gestation. Singleton fetuses with low body weight and an impoverished appearance, described as IUGR, occur in conjunction with placental problems, for example, placentitis. Most IUGR fetuses die *in utero* but some show a remarkable ability to survive and appear clinically mature even when born many weeks before full term, suggesting they have achieved physical and functional maturity. The pathology of IUGR and other intra-uterine conditions has been described previously in equine fetuses (Platt 1978; Whitwell 1987) but the effects of such conditions on fetal organ development is not clear.

In order to understand more fully how intra-uterine disturbances affect fetal development, the technique of stereology was used to provide quantitative, 3-dimensional estimates of microstructural components of organs and tissues, and a greater insight into organ function than that obtained using conventional histological techniques (Howard and Reed 1998). This technique has been used to identify microstructural deficiencies in organs from infants with IUGR and Sudden Infant Death Syndrome and in piglets and lambs with IUGR (Hinchcliffe *et al.* 1992; Ansari *et al.* 1995; Sibbons *et al.* 1996; Beech *et al.* 2000, 2001a). The aim of this study was to determine whether perturbations in placental or umbilical cord function would alter fetal organ development as determined by stereological techniques.

MATERIALS AND METHODS

Data were collected from 45 TB fetuses which died *in utero* or were aborted spontaneously

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between 110 and 356 days gestation. They were sub-divided according to primary cause of death:- placental pathology (Group 1, n=13); low birth weight (LBW) or twins (Group 2, n=7); cord abnormalities (Group 3, n=9); or controls (Group 4, n=16). Placental pathology included placentitis, placental haemorrhage/oedema/thickening or premature separation, amnion thickened or destroyed. Damage to the umbilical cord included constriction, haemorrhage, bruising, excessively long (> 80 cm) or short (< 40 cm) cords. Low birth weight fetuses had placentitis, were emaciated and were more than 2 sd below the mean body weight (bwt) for controls at a given gestational age. Controls fetuses were from mares subjected to euthanasia due to colic, laminitis, orthopaedic accidents or dysautonomia. At post mortem, the fetus was weighed and the left lung, left kidney, left adrenal and brain were collected, weighed and stored in 10% neutral buffered formalin. Organ development was assessed using stereological techniques as described by Beech *et al.* (2001b).

RESULTS

Fetal body weight increased exponentially in control fetuses with greatest growth during the last trimester (>250 days). Group 2 fetuses had lower body weights than controls after approximately 180 days. Some Group 1 and 3 fetuses had low body weights particularly towards term. Because of differences in body weight, stereological data have been expressed per kg body weight. Lung and kidney weights were in proportion to body weight throughout gestation in Group 4 (control) fetuses and in most Group 1 and 3 fetuses. Group 2 (LBW) fetuses had proportionally higher lung weights than controls. Kidney weight, when considered in relation to fetal body weight rather than gestational age, was greater in abnormal fetuses compared with controls. Adrenal weights were proportional to body weight throughout gestation but were higher in the majority of abnormal fetuses compared with controls. Brain weight was proportional to body weight in all groups but was substantially higher in one twin fetus.

Stereological analyses showed that, in control fetuses, lung volume decreased in proportion to body weight with increasing gestational age whereas gas exchange surface area (GESA)/kg bwt continued to increase with gestational age. Lung volumes, terminal bronchiolar duct ending (TBDE) numbers and GESA were proportionally reduced in some Group 1 and 3 fetuses compared with controls, particularly at term. Two Group 2 fetuses had increased lung volumes compared with controls but GESA and TBDE are still to be determined. Lungs from all fetuses aged <180 days gestation were immature but some older (>300 days) fetuses, including controls, also had immature lungs. The kidney volumes/kg bwt (total, medulla, cortex) were comparable between groups except 2 Group 1 individuals with lower kidney medulla volumes than controls. Glomerular number/kg bwt was reduced in some Group 3 fetuses compared with controls at term. Adrenal volumes/kg bwt were relatively constant in Group 4 (control) fetuses throughout gestation. Adrenal volumes (total and cortex) were elevated in the majority of abnormal fetuses at all gestational ages.

DISCUSSION AND CONCLUSIONS

These data describe in detail the development of microstructural components in various organs from apparently normal and abnormal fetuses throughout gestation. The development of the fetal lung in control TB fetuses is similar to that described by Beech *et al.* (2001b). The apparent immaturity of some control fetal lungs at term may be due to single or groups of intermittent placental compromise at a time of particularly active lung organogenesis. Lung microstructure was compromised in some full term fetuses with pathology of the cord or placenta implying that these conditions may disrupt pulmonary function; certainly respiratory problems are common in sick newborn foals (Dwyer 1998). In contrast, lung volume per kg bwt was increased in the 2 LBW/twin fetuses analysed to date. If substantiated, these data may help to explain why some fetuses with low body weights are able to survive when born before full term.

The development of the fetal kidney did not appear to be compromised in the majority of abnormal fetuses. Some Group 3 (cord) fetuses had reduced glomerular numbers/kg bwt and this may have been related to altered haemodynamics within the umbilical cord. In infants and lambs suffering from IUGR, glomerular numbers are reduced (Hinchcliffe *et al.* 1992; Bains *et al.* 1996; Beech *et al.* 2000). However, there was no evidence of this in the 2 LBW equine fetuses from the present study or previously (Holdstock *et al.* 2000).

The adrenal gland weight and volume (total, cortex, medulla)/kg bwt was lower in controls than abnormal fetuses. Adrenal weight in controls >300 days was similar (≈ 60 mg/kg) to that described previously for equine fetuses prior to the pre-partum surge in fetal cortisol (Fowden and Silver 1995). The higher adrenocortical volumes in the majority of abnormal fetuses imply a degree of intra-uterine stress; several fetuses with cord pathology were meconium stained which is considered pathognomonic of fetal stress. The highest adrenocortical volume was in a 6.8 kg twin fetus (269 days) which clearly was stressed *in utero;* its sibling had died some time previously because its tissues were autolysed. The brain weight of this fetus was also high compared with controls indicating that brain growth was maintained at the expense of body mass. Brain weight in other abnormal fetuses, which were not severely growth retarded, was proportional to body weight.

These data provide an insight into how pathology of the placental or umbilical cord adversely affects growth and microstructural development of various organs in the TB fetus. The results suggest that lung function, in particular, may be affected and that many fetuses are stressed *in utero*. These data provide information about the potential functional deficits which may manifest themselves during post natal life and may have important implications for the care of sick neonatal foals.

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SESSION VII:

Chairman:

R. H. F. Hunter

ROLE OF THE IGF SYSTEM IN MEDIATING NUTRITIONAL EFFECTS ON PLACENTAL AND FETAL DEVELOPMENT

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INTRODUCTION

The intra-uterine environment, in particular the nutrient supply, is a major determinant of fetal growth. A compromised supply will result in low birthweight animals, which suffer an increased risk of perinatal mortality. Post natal development may also be affected adversely in a number of ways leading, for example, to an increased risk of diabetes in man (Barker 1998) and reduced reproductive performance in sheep (Rhind *et al.* 2001). Many studies have implicated the insulin-insulin like growth factor (IGF) axis in the regulation of nutrient delivery. Maternal insulin and IGF-I concentrations are both affected by the maternal nutrient status and both play a role in partitioning the supply of nutrients between the mother and the fetoplacental unit (Kniss *et al.* 1994; Liu *et al.* 1994).

NUTRITIONAL EFFECTS OF THE INSULIN-IGF SYSTEM IN THE MOTHER AND FETUS

Nutrients provided from the maternal compartment to the fetoplacental unit are derived either from the products of digestion or as mobilised body reserves. The fetus also develops its own endogenous reserves and endocrine system, which can respond to nutritional stimuli (Fowden 1997). The relative contributions of maternal nutrient supply and body condition was investigated in Welsh mountain ewes carrying singleton pregnancies. In the first study (Osgerby *et al.* 2002), ewes with a standard body condition score (BCS) of 2.5 at mating were placed on rations which provided either 100% or 70% of their calculated maintenance requirements throughout gestation. Ewes on the 70% ration had lower glucose concentrations throughout pregnancy. In contrast, in the first half of gestation, both insulin and IGF-I levels tended to be slightly higher. In later gestation, as the nutrient requirements of the fetus were increasing, this trend was reversed and metabolic hormone concentrations were lower (Fig 1).

In a second study (Osgerby *et al.* 2003a) the BCS was adjusted well before mating so that ewes were of either high (3.5) or low (2.0) BCS at service. Animals in each group were then fed either the 100% or 70% ration. Tissues were collected on Day 65 of gestation. In the first half of pregnancy both insulin and IGF-I were significantly higher in the high BCS ewes, but ration had no effect.

Measurements in the fetal circulation in both experiments were broadly in line with maternal values. In the first experiment, IGF-I, insulin and glucose were all reduced at Day 135 of gestation in fetuses of mothers on the restricted rations. Dietary restriction and/or low BCS also reduced fetal glucose levels at Days 65 and 90.

In summary, BCS had the greatest influence on maternal metabolic hormone concentrations in early pregnancy, but the ration became limiting in the last trimester of gestation. Maternal reductions in glucose concentrations were reflected in lower circulating glucose values in the fetus.

RELATIONSHIP BETWEEN MATERNAL METABOLIC HORMONE CONCENTRATIONS AND PLACENTAL DEVELOPMENT

Placental size is a major determinant of fetal size, and a small placenta will limit fetal growth in late gestation. Nutritional modulations clearly influence placental development in the ewe. The precise effects are, however, influenced by the extent and timing of the dietary change, maternal BCS at mating and age and breed of the mother. In the 2 experiments described here, placental weight

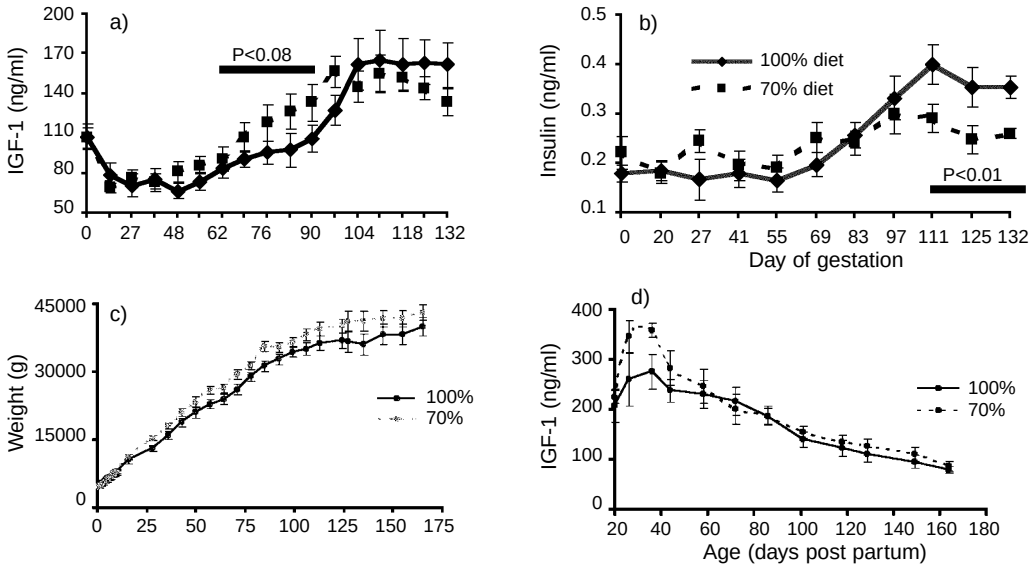

Fig 1: Changes in metabolic hormone concentrations and growth rate associated with mild undernutrition during pregnancy in the Welsh Mountain ewe. Ewes were placed on rations calculated to provide 100% (solid line) or 70% (dashed line) maintenance requirements from Day 22 of gestation. The graphs illustrate maternal plasma concentrations of: a) IGF-I; b) insulin during pregnancy; c) lamb weight; and (d) lamb IGF-I concentrations post natally. In the ewe, both IGF-I and insulin tended to be higher in mid gestation on the restricted diet, but then switched to becoming lower in the last third of pregnancy. Lambs were born non-significantly lighter, but showed a significant increase in weight from Days 16 to 132. This was accompanied by raised IGF-I concentrations in the first 2 months of life. Data from Osgerby et al. *(2002) and unpublished.*

was maintained between Days 90–135 on the 100% ration, but decreased over this period on the 70% ration. Mean placentome weight was higher in low BCS ewes on Day 65. These results are in agreement with other studies (eg Heasman *et al.* 1998) in showing that maternal nutrient restriction during the period of rapid placental development can stimulate placental growth. Later in pregnancy, however, the placental tissue may be mobilised if nutrient supply becomes limiting, thus reducing placental mass.

The IGF system within the ovine placenta has been studied to investigate possible mechanisms whereby it might regulate placental growth. Gene deletion studies in mice have established that placentally derived IGF-II is a major determinant of placental size (Baker *et al.* 1993; Constancia *et al.* 2002). However, Osgerby *et al.* (2003a,b) failed to establish any positive relationship between placental IGF-II expression and placental weight. Localisation studies using *in situ* hybridisation have shown that a variety of IGF binding proteins are expressed on the maternal side of the placenta with IGFBP-2, -3 and -6 present in both the caruncular stroma of the maternal villi and the surrounding placentome capsule (Fig 2). The

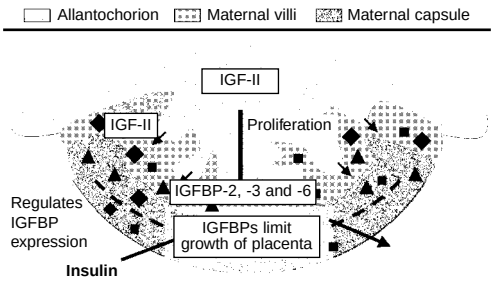

Fig 2: The suggested mechanism whereby nutritional changes can regulate growth of the ovine placentome. Maternal insulin concentrations alter in response to maternal body condition, age and diet. Insulin and other metabolic factors can regulate expression of IGFBP-2 and -3, which are present in both the maternal villi and in the surrounding placentome capsule. These binding proteins may limit the proliferative action of IGF-II. IGF-II mRNA is highly expressed in the fetal villi and to a lesser extent on the maternal side of the placenta but its concentration does not generally vary in relation to nutritional status. Data from Osgerby et al. *(2003a,b).*

expression of these IGFBPs can alter in response to maternal nutrient status. At Day 65, the period of maximum proliferation, IGFBP-2 and -3 mRNA concentrations were consistently highest in the

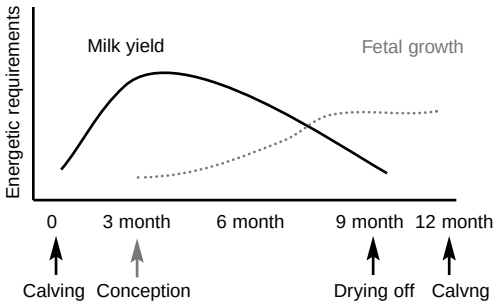

Fig 3: Energetic requirements of the dairy cow for milk production and fetal development. Milk yield peaks at about 2–3 months at approximately the same time that the cow is expected to conceive again. During the early stages of embryo and fetal development nutrients may be preferentially partitioned towards the mammary gland, thus reducing nutrient availability for the conceptus.

high BCS ewes fed the 100% ration which had the lowest mean placentome weight. Regression analysis revealed negative relationships between placental weight and both maternal insulin and placental IGFBP-3 mRNA concentrations, whereas there was a positive relationship between maternal insulin and IGFBP-3.

These data suggest that high insulin concentrations stimulate IGFBP-3 expression in the maternal component of the placenta and this then limits the proliferative action of IGF-II. This would accord with the observation that placental growth is restricted in well nourished animals. It should also be noted that there is nutritional control of IGF binding protein expression in the intercotyledonary region of the endometrium (Osgerby *et al.* 2003a,b). In this region IGFBP-3, IGFBP-5 and uterine milk protein (UTMP) mRNAs are all expressed in the glandular and luminal epithelium. There was a negative relationship between glandular UTMP expression and both maternal weight and insulin levels. IGFBP-3 and -5 expression in the glands was positively correlated with maternal weight, so that lean ewes expressed lower concentrations than fat ewes. The authors believe that, in this region, maternally derived IGF-I influences the secretory activity of the endometrial glands and that this represents another mechanism whereby maternal metabolic status can influence nutrient supply to the fetus. Glandular secretion may be up-regulated when the ewe is in poor nutritional status. This is likely to be particularly important in early pregnancy, before the placentomes are fully developed.

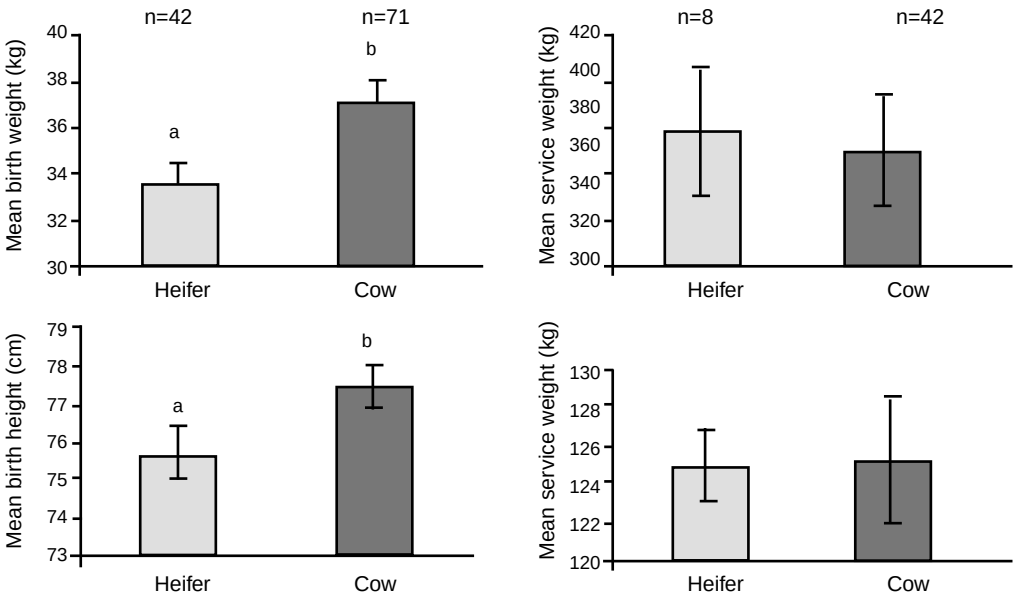

Fig 4: The female offspring of heifers (cows which are calving for the first time) are born significantly lighter and shorter than the offspring of multiparous cows (which were lactating during most of their pregnancy). The heifer offspring then show catch-up growth and so reach an equivalent size at 15 months of age when they are served for the first time. a<b P<0.05 A. Swali, D.E. Beever and D.C. Wathes, unpublished observations.

POST NATAL EFFECTS ON THE INSULIN-IGF SYSTEM

In our sheep experiments, lambs on the 70% restricted ration tended to be slightly lighter at Day 135 and at birth, but this did not achieve statistical significance. At Day 135 a wide variety of organs were, however, growth restricted, including the gut, thymus, heart, kidneys and pancreas (Osgerby *et al.* 2002). Post natally the growth rate of the restricted offspring was increased over the first 5 months, indicative of catch up growth. This was associated with a temporary increase in circulating IGF-I in the first 2 months of life (Fig 1).

More recently, the insulin-IGF system in dairy calves has been investigated. Cows selected for increased milk yield partition nutrients preferentially into milk. The dams are expected to conceive again in early lactation, when both IGF-I and insulin concentrations are reduced (Wathes *et al.* 2003). It is hypothesised that this may limit the nutrient supply to the fetus (Fig 3). Results to date have shown that the offspring of cows pregnant for the first time are born smaller. These calves were developing *in utero* while their mothers were still growing but not lactating. Such calves then exhibited post natal 'catch-up' growth (Fig 4), which was again associated with an increased concentration of IGF-I, measured at 6 months of age. In contrast, small offspring of multiparous dams did not show catch up growth and had lower IGF-I than their larger/heavier counterparts. These calves would have developed *in utero* while their dams were lactating.

These studies both support the evidence obtained from previous work (reviewed by Barker 1998) that the post natal growth axis can be altered by the maternal uterine environment. In dairy cows, this may have important consequences for the way in which the individual cow will later respond to the metabolic stress of lactation, thus potentially influencing both milk yield and fertility.

ACKNOWLEDGEMENTS

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IS LEPTIN A NUTRITIONAL SIGNAL IN THE FETUS?

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In adult animals, leptin is a polypeptide hormone synthesised and secreted primarily by adipose tissue. At least 6 isoforms of the leptin receptor (Ob-Ra to Ob-Rf) are found in a variety of tissues, including the hypothalamus; Ob-Rb is the signaling, long-form of the leptin receptor (Ahima and Flier 2000). Leptin has an important role in the control of appetite and energy expenditure, and therefore, in the coordination of metabolism with nutrient availability (Ahima and Flier 2000). Leptin has been detected in the fetal circulation and leptin receptors are present in the placenta and many fetal tissues. However, its function in the fetus is poorly understood.

EXPRESSION OF LEPTIN AND LEPTIN RECEPTORS IN THE FETUS

Leptin has been measured in the circulation of human, ovine and porcine fetuses from mid-gestation (Jaquet *et al.* 1998; Chen *et al.* 2000; Forhead *et al.* 2002). Plasma leptin concentration and adipose leptin mRNA abundance increase towards term in the ovine fetus (Yuen *et al.* 1999; Forhead *et al.* 2002) which suggests that fetal adipose tissue is a major source of circulating leptin *in utero.* However, plasma leptin may also originate from a range of other fetal and placental tissues. Table 1 shows the widespread distribution of the genes and proteins for leptin and the leptin receptor in different species. In all species studied to date, leptin and/or leptin receptors are expressed in the placenta to varying extents, often in close association with both the maternal and fetal circulations. In mouse embryos, leptin and the leptin receptor have been localised in a variety of tissues, particularly in developing bone and skin (Hoggard *et al.* 1997, 2000). Preliminary studies in fetal sheep have shown a similar distribution of leptin and leptin receptor proteins in adipose tissue and the placenta (Fig 1). In addition, leptin and leptin receptor immunoreactivity is especially intense in epithelial cells of the skin, small intestine and renal tubules (Fig 1).

RELATIONSHIPS BETWEEN LEPTIN LEVELS AND FETAL BODY SIZE

In human infants at delivery, umbilical leptin concentration has been correlated with birthweight, adiposity and placental weight (Helland *et al.* 1998; Varvarigou *et al.* 1999). Circulating leptin *in utero* may reflect adipose and placental tissue mass. Furthermore, adipose leptin mRNA abundance in sheep fetuses has been shown to correlate with fetal bodyweight at mid and late gestation (Yuen *et al.* 1999). These observations have led to the suggestion that leptin may be involved in the regulation of fetal growth. Low concentrations of circulating and placental leptin have been reported in babies with intra-uterine growth retardation and high concentrations in overgrown babies of diabetic mothers (Lea *et al.* 2000). Furthermore, in human infants at term, positive relationships have been identified between umbilical leptin and other hormones known to be associated with nutritional status, such as insulin, cortisol and insulin-like growth factor-I (Maffeis *et al.* 1999).

EFFECT OF LEPTIN ON FETAL GROWTH AND METABOLISM

Few studies have investigated the effect of changes in leptin activity on growth and metabolism of the fetus. In sheep fetuses, intraventricular leptin administration has no effect on plasma glucose or insulin concentrations, or on glucose clearance or

TABLE 1: Expression of leptin and the leptin receptor in fetal and placental tissues in different animal species

Species	Leptin		Leptin receptor		References
	Protein	mRNA	Protein	mRNA	
Man	Placenta (syncytio- and cytotrophoblast) Adipose tissue	Placenta (syncytio- and cytotrophoblast) Adipose tissue	Placenta (syncytiotro-phoblast)	Placenta Adipose tissue Anterior pituitary	Hassink *et al.* (1997) Hoggard *et al.* (1997) Shimon *et al.* (1998) Lea *et al.* (2000) Lepercq *et al.* (2001)
Baboon		Placenta (syncytiotrophoblast)			Henson *et al.* (1999)
Sheep	Placenta	Placenta Adipose tissue Brain Heart Liver Kidney Skeletal muscle		Placenta Heart Liver Kidney Skeletal muscle	Hoggard *et al.* (1997) Yuen *et al.* (1999) Buchbinder *et al.* (2001) Thomas *et al.* 2001 Ehrhardt *et al.* 2002
Pig	Placenta	Adipose tissue		Adipose tissue Umbilical cord Brain Intestines Heart Liver Muscle	Chen *et al.* (2000) Lin *et al.* (2000)
Mouse	Placenta Cartilage and bone Heart Liver Hair follicles	Placenta Cartilage and bone Heart Liver Hair follicles	Placenta Cartilage and bone Hair follicles Brain Lung Kidney	Placenta Cartilage and bone Hair follicles Brain Lung Kidney Testis	Hoggard *et al.* (1997) Hoggard *et al.* (2000)
Rat	Placenta		Placenta	Placenta	Hoggard *et al.* 1997 Smith and Waddell (2002)

insulin responsiveness to a glucose challenge (Howe *et al.* 2002). However, leptin suppresses the pulsatile secretion of adrenocorticotrophin-releasing hormone and cortisol close to term which may have implications for the onset of parturition in this species (Howe *et al.* 2002).

Genetic mutations in leptin or the leptin receptor in rodents appear to have little direct effect on fetal size although more detailed morphological analyses are required. In mice deficient in leptin (ob/ob mice), the establishment of pregnancy is leptin-dependent, but withdrawal of leptin replacement from 0.5 days of gestation has no effect on litter size or fetal weight (Mounzih *et al.* 1998). In mice heterozygous for a mutation in the leptin receptor ($db^{\pm}$), all fetuses are heavier than those in control animals; however, $db^{\pm}$ and wild type fetuses within the same litter do not differ in birthweight (Yamashita *et al.* 2001). Placental leptin levels, but not receptor expression, are greater in $db^{\pm}$ mothers and may be responsible for the overall increase in fetal growth. Indeed, treatment of normal pregnant mice with leptin decreases placental leptin, and causes reductions in both placental and fetal birthweights (Yamashita *et al.* 2001).

Placental leptin may modulate fetal growth and metabolism via effects on placental function. Recent *in vitro* studies have shown that leptin stimulates the activity of the amino acid transporter system A in human placental villous fragments (Jansson *et al.* 2003). This sodium-dependent transporter is responsible for the placental transfer of neutral amino acids to the fetus, and is known to be upregulated in diabetic, and downregulated in intra-uterine growth retardation, pregnancies (Jansson *et al.* 2003). In addition, placental leptin has been proposed to have a role in the control of trophoblast invasion, angiogenesis and immunomodulation (Bajoria *et al.* 2002).

EFFECT OF INTRA-UTERINE NUTRITION ON LEPTIN EXPRESSION IN THE FETUS

Moderate increases or decreases in maternal nutrition do not appear to alter tissue leptin expression or circulating leptin in the ovine fetus

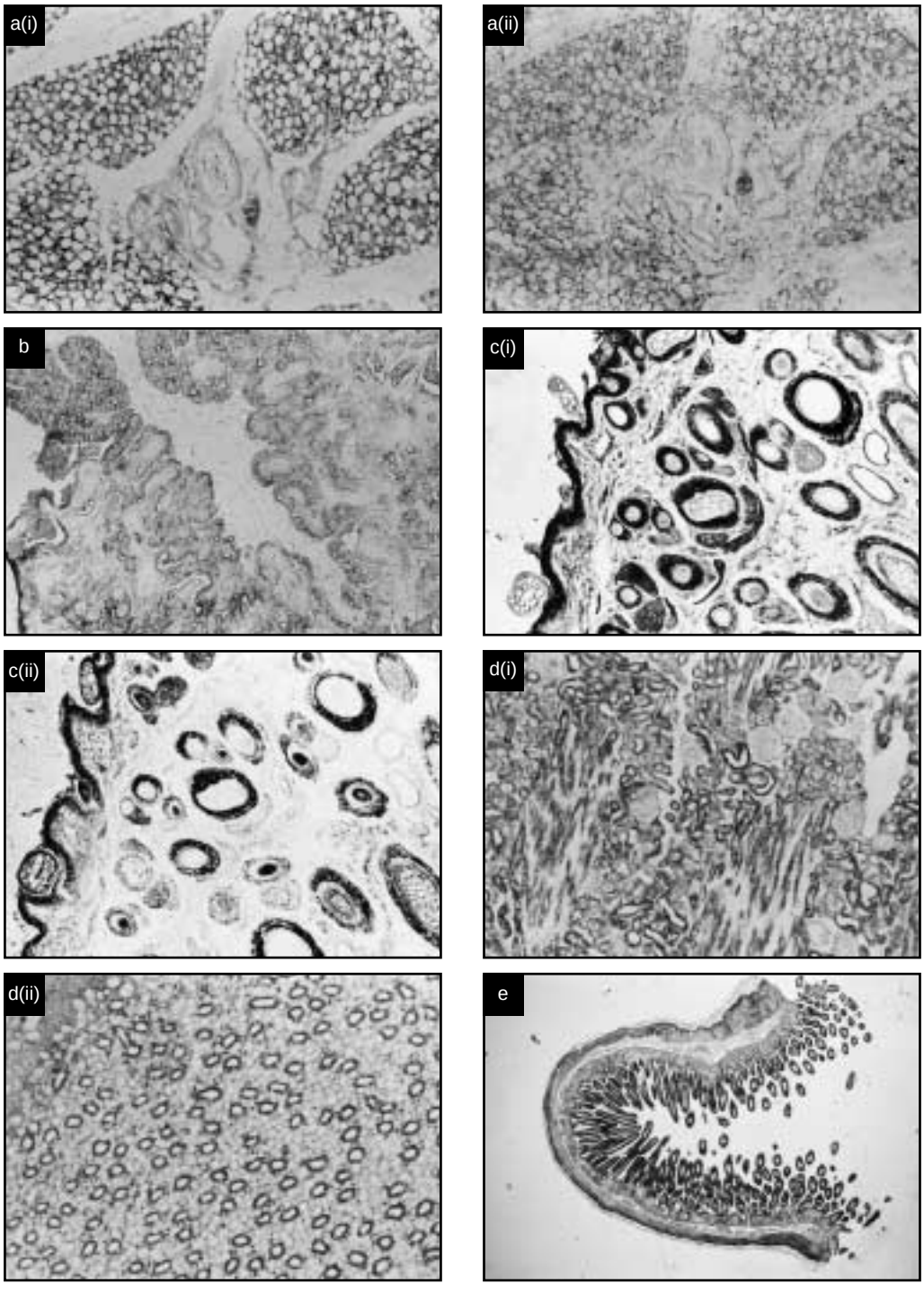

Fig 1: Expression of leptin and leptin receptor proteins in ovine fetal tissues: a) Perirenal adipose tissue stained for (i) leptin and (ii) the leptin receptor; b) Leptin immunoreactivity in the placentome; c) Skin stained for (i) leptin and (ii) the leptin receptor; d) Immunoreactivity for leptin in (i) the renal cortex and (ii) the renal medulla; e) Leptin immunoreactivity in the duodenum.

TABLE 2: Effect of manipulation of the intra-uterine environment on leptin expression in the fetus

Intra-uterine manipulation	Effect on leptin expression	Reference
Maternal undernutrition (55% for 13 days)	No change in plasma leptin	Ehrhardt *et al.* (2002)
Maternal undernutrition (50% for 30 days)	No change in adipose leptin mRNA	Yuen *et al.* (2002)
Maternal over-nutrition (155% for 25 days)	No change in plasma leptin	Mühlhäusler *et al.* (2002)
Chronic fetal hypoglycemia and hypoinsulinemia (36–76 days)	50% decrease in adipose leptin mRNA	Devaskar *et al.* (2002)
Chronic fetal hyperglycemia and hyperinsulinemia (14–20 days)	40% increase in adipose leptin mRNA	Devaskar *et al.* (2002)
Acute fetal hyperinsulinemia and euglycaemia (24 h)	100% increase in adipose leptin mRNA	Devaskar *et al.* (2002)
Acute fetal hyperglycemia and euinsulinemia (24 h)	No change in adipose leptin mRNA	Devaskar *et al.* (2002)
Chronic 50% reduction in uterine blood flow	45% increase in plasma leptin	Buchbinder *et al.* (2001)
Fetal cortisol or dexamethasone infusion (2–5 days)	Transient increase in plasma leptin	Forhead *et al.* (2002)

(Table 2). Although maternal nutritional status influences maternal plasma leptin, and fetal glucose and insulin concentrations, there is little effect on plasma leptin and adipose leptin mRNA abundance in the fetus (Table 2; Yuen *et al.* 2002; Ehrhardt *et al.* 2002; Mühlhäusler *et al.* 2002). However, leptin gene expression in fetal adipose tissue has been shown to be influenced by circulating insulin concentrations *in utero.* In fetal sheep, chronic hyperglycaemia and hyperinsulinaemia cause an increase in adipose leptin mRNA, whereas hypoglycaemia and hypoinsulinaemia lead to a reduction in adipose leptin mRNA levels; both these changes coincide with changes in fetal bodyweight (Table 2; Devaskar *et al.* 2002). In acute studies, hyperinsulinaemia with euglycaemia cause a 2-fold increase in leptin gene expression in fetal adipose tissue, whereas hyperglycaemia with euinsulinaemia does not change adipose leptin mRNA abundance (Table 2; Devaskar *et al.* 2002).

Chronic undernutrition of the ovine fetus by occlusion of the uterine artery and a reduction in uterine blood flow leads to a rise in plasma leptin concentration (Table 2; Buchbinder *et al.* 2001). Likewise, elevated plasma leptin has been reported in growth-retarded human fetuses with lactacidaemia and abnormal Doppler imaging of the umbilical cord (Cetin *et al.* 2000). Stimulation of plasma leptin during fetal distress may be related to changes in oxygen availability and/or adrenocortical activity, because plasma leptin in sheep fetuses has been shown to correlate inversely with the arterial partial pressure of oxygen and positively with plasma cortisol concentration (Forhead *et al.* 2002). In addition, plasma leptin is increased by synthetic and endogenous glucocorticoid administration (Table 2; Forhead *et al.* 2002).

Therefore, circulating leptin *in utero* may act as an endocrine index of both fetal and placental size, and may signal the long-term nutritional status of the fetus to the mother, the placenta and to various fetal tissues. The relationships between plasma leptin and both plasma insulin and fetal size suggest that leptin may be involved in the control of fetal growth. Furthermore, the release of leptin in stressful situations and during glucocorticoid treatment may modulate fetal metabolism and the partitioning of nutrients. The actions of fetal and placental leptin on the development of the fetus in normal and adverse intra-uterine conditions remain to be established.

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WORKSHOP SUMMARY

WORKSHOP SUMMARY

The formidable task of summing up this excellent workshop presented the speaker with a major dilemma and imposed a useful degree of discipline. Concerning the latter, one must listen diligently to all lectures and not miss a single word, no matter what the topic may be. As to the former, should one strive to be comprehensive or merely touch on highlights? Happily, after splendid Italian hospitality and perhaps a glass or two of wine, such matters are resolved more easily than anticipated. There remains, of course, the concern that an individual speaker may feel that his or her delivery has not been adequately assessed or even mentioned at all. The remarks that follow will not include the names of speakers, but careful perusal should suggest that the summary attempts to be comprehensive. None of this stops me from feeling something of an imposter – a Fallopian tube and fertilisation enthusiast commenting upon tissues within a gravid uterus.

However, to indulge in native territory, the opening proposal was that exposure of an oocyte or developing embryo to fluids in the lumen of the Fallopian tubes could exert a carry-over influence on fetoplacental development. The physiology of fluid accumulation in the tubal lumen was briefly recalled, not least the cyclic variation in volume of luminal fluid and the variation in its composition according to region of the duct being sampled and stage of the oestrous cycle. Male gametes that bind to organelles of the Fallopian tube isthmus can alter the nature of tubal secretions, as may follicular fluid and the suspension of ovarian follicular cells that accompanies the oocyte(s) to the site of fertilisation. Depriving *in vitro* matured and fertilised oocytes of this tubal environment before transplantation into the uterus may compromise the normal pattern of development and lead to a low incidence of pregnancy. In the case of transgenic embryos and those derived from cloning procedures, again not exposed to the Fallopian tubes, the incidence of tissue abnormalities may be high.

The second presentation lent general support to these points with the welcome addition of specific data! Although development was approached from diverse angles, nutritional considerations *vis à vis* the pre-implantation embryo became uppermost and the role of various toxins was touched upon. The conclusion could not be avoided that pre-implantation embryos respond to their fluid environment with sensitivity, and can certainly distinguish between a sojourn *in vitro* and one *in vivo.* Epigenetic events in young embryos were recalled, and evidence for molecular perturbations after a spell of *in vitro* culture assembled, notably from work on sheep or cow embryos. There was inevitable mention of the Large Offspring Syndrome in ruminants and also components of the fashionable IGF system – a theme to be repeated in various presentations. There was also the intriguing suggestion that ovarian oocytes and young embryos may be able to recall environmental conditions, especially dietary ones, when they achieve subsequent developmental stages.

To keep participants on their intellectual toes, attention turned to findings on sex ratio in the progeny of rodents, as seemingly influenced by maternal nutrition. Putting statistical analysis of the results to one side, the manner whereby diet during pregnancy might modify sex ratio in mammals remains unknown. As one who wrote a detailed book on mechanisms of sex determination and differentiation in mammals not many years ago (Cambridge University Press 1995), this reviewer was both excited and perplexed by the results. How could nutritional treatments be acting to influence relevant gene pathways? Speculation was rife and a little more might be helpful! Because male embryos develop more rapidly than females, diet might be acting via rates of growth, thereby facilitating preferential development of

male embryos. Another possibility could involve dietary influences on temperature in the genital tract. It is slowly being appreciated that such temperatures are not constant, that gradients do exist, and that male and female embryos may respond differentially to local changes in temperature.

The gravid uterus was next examined from the maternal side of the placenta, especially with regard to morphogenesis and function of endometrial glands. Histotrophe was noted to complement haemotrophe. Diverse aspects of endometrial gland growth were correlated with subsequent growth of the fetus. The life cycle of uterine glands was outlined in sheep, and a scholarly aspect introduced with reference to the studies of Wimsatt, William Harvey and Aristotle. There was much discussion of a uterine gland 'knock-out' model, one that shows luteolytic problems due to insufficient $PGF_{2\alpha}$ generation and insufficient secretion overall. Endocrine features of the model were presented in some detail.

Placental considerations then focused on the time-course and pathways of trophoblast invasion, including details of the spiral arteries and their pattern of remodelling. Trophoblast invasion demonstrated an interstitial as well as an endovascular pathway. The nature of association between blood vessel and uterine tissue received close attention, with primates and rodents being models of choice. Vascular invasion in man and rats requires endothelial disruption, breakdown of medial smooth muscle and, eventually, reestablishment of the endothelium. Defects of trophoblast invasion in man are typical of pregnancy complications such as pre-eclampsia, a point leading to discussion of a pre-eclamptic rat model.

Next came a detailed commentary on placental circulation, with emphasis on the period of 10 weeks required for full, direct, interplacental communication in man. Little maternal blood flowed into the intervillous space, but what does enter may be broken down to form histotrophe supplemented by uterine gland secretions. The change in oxygenation at 10–12 weeks of gestation was correlated with measurements of blood flow. In fact, it was emphasised that the human placenta was not truly haemochorial until the end of the first trimester. Together with characterisation of the endometrium, assessment of secretory activity of endometrial glands was made up to this stage with special reference to growth factors and transport proteins. Scholarly aspects were once again pursued with mention of the work of G.W. Corner and G.L. Streeter (both sometime of the Carnegie Institution) and to that of Boyd in Cambridge whose catalogued collection of human embryos underpins so many important studies.

As a fascinating feature of the equine blastocyst, attention turned to formation of the so-called capsule. This acellular structure lies between the trophectoderm and enveloping zona pellucida and begins to form 6–7 days after ovulation. Although primarily derived from trophoblast glycoprotein secretion, a strong argument for a uterine contribution or influence comes from *in vitro* derived embryos that lack a conspicuous capsule. Fluorescent antibody techniques were employed to analyse the nature of capsule expression in both *in vitro* and *in vivo* embryos. Whereas the capsule of suitably-staged *in vivo* derived embryos showed a typical bilaminar appearance, those produced *in vitro* were devoid of a capsule and unable to develop appropriately. The anomaly was thought to reside in trophectoderm activity. The uterine environment was shown to stimulate cross-linkage and coalescence of secreted glycoproteins on the trophectoderm surface. This elegant analysis apart, the capsule's putative function of protecting the embryo during its displacement throughout the uterine lumen might be coupled with one of attracting and sequestering diverse molecules, possibly in a context of suppressing luteolytic activity. Be that as it may, there would seem to be scope for co-culture studies of blastocysts with and without a capsule, but placed in close contact in the region of punctured or drilled zonae. There could also be scope for a more direct micromanipulation of fluids in the perivitelline space, such as aspiration from a competent *in vivo* derived blastocyst and injection into an *in vitro* preparation.

Large-scale studies from Alberta employed a pig model to demonstrate the influence of nutritional factors on pre-natal loss. The notion of uterine crowding (overcrowding) was explored as one regulator of potential litter size. Pin-pointing the actual mechanisms of action was not straightforward in these studies of embryonic or subsequent loss and there remains the notion that, within reasonable limits, uterine tissues should be able to grow to accommodate the viable products of conception. After all, growth and distension are

substantial towards the end of gestation, so in the present context factors more subtle than simple physical growth or overcrowding must surely be involved in early pre-natal loss. Endocrine aspects were not overlooked and the classical gonadal hormones, oestradiol and progesterone, were much in evidence together with growth factors and insulin. Especially pleasing to this reviewer was mention afresh of local counter-current transfer of ovarian hormones to influence physiology of the Fallopian tube, a mechanism first demonstrated in Edinburgh in the early 1980s. And, reinforcing an emerging theme in the workshop, there was further evidence of metabolic 'imprinting' of very young embryos and perhaps also of oocytes. Moreover, the Fallopian tube may have a nutritional memory in terms of its protein secretions.

Continuing with the pig model, placental growth and efficiency were discussed in breeds with exceptionally high ovulation rates, such as the Meishan from China. Growth of the young embryo was examined, and such studies extended to growth of the conceptus and eventually to that of the fetoplacental unit. Evidence for both maternal and conceptus effects was presented, and reciprocal embryo transplants used to bring out differences. Further discussion centred on vascular endothelial growth factor (VEGF) and placental efficiency, although without a rigorous definition of the latter term. Blood flow would represent only one component of such efficiency. Ultimately, the dynamics of membrane transport for a wide range of substances would be involved – an intimidating prospect for experimental work even in this age of computerised analysis. At least as important a consideration, placental efficiency will undoubtedly vary enormously with time (minutes, hours, days, weeks) and as influenced by the activity of neighbouring placental and uterine tissues, including the myometrium. This last point is made in spite of the classical dogma of Csapo's progesterone block of myometrial activity.

The workshop returned from the polytocous pig to unique aspects of the equine embryo, notably the role of the allantoic mesenchyme in formation of the chorionic girdle. This was a sphere to which the late Francesca Stewart dedicated much of her professional life. The intellectual interest remains in part associated with the subsequent gonadotrophic function of tissues arising from this girdle region. The presumptive girdle was always located between the allantois and regressing yolk sac, its cells becoming binucleate as a prerequisite for invasion of the uterine stroma. Lymphocytes accumulate around the invading girdle. Of course, tissue movements and reorganisation are proceeding apace in 25–35-day-old embryos and, even with modern techniques, it will be difficult to account for all cellular contributions to a structure so specialised as the chorionic girdle. Further relevant details must remain one of the remits of 'Veterinary Science Tomorrow', not least molecular contributions from migrating lymphocytes, including a spectrum of cytokines and local growth factors.

Moving to the wonderful world of scanning electron microscopy, images of the highest quality were used to demonstrate the microvasculature of the equine placenta. Attention focused on microcotyledons and microcaruncles, with some emphasis on the honeycombed appearance at the side and base of microcaruncles. Each microcotyledon received a single artery which followed a straight course before subdividing, and details of the corresponding veins were given. Blood supply to the villous structures was also noted, with detail on the 3–5 capillary loops formed from the terminal villi. Over and above such extensive findings on the vascular microarchitecture, a conclusion was presented of much interest to vascular physiologists, viz. that the relevant fetal and maternal vessels are arranged largely in parallel, especially at the terminal villi, with blood flowing in opposite directions. This would suggest a counter-current system of exchange. Despite the excellence of the scanning electron micrographs, one must remember that they illustrate potential pathways and not necessarily what is happening within at a given time.

Due to insufficient self-discipline at the lunch and dinner tables, specifically with regard to intake of fluids, this reviewer's attempts at note taking suffered a serious nose dive from this stage onwards. Some readers of these pages, if not workshop participants, may find this welcome. Accordingly, the remaining contributions will be recorded more concisely.

The role of nutrient intake, restriction or deprivation at different stages of gestation was the principal topic for the rest of the meeting, with sheep being the predominant model species. This was in spite of the fact that hill sheep in particular have evolved to cope with phases of inadequate

nutrition during pregnancy (in winter). Elegant experiments were reported, not least those involving catheterised fetal preparations. The number and nature of placentomes received detailed comment, and changes in placentome morphology were noted under different nutritional regimes. Much detail on fetal organ weight was marshalled and on placental fluid volume. Lung development and function received serious comment because of the particular relevance to equids. Insulin, the IGF system and its numerous regulatory binding proteins were much in evidence, and there was also examination of blood glucose and placental glucose transport; downstream influences of glucose availability were touched on.

Sheep with manipulated nutrient intake were used to shed light on perturbations during human adolescent nutrition, although the major limitations in such an extrapolation model must be kept in mind. Cardio-vascular function in offspring arising from wayward parental nutrition attracted much attention, and the possibility of trans-generational 'programming', 'imprinting' or other expressions of genomic memory riveted the audience. Endothelial function received specific comment and, for the two members of Sidney Sussex College present, mention of the work of Widdowson and McCance brought back memories of a Cambridge before the excellence of British university education was so seriously undermined.

Appropriately, experimental work in equids regained the stage shortly before the end of the workshop and we galloped home in fine fettle, despite extensive portrayal of the consequences of nutritional inadequacy. Much was reminiscent here of Hammond's lectures on placentation and of his classical Shire Shetland breeding experiments. The penultimate paper took us back to the IGF system in ruminants, thyroid function seemed to have slipped from sight, and we closed with some splendid studies on the role of leptin in fetal metabolism.

Having prepared these various sketches, this reviewer remains apprehensive. Participants may recall that the Roman writer Seneca said:

> *"Show me a man who is not a slave; one is a slave to lust, another to greed, another to ambition, and all men are slaves to fear."*

Reviewers suffer – or should suffer – from this last emotion and, in the present instance, be concerned that the task of rendering many sessions of excitement into a few pages of printed word is indeed formidable. Nonetheless, what can be stated with confidence is that the workshop – and others in the series – provided more stimulation in three days than some large research organisations provide in three months or, in some notable cases, in three years.

We must close by expressing gratitude to those behind the scenes, to Sandra Wilsher and Twink Allen for quietly hatching an excellent programme, to Jan Wade and her staff for making impeccable arrangements for all aspects of the workshop, and to President Gene Pranzo and his Havemeyer Foundation for generous hospitality and a continuing enthusiasm. Now that the series of attractive Havemeyer monographs is established and becoming quite widely known, both Gene Pranzo and Jan Wade must feel that this particular entry into publishing is bearing fruit. Timely appearance of workshop proceedings is an essential feature of fructification.

R.H.F. Hunter
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