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EDITORS' FOREWORD

Dorothy Russell Havemeyer founded the Havemeyer Foundation in 1979 and was President until her death in 1985. During her lifetime, and subsequently with the enthusiasm and encouragement of the current President, Mr Gene Pranzo, the Foundation has provided generous support for equine research in the form of Scientific Workshops. These meetings are held in high regard within the equine scientific community and provide an unparalleled opportunity for a small number of highly specialist participants to discuss at length, recent scientific knowledge from both equine and comparative disciplines.

Topics for workshops have been diverse, but the Foundation has always shown strong support for equine reproduction. In 2003 it kindly sponsored the first workshop on 'Embryonic and Fetal Nutrition' in which the role of maternal nutrition in both placental development and fetal nourishment *in utero* was examined. Because the horse is rarely used as a model for extrapolation in other species, and is not farmed intensively, knowledge of the effects of nutrition on equine embryonic and fetal development is meagre compared to, for example, the pig and sheep. The first workshop in 2003 helped to bring forward ideas that were not only fruitful for the participating equine scientists but also for those providing imput from comparative species. It is hoped that this second workshop on Embryonic and Fetal Nutrition will build on the foundations and discussion of 2003, and will generate useful new ideas and enjoyable musings.

Sandra Wilsher and Twink Allen
Workshop Organisers

HAVEMEYER SCIENTIFIC WORKSHOPS

1981	**First International Workshop on Lymphocyte Alloantigens of the Horse** October - New York City, USA *Organiser: Dr D. F. Antczak*
1982	**Second International Workshop on Lymphocyte Alloantigens of the Horse** October - Cornell University, Ithaca, New York, USA *Organiser: Dr D. F. Antczak*
1983	**Third International Workshop on Lymphocyte Alloantigens of the Horse** April - New Bolton Center, University of Pennsylvania, USA *Organiser: Dr D. F. Antczak*
1984	**First International Symposium on Equine Embryo Transfer** October - Cornell University, Ithaca, New York, USA *Organisers: Drs D. F. Antczak and W. R. Allen*
1985	**Fourth International Workshop on Lymphocyte Alloantigens of the Horse** October - University of Kentucky, USA *Organisers: Drs D. F. Antczak and E. Bailey*
1986	**Workshop on *Corynebacterium equi* Pneumonia of Foals** July - University of Guelph, Canada *Organiser: Dr J. F. Prescott*
1987	**Fifth International Workshop on Lymphocyte Alloantigens of the Horse** October - Louisiana State University, USA *Organisers: Drs D. F. Antczak and J. McClure*
1989	**Second International Symposium on Equine Embryo Transfer** February - Banff, Alberta, Canada *Organisers: Drs D. F. Antczak and W. R. Allen*
1990	**International Workshop on Equine Sarcoids** April - Interlaken, Switzerland *Organisers: Dr D. F. Antczak and Professor S. Lazary*
1992	**Workshop on Equine Neonatal Medicine** January - Naples, Florida *Organisers: Drs D. F. Antczak and P. D. Rossdale*

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February - Buenos Aires, Argentina
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1995 **Equine Perinatology**
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Organisers: Drs D. F. Antczak, S. Lazary and E. Marti

Equine Placentitis Workshop
October - Lexington, Kentucky, USA
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Septicemia II Workshop
November - Boston, Massachusetts, USA
Organiser: Dr M. R. Paradis

1999

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Organisers: Drs D. F. Antczak and E. Bailey

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

Fetomaternal Control of Pregnancy
November - Barbados, West Indies
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2000

Equine Genome Project
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Fifth International Gene Mapping Workshop
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Organisers: E. Baily and E. Vandyke

Equine Recurrent Laryngeal Neuropathy
September - Stratford-upon-Avon, UK
Organisers: P. Dixon and E. Robinson

Transporting Gametes and Embryos
October - Brewster, Massachusetts, USA
Organiser: E. Squires

Third Meeting of the European Gamete Group (EEGG)
October - Pardubice, Czech Republic
Organisers: J. and Z. Müller

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October - Lexington, USA
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2004

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January - San Diego, California, USA

Equine Viral Herpes Virus Workshop
June/July - Tuscany, Italy
Organiser: P. Lunn

Equine Embryo Transfer 6th Workshop
August - Rio de Janiero, Brazil
Organiser: M. Alvarenga

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November - Barbados, West Indies
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2005

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SESSION I:

Chairman:
Twink Allen

INFLUENCE OF ENVIRONMENTAL POLLUTANTS DURING OOCYTE MATURATION ON THE QUALITY OF CATTLE AND PIG EMBRYOS

H. Alm and H. Torner

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INTRODUCTION

It is generally accepted that mammalian pre-implantation embryos are sensitive to their environment and that conditions of culture can affect future growth and developmental potential both pre- and post natally. Evidence suggests that while culture conditions during bovine *in vitro* embryo production can impact somewhat on the developmental potential of the early embryo, the intrinsic quality of the oocyte is the key factor determining the proportion of oocytes developing to the blastocyst stage.

Pollutants are available substances or substance mixtures in the environment, which can be harmful to people, animals, plants and/or other organisms as well as whole ecosystems. Pollutants are distinguished in 2 groups: natural pollutants, such as dust or mycotoxins, and artificial pollutants, mostly of anthropogenic origin, ie caused and released by people.

Within the artificial pollutants organochlorine pesticides play an important role. They are widely used in a variety of agricultural fertilisers, household insecticide sprays, human parasitic medications and animal parasitic solutions.

Fusarium mycotoxins, secondary metabolites produced by moulds, occur worldwide in cereal grains and animal feed and cause outbreaks of fusarium mycotoxicoses in humans and animals (Kuiper-Goodman *et al.* 1987).

Both pesticides and mycotoxins can influence the reproductive success and fetal development, and thereby impair growth and/or reproductive efficiency.

It is unknown whether the pesticides impair fertility by direct action on oocyte meiotic progression or on early embryonic development, or whether these environmental pollutants exert an indirect effect on the genital tract. Therefore, an *in vitro* system was selected, in which we evaluated the influence of pesticides on both oocyte maturation and post fertilisation development of bovine oocytes.

The effect of mycotoxins on oocyte and embryo development was investigated in pigs both *in vitro* and *in vivo*.

MATERIALS AND METHODS

The *in vitro* investigations were carried out on oocytes from slaughtered cattle and pigs. The cumulus oocyte complexes (COCs) were collected by follicle aspiration. Oocytes with compact cumulus investment were selected and the bovine COCs were matured for 24 h in TCM 199, while the porcine COCs were matured for 48 h in NCSU-23 medium (Petters and Wells, 1993) at 38°C under 5% CO_2 in 100% humidified air.

The organochlorine pesticides [dichloro-diphenyltrichloroethane (DDT), lindane, the γ-isomer of hexachlorocyclohexane (γHCH) and methoxychlor (MXC)] were added in an increasing level (0, 7.25, 14.5, 29.0 µg/ml) to the culture medium during maturation of bovine COCs. The developmental competence of these treated COCs was evaluated after IVF and subsequent culture (Alm *et al.* 1998).

Different concentrations of the mycotoxins deoxynivalenol (DON, 0–7.5 µM) and zearalenone (ZON, 0–90.0 µM) were added during maturation and embryonic development of porcine oocytes and zygotes *in vitro* (Alm *et al.* 2002), and after feeding *in vivo* (0.2 to 9.57 mg DON/kg and 0.004–0.358 mg ZON/kg) (Alm *et al.* 2006).

TABLE 1: Influence of DDT, γHCH and MXC at various concentrations during maturation *in vitro* on fertilisation and embryonic development of bovine oocytes *in vitro* (mean ± SE)

Treatment group	Number of oocytes exposed	% of oocytes fertilised	% of cleaved embryos	% of morulae/ blastocysts
Control	190	87.2 ± 2.86[a]	78.8 ± 3.75[a]	34.6 ± 2.57[a]
DDT	194	88.6 ± 2.72[a]	80.5 ± 5.20[a]	23.5 ± 2.66[b]
γHCH	187	93.8 ± 1.66[a]	84.1 ± 2.20[a]	23.7 ± 3.27[b]
MXC	194	85.3 ± 3.87[a]	79.3 ± 5.26[a]	29.3 ± 2.49[a,b]

[a-b] Different superscripts in the same column indicate significant differences within groups (P<0.05)

TABLE 2: Influence of α-zearalenol at various concentrations on embryonic development of porcine zygotes after 6 days of *in vitro* culture (mean ± SE)

Concentration of α-zearalenol (μM)	Number of zygotes exposed	% of blastocysts
0	42	61.9 ± 10.0[a]
3.75	30	60.9 ± 6.5[a]
7.50	29	45.4 ± 8.6[a. b]
15.0	32	26.5 ± 9.2[b]
30.0	33	6.1 ± 3.6[c]

a-c Different superscripts in the same column indicate significant differences within groups (P<0.05)

TABLE 3: Maturation rate in compact porcine COCs after 48 h of culture (n=111)

Treatment group (mg DON+ZON/kg feed)	Number of oocytes	Maturation rate, n (%)
0.21 + 0.004 (control)	28	24 (85.7)
3.07 + 0.088	29	18 (62.1)
6.10 + 0.235	28	14 (50.0) *
9.57 + 0.358	26	15 (57.7) *

*Significant differences between the control and experimental group

RESULTS

All investigated pesticides affected maturation and degeneration rates of bovine oocytes in a dose-dependent manner. Under the influence of DDT at 29.0 μg/ml the frequency of TI/MII was significantly lower (32.0%) than in the unexposed control (80.3%) or those exposed to lower concentrations (7.25 μg/ml – 67.2%; 14.5 μg/ml – 67.7%). Similar effects were observed after addition of γHCH or MXC. Higher concentrations of pesticides were associated with higher rates of chromatin degeneration. Because the maturation of bovine oocytes was depressed in a dose-dependent manner, the fertilisability and further embryonic development of *in vitro* matured oocytes was studied at the lowest previously tested concentration (7.25 μg/ml) only. No significant difference in fertilisation rates was seen between unexposed control and treated groups. The cleavage rates did not differ among groups 48 h after IVF. The number of morulae and blastocysts on Day 7–8 after IVF, which is commonly used as a parameter for normal development, was significantly different between control and DDT and γHCH treated groups, but not between the control and MXC groups.

These results show that the tested pesticides decrease the rate of normal oocyte maturation *in vitro* in a dose-dependent manner. The effect of the lowest concentration of pesticides is seen only after Day 7 of embryo development (Table 1).

All investigated specific mycotoxins affected maturation and degeneration rates also in a dose-dependent manner, but to different extents. The metabolites of ZON, α- and β-zearalenol (α- and

β-ZOL) showed significant influence at different concentrations. So significant differences could already be obtained at a concentration of 7.5 µM α-ZOL. β-ZOL negatively affected the process of oocyte development beginning at a concentration of 30.0 µM ($P<0.05$). DON had significant influence on oocyte maturation at a concentration of 1.88 µM (31.4 vs. 79.3% - for control).

Differences in embryonic development *in vitro* were obtained at concentrations of 15 and 30 µM α-zearalenol ($P<0.05$) (Table 2). Not only the blastocyst rate but also the number of nuclei in the developed blastocysts were significantly decreased at both these concentrations.

In vivo, after feeding of wheat naturally contaminated with the *Fusarium*-toxins DON and ZON, the proportion of oocytes with intact immature chromatin was reduced in the higher contaminated groups. The proportion of oocytes having degenerated meiotic chromatin was significantly higher in the group with the highest investigated contamination. The proportion of oocytes reaching metaphase II in culture was significantly lower in the groups with 6.1 and 9.57 mg DON and 0.235 and 0.358 mg ZON/kg (Table 3). It is concluded that oocyte quality is significantly reduced by feeding of *Fusarium* toxins to gilts.

Discussion

The results of the present studies indicate that maturation and embryonic development of bovine oocytes *in vitro* are influenced by DDT, γHCH and MXC in a dose-dependent manner. DDT and γHCH were most toxic. MXC demonstrated a weaker inhibition of embryonic development. The data showed that DDT, γHCH or MXC in a concentration of 29.0 µg/mL resulted in more than 50% degeneration of the exposed COCs. Lower levels of pesticide concentrations showed less degeneration and higher levels of nuclear maturation.

In a previous study, the influence of the same pesticides on embryonic development of 8-cell mouse embryos *in vitro* was investigated. The proportion of blastocysts which hatched decreased with increasing concentration of each of the 3 pesticides (Alm *et al.* 1996).

Similar results showing dose-dependent influence of polychlorinated biphenyls (PCB) on embryonic development of rabbit embryos were obtained by Lindenau and Fischer (1996). Exposure to 50 µg PCB/mL led to an almost complete degeneration of morula stage embryos, associated with dense cytoplasm.

Significant toxicity of these agents has been shown not only during the first cleavage stages but also in the early embryogenesis stage. Direct exposure of gestational Day 10 rat conceptus to γHCH resulted in a dose- and time-dependent increase in mortality and in decreased growth parameters (McNutt and Harris *et al.* 1994).

Mycotoxins influenced the maturation of pig oocytes both *in vitro* and *in vivo*, in which α-zearalenol *in vitro* affected at a concentration of 7.5 µM whereas β-zearalenol showed a significant effect only at 30 µM. Almost half of the exposed oocytes were degenerated. These results are in agreement with data from Minervini *et al.* (2001) who demonstrated a negative effect at 9.4 µM, and an almost total degeneration of oocytes at 94 µM. An explanation for the greater toxicity of α-zearalenol compared with β-zearalenol could be their different oestrogenicity. In investigations carried out by Tiemann *et al.* (2003), it was found that α-zearalenol is more potent in its respective affinities to oestradiol receptors in endometrial cytosol from pig. The mechanism of action of α-zearalenol on pig endometrial cells can be explained by the fact that it exerts its effect by competing with oestrogen for cytosolic receptor on cells in target tissue. Thigpen *et al.* (1987) reported on the oestrogenic activity of zea α found in *in vivo* experiments, which is due to a direct action of this compound on the uterus.

In vivo feeding of *Fusarium* toxin at high concentrations was associated with oocyte degeneration and reduced meiotic competence of compact COCs after IVM.

Blastocyst development and cell number are valuable indicators of embryo viability. Although the blastocyst rate in the bovine was influenced by the pesticides, all embryos reaching this stage were intact and had numbers of nuclei comparable to results observed by other authors (Farin *et al.* 1995).

In the pig comparable nuclei numbers were found after exposure to α-zearalenol at concentrations of 3.75 and 7.5 µM. However, concentrations of 15 and 30 µM decreased both the percentage of zygotes reaching the blastocyst stage and the number of cell nuclei per blastocyst.

These results suggest that the reproductive failure associated with ingestion of these

mycotoxins may be due to a direct effect of the toxins on oocyte and embryo development, in addition to the overall systemic and reproductive effects of the toxins on the animals consuming these mycotoxins.

ACKNOWLEDGEMENTS

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CONSEQUENCES OF *IN VITRO* PRODUCTION ON GENE EXPRESSION PATTERNS IN PRE-IMPLANTATION BOVINE EMBRYOS

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Current *in vitro* production (IVP) and somatic nuclear transfer (sNT) technologies have been significantly improved in cattle. But the *in vivo* situation still cannot be mimicked sufficiently well. Morulae and blastocysts can be produced with reasonable efficiency. However, generated embryos display a number of marked differences compared to their *in vivo* counterparts including different gene expression patterns. It has been suggested that persistent alterations from the normal expression patterns may have detrimental effects on embryonic development and contribute to the incidence of the 'Large offspring syndrome (LOS)', of which a high birth weight and an extended gestation length are the predominant features (Walker *et al.* 1996; Kruip and den Daas, 1997). The underlying mechanisms are largely unknown at present, but alterations of epigenetic modifications of embryonic and fetal gene expression patterns, primarily caused by alterations in DNA methylation, are thought to be involved (Wrenzycki *et al.* 2005a; Fig 1).

The pre-implantation bovine embryo is initially under the control of maternal genomic information that is accumulated during oogenesis. Soon, the genetic program of development becomes dependent upon new transcripts derived from activation of the embryonic genome. The early steps in development, including timing of first cleavage, activation of the embryonic genome, compaction, and blastocyst formation, can be affected by the culture media and conditions as well as the production procedure itself. These perturbations can possibly result in a dramatic decrease of the quality of the resulting blastocysts, and may even affect the viability of offspring born after transfer.

Analysis of mRNA expression patterns of developmentally important genes essential in early development provides a useful tool to assess the normality of the embryos produced and to

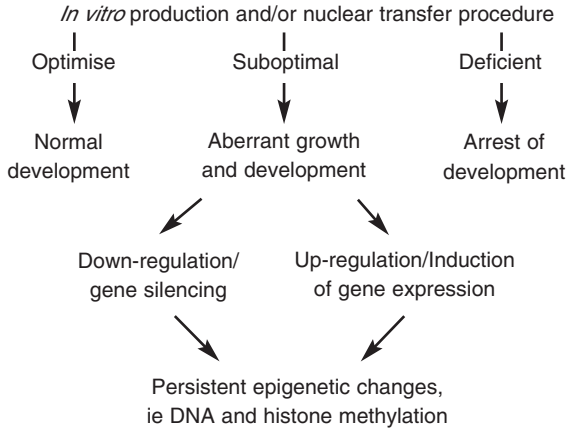

Fig 1: Effects of in vitro production and/or nuclear transfer on gene expression patterns.

optimise assisted reproduction technologies. Messenger RNA studies in bovine embryos has emerged as a rapidly moving field and a large body of literature exists already (Wrenzycki *et al.* 2005b; Lonergan *et al.* 2006). Numerous aberrations have been found ranging from suppression of expression to *de novo* overexpression or more frequently to a significant up- or down-regulation of a specific gene (Wrenzycki *et al.* 2005b). Defining the expression patterns of specific genes critically involved in pre-implantation development will aid in selecting markers for determining embryo quality. The identification and characterisation of the short-term effects raises the question about long-term consequences and safety of assisted reproductive technologies. Recent reports indicate that *in vitro* culture of murine embryos can have irreversibly long-term consequences of post natal development, growth, physiology, and behaviour in resulting offspring (Ecker *et al.* 2004; Fernandez-Gonzalez *et al.* 2004).

The molecular deviations observed in studies with farm and laboratory animals emphasise the need for further studies to gain insight into the expression patterns correlated with an undisturbed embryonic and fetal development. Understanding the molecular mechanisms will aid to improve biotechnologies applied to early embryos in all species, including humans. The bovine embryo could serve as a useful model as there is growing evidence that the bovine is a good model for human pre-implantation development.

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FACTORS AFFECTING *IN VITRO* FERTILISATION AND EARLY DEVELOPMENT OF HORSE EMBRYOS

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INTRODUCTION

The development of assisted reproductive techniques such as artificial insemination and embryo transfer has revolutionised genetic improvement within the equine breeding industry. By contrast, the commercial application of techniques involving the production of horse embryos *in vitro* (IVP) has been restricted by low efficiency (Allen 2005). With regard to the reasons for the poor success of equine IVP, it is difficult to disentangle the roles of suboptimal oocyte maturation and/or abnormalities arising during fertilisation or early embryonic development in the failure to yield a developmentally competent blastocyst or viable foal. In this paper the authors focus on cellular events critical to oocyte maturation, sperm-oocyte interaction or early embryonic development that are disturbed *in vitro*, with the aim of highlighting areas where IVP of horse embryos can be improved.

OOCYTE MATURATION: ENSURING CYTOPLASMIC MATURITY

Oocyte quality is critical to the development of a viable pregnancy. During folliculogenesis and oocyte maturation, an oocyte acquires 'developmental competence' by growing and accumulating a store of mRNAs. Although live foals have been produced from *in vitro* matured (IVM) oocytes, only a small proportion of such oocytes yield blastocysts, presumably because current IVM systems fail to adequately support the acquisition of developmental competence. In this respect, it appears that while cultured oocytes readily resume meiosis and complete nuclear maturation, they fail to undergo a number of cytoplasmic changes essential for normal post fertilisation development. It has been suggested that pre-culturing immature oocytes under conditions that delay the onset of meiosis, while allowing cytoplasmic maturation to proceed, might improve developmental competence (Sirard 2001). Using this rationale, the authors examined the ability of different components of the follicle wall to inhibit the resumption of meiosis in cultured immature horse oocytes. Cumulus oocyte complexes (COCs) were cultured for 38 h in M199 still attached to either a full-thickness piece of follicle wall or to membrana granulosa alone; additionally, COCs attached to membrana granulosa (COCGs) were co-cultured with separate pieces of follicle wall, sheets of theca cells or theca-cell conditioned medium. The majority of oocytes attached to intact follicle wall did not undergo germinal vesicle breakdown (GVBD) and resumed meiosis during culture (41/52; 79%). By contrast, only 15% (19/125) of controls (COC alone) and 21% (11/52) of COCGs co-cultured with separate follicle wall remained in meiotic arrest, while the majority reached metaphase II (75% and 66% for control and follicle wall co-culture, respectively). When COCGs were co-cultured with theca cells or in theca-cell conditioned medium, however, the majority of oocytes did arrest at the GV stage (64% and 52%, respectively). In short, theca cells appear to play a critical role in maintaining meiotic arrest, probably via a secreted inhibitory factor whose effect on oocyte activity must be transmitted by attached granulosa cells (Tremoleda *et al.* 2003). A combination of attached granulosa cells and theca cell secretions could therefore form the basis of an oocyte pre-maturation system to increase the time available for, and thereby improve the quality, of cytoplasmic maturation.

TABLE 1: Developmental and morphological features of Day 7 horse embryos produced *in vivo* or *ex vivo*. *Ex vivo* embryos were produced by *in vitro* oocyte maturation followed by ICSI of either oocytes derived from compact and expanded COCs and subsequently cultured in SO, or oocytes from compact COCs and 'incubated' in the oviduct of a progesterone-treated sheep

Embryo origin	No. oocytes	No. cleaved	Cleavage rate	No Day 7 embryos	Embryos/ oocyte (%)	Embryos/ cleavage (%)	No. analysed	Diameter (μm)	Number of cells (mean ± SEM)
SOF-IVC (Comp-COCs)	349	214	61.3 %	11	6.3 ± 1.9[a]	12.6 ± 4[c]	11	140.3 ± 3.2[f]	82.91 ± 30.7[c]
Sheep oviduct (Comp-COCs)			28	16 ± 3.3[b*]	23.72 ± 5.9[d*]	15	138.1 ± 2.5[f]		85.8 ± 9.3[c]
SOF-IVC (Exp-COCs)	317	201	63.4 %	30	9.4 ± 1.3[a]	15 ± 1.5[c,d]	21	139.7 ± 1.7[f]	132.9 ± 15.9[d]
In vivo	-	-	-	-	-	-	10	374 ± 64.1[g]	1736 ± 567.9[e]

Within a column, values with a different superscript differed significantly ([a,b] P< 0.01; [c, d, e] P< 0.05; [f, g] P<0.001). *The embryo development rate was adjusted to account for the number of cleaved embryos actually transferred into sheep oviducts (n=118).

CONVENTIONAL *IN VITRO* FERTILISATION: AN UNSOLVED ENIGMA

The major obstacle to developing a practical and cost-effective system for producing horse embryos *in vitro* is the poor success of conventional IVF. To date, only 2 conventional IVF foals have been born, and both were from oocytes matured *in vivo* (Bezard 1992). The reasons for fertilisation failure appear to rest primarily on an inability of stallion sperm to penetrate the zona pellucida *in vitro*, although the precise cause (eg culture-induced changes in zona hardness and/or inadequate activation of sperm) is unresolved. To determine whether sperm activation is the limiting factor, the authors examined whether sperm binding, penetration and fertilisation of *in vitro* and *in vivo* matured oocytes could be influenced by supplementation with progesterone, a physiological inducer of the acrosome reaction (Rathi *et al.* 2003). To exclude stallion and semen preservation effects, IVF was performed with fresh and frozen-thawed sperm from 2 fertile stallions. In an initial experiment, IVM oocytes with or without a cumulus investment were incubated with stallion sperm in the presence or absence of 150 ng/ml of progesterone. Subsequently, *in vivo* matured oocytes recovered from pre-ovulatory follicles by transvaginal ultrasound-guided aspiration were incubated with sperm, plus or minus progesterone. Sperm-oocyte interaction was assessed using confocal microscopy combined with stains for sperm viability (Calcein-AM and EthdD-1/PI: Live/Dead Molecular Probes) and acrosome status (FITC-PNA: an outer acrosomal membrane marker). In all experimental conditions, sperm bound to the zona pellucida but failed to complete the acrosome reaction, suggesting that failure of IVF is due primarily to inadequate sperm activation. Moreover, since the majority of IVM oocytes are fertilised if they are transferred to the oviduct of an inseminated mare (Hinrichs *et al.* 2002), it appears that deficiencies accumulated during oocyte culture can be rectified by an appropriate (oviductal) environment. Studies to identify oviductal factors that regulate sperm capacitation, zona binding and oocyte penetration may, therefore, lead to improved IVF media.

IN VITRO CULTURE OF HORSE EMBRYOS

The poor success of conventional IVF has led to intracytoplasmic sperm injection (ICSI) becoming the method of choice for fertilising horse oocytes *in vitro*. However, while several foals have been produced by ICSI (for review see Allen 2005), the rates of blastocyst production *in vitro* and subsequent embryo survival *in vivo* are disappointing, presumably largely because of culture-induced abnormalities. To determine what goes wrong when horse embryos develop *ex vivo*, the authors examined the impact of IVP on the morphological, ultrastructural (microfilament organisation) and developmental (cell number,

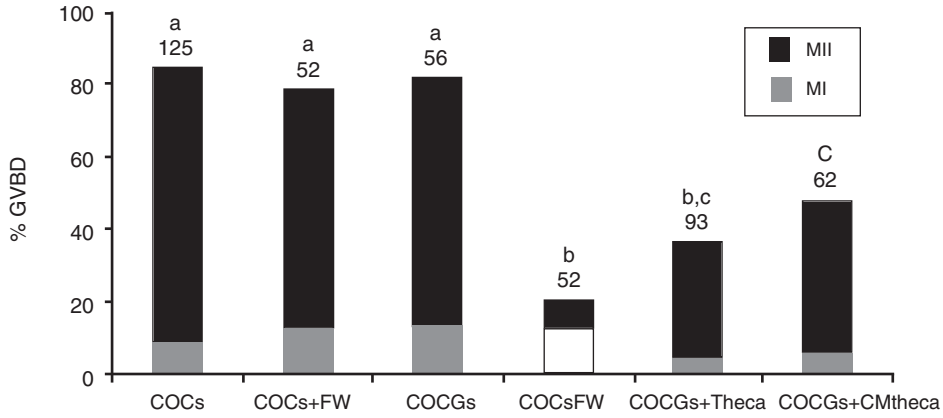

Fig 1: The effect of follicle wall components on the progression of meiosis in horse oocytes matured in vitro. Oocytes were cultured for 38 h in M199 in the form of: cumulus-oocyte complexes (COCs), COCs attached to follicle wall (COCsFW), COCs co-incubated with loose follicle wall (COCs+FW), COCs attachted to membrana granulosa (COCGs), COCGs in the presence of theca cells (COCGs+theca) and COCGs in theca-cell conditioned medium (CMtheca). The percentages of oocytes that reached the MI (metaphase I) or MII (metahase II) stages are depicted, and the number of oocytes analysed per group is indicated in parentheses. a, b, c Bars with different letters differ significantly with respect to the percentage of oocytes that underwent germinal vesicle breakdown (GVBD) (P<0.05).

apoptosis rate, formation of the glycoprotein capsule) characteristics of resulting embryos. Embryos were produced by ICSI of IVM oocytes, followed by either culture in synthetic oviductal fluid (SOF: Galli *et al.* 2001) or temporary transfer to the oviduct of a progesterone-treated ewe. Control *in vivo* embryos were flushed from the uterus of inseminated mares 6–9 days after ovulation. In summary, IVP embryos were smaller with significantly fewer cells (Table 1), a smaller blastocoele, and a less distinct inner cell mass than *in vivo* embryos of a similar age. In addition, IVP embryos contained a higher percentage of apoptotic cells, irregularities in cell size and shape, a disturbed pattern of microfilament distribution, and they hatched aberrantly (Tremoleda *et al.* 2003). The influence of culture on formation of the blastocyst capsule was examined using the capsular-glycoprotein specific monoclonal antibody OC-1 (Oriol *et al.* 1993). It transpired that while IVP embryos secrete capsular glycoproteins, the latter fail to coalesce into a confluent layer enveloping the embryo. Since absence of a capsule compromises embryo survival *in utero*, but transferred Day 7 IVP embryos can develop into normal pregnancies, it must be assumed that capsule coalescence can still occur if IVP embryos are transferred early enough into a mare´s uterus. Certainly, this study established a useful set of baseline embryo quality parameters for examining how well culture conditions mimic the uterine environment.

Conclusions

Despite the inadequacies of current equine IVP systems, normal foals have been produced and blastocyst production rates are continuing to improve. This suggests that IVP has a future in horse breeding for accelerating genetic progress, salvaging endangered germ-lines and investigating or treating sub-fertility. However, before IVP is ready for widespread commercial use, efficiency needs to be improved and further studies need to establish that carry-over effects on the health of resulting foals are minimal.

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SESSION II:

Chairman:
Pascale Chavatte-Palmer

METABOLIC EFFECTS ON THE REPRODUCTIVE TRACT ENVIRONMENT AND CONCEPTION RATES IN THE DAIRY COW

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Timing and extent of embryo and fetal losses in the dairy cow

Dairy cows generally calve for the first time at about 23–27 months of age. They are subsequently inseminated from about 8 weeks into each lactation with the aim of maintaining an annual calving interval. Pregnancy must therefore be initiated while the animal is concurrently growing and/or lactating. These demands will therefore compete with the reproductive tract for the supply of nutrients.

Over the past 50 years intense genetic selection in the dairy industry has produced an animal with a greater ability to produce milk. However both fertility and longevity have declined over the same period. Failure to establish a pregnancy is the main reason for involuntary culling and calving rates to a single insemination at observed oestrus are generally below 40%. Table 1 summarises the losses reported at different stages of pregnancy, with most (around 40%) occurring in early gestation. Flushing/slaughter experiments indicate that some of these embryos are already degenerating within the first week. Others are still present at Day 16, but have not expanded greatly by this time. Such small embryos produce significantly less interferon τ (the maternal pregnancy recognition signal) and may in consequence be unable to inhibit luteolysis (Robinson *et al.* 2006). The increased use of ultrasound scanning in recent years has suggested that embryo losses in the second month of gestation have also increased to about 20%. The reasons for this are largely unknown, but may be due to genetic defects, disease, or poor placental development, which would in turn be associated with an inadequate nutrient supply to the developing conceptus.

Metabolic effects on fertility

Many studies which have measured blood metabolites around the time of insemination have failed to provide strong associations between the current metabolic indices and the ability to conceive. Instead, attention is increasingly focussing on the time immediately before and after calving when the cow enters a period of negative energy balance (NEB) from which it may take her many weeks to recover. In late gestation and early lactation the nutrient requirements for fetal growth and milk synthesis increase dramatically and the cow is unable to meet the energetic demands for glucose, amino acids and fatty acids from her feed. The deficit is dealt with by mobilising lipid and muscle. Poor EB status in the peripartum period can have carry over effects on fertility some months later.

In order to investigate the possible relationships between metabolic status and fertility a dataset has been compiled of 500 lactations in which metabolic traits were measured in all cows at 4 time points in relation to calving: -1 week and +2, +4 and +7 weeks (Wathes *et al.* 2007a, 2007b). Stepwise multiple regression analyses were used to establish those traits which made a significant contribution to explaining the variations in fertility. Both long calving intervals and failure to conceive were associated with altered concentrations of urea measured at weeks -1 and +7 and of IGF-I in weeks -1 to +4 post partum. However the pattern differed considerably between first lactation and older cows (Table 2). First lactation cows produce significantly less milk and are still growing. In these animals IGF-I concentrations were consistently much higher than in older cows and were not strongly related to fertility, whereas in

TABLE 1: Summary of timing of embryo and fetal losses in dairy cows from conception to birth

Stage of life	Starting No.	% died	Reasons
Insemination	100		
Fertilisation	90	10%	Fertilisation failure, wrong time AI
Pregnant at 24 days	54	40%	Early embryo loss
Pregnant at 2 months	43	20%	Late embryo loss
Late gestation	41	5%	Abortion
Alive at 24 h post partum	38	7%	Perinatal mortality
Live heifer	19	50%	Male calves

Data from Wathes (1992); Roy (1990), Mann *et al.* (1999), Lucy (2001).

TABLE 2: Metabolic traits measured at particular times in relation to calving associated with poor fertility in dairy cows*

Time relative to calving	Primiparous	Multiparous	Multiparous
	Long interval to conception		Failure to conceive
-1 week	High BCS High urea	High leptin Low NEFA Low urea	Low IGF-I
+2 to +4 weeks		Low IGF-I High PMY	Low urea
+7 weeks	High urea Low BCS	High urea High PMY Low BCS	Low urea

*Data from Wathes *et al.* (2007a, 2007b).
BCS, body condition score; PMY, peak milk yield.

multiparous cows lower IGF-I concentrations were associated with both longer intervals to conception and failure to conceive. In both age groups high blood urea concentrations were associated with longer intervals to conception, whereas in multiparous cows lower circulating urea levels were found in cows which failed to conceive at all.

Poor fertility was thus associated with altered profiles of IGF-I and urea in the peripartum period. Regulation of IGF-I production is discussed below. High urea concentrations are predominantly associated with an excess intake of degradeable protein over energy, which leads to increased ammonia production by rumen microbes. This is transported to the liver and metabolised to urea. Blood urea may also rise as a consequence of muscle proteolysis, which can occur to meet a nutrient deficit. Low urea may be due to inadequate protein intake, which may be caused by low feed consumption or provision of poor quality forage (Moore and Varga 1996).

Effects of negative energy balance on the liver

In order to investigate the effects of energy balance status on the reproductive tract a model which produces changes in EB in early lactation cows was used. At calving cows were randomly allocated to mild (mNEB) or severe (sNEB) EB groups (each n=6). mNEB cows were fed ad libitum grass silage and 8 kg/day concentrates and milked x 1 daily whereas sNEB cows were restricted to 25 kg/day silage and 4 kg/day concentrate and milked x 3 daily (Patton *et al.* 2006). Tissues were collected 2 weeks post partum, at the depth of the NEB nadir. These treatments resulted in decreases in actual EB and blood glucose measured, whereas circulating concentrations of NEFAs, BHB and urea all increased in the sNEB cows.

EB is well known to have a profound effect on the IGF system within the liver. In a recent study (Fenwick *et al.* 2008, Table 3) the most dramatic

TABLE 3: Summary of measured effects of severe negative energy balance on expression of the IGF system in the liver and reproductive tract of dairy cows at 2 weeks post partum+#

mRNA *	IGF-I	IGF-II	IGF-1R	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Relative affinity				I=II	II>I	I=II	I=II	II>I	II>I
Liver (PCR)	↓↓	=	↓	=	↑	↓	↓	↓	↓
Ovary, 1-5 mm follicles (ISH)	ND	=	=	ND	↓	ND	=	=	=
Oviduct (PCR & ISH)	=	=	=	ND	↓	=	=	↑	↓
Uterine endometrium (ISH)	ND	=	=	ND	↑	=	↓	=	↓

*Measured by quantitative PCR and/or *in situ* hybridisation (ISH). ND = not detectable.
+ The comparison was between cows in mild or severe NEB, with 6 cows per group.
# Data from Fenwick et al. (2006); Llewellyn *et al.* (2006) and same authors unpublished.

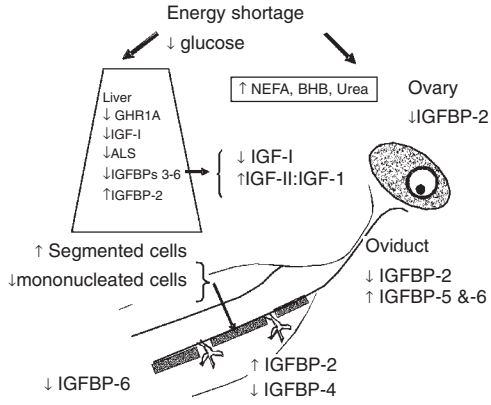

Fig 1: Diagram summarising the main changes detected in the blood and tissues of dairy cows measured at 14 days post partum when the animals were in severe negative energy balance.

changes in RNA expression (measured by quantitative PCR) were a 27 fold decrease in the liver specific GH receptor (GHR-1A), a 14 fold decrease in the acid labile subunit (ALS) of IGFBP-3 and a 10 fold decrease in IGF-I. Expression of both the type 1 and 2 IGF receptors and of IGFBPs 3-6 all decreased about 2-3 fold in the severe NEB group. In contrast, expression of IGFBP-2 mRNA increased 2 fold. These changes led to a 5 fold decrease in circulating IGF-I. The changes in expression would predict a shift in relative importance of the trinary complex of IGFBP-3 (which includes the ALS) to the binary complexes, in particular IGFBP-2, which would in turn reduce the half life of IGF-I in the circulation.

Effects of negative energy balance on the reproductive tract

Within the reproductive tract we have used a combination of quantitative PCR and in situ hybridisation to determine relative changes in expression of the IGF system (Table 3 and Fig 1). Within antral follicles, IGF-II is highly expressed by the theca whereas the IGF-I in ovine follicular fluid is derived from the circulation. IGFBPs -2,-4,-5 and -6 are all expressed in the theca and/or granulosa where they are thought to regulate the bioavilability of the IGFs (Wathes *et al.* 2003). IGFs in follicles can stimulate both proliferation and steroidogenesis. Cows in sNEB had significantly reduced expression of IGFBP-2 mRNA in the walls of the 1–5 mm follicles (Llewellyn *et al.* 2007).

In the oviduct IGFBP-2 mRNA expression was also reduced by sNEB whereas concentrations of both IGFBP-5 and -6 mRNAs were increased. The uterus was more similar to the liver in that IGFBP-4 and -6 mRNAs were reduced, whereas expression of IGFBP-2 increased in response to sNEB.

Histological sections taken from both uterine horns were also examined for the presence of immune cells. Cows in the sNEB group had more segmented inflammatory cells but fewer mononuclear cells in both the stratum compactum and the luminal epithelium. There were also more capillaries in the stratum compactum and fewer glands. These differences suggest that a poor EB status is associated with a greater degree of uterine inflammation following calving and a slowing of

the repair process. Bonnett and Martin (1995) previously reported that similar histological findings were associated with poor reproductive performance.

Summary of effects of NEB on the reproductive tract

In summary, it has been shown that delays or failure of conception in post partum dairy cows are associated with reduced IGF-I concentrations and either higher or lower urea values. Both liver mRNA and circulating concentrations of IGF-I were highly correlated in individual cows and were significantly reduced by sNEB. In contrast, the EB status did not alter the expression of IGF-II in the liver, ovary, oviduct or uterus. It was also noticeable that those IGFBPs whose expression altered in the reproductive tract (IGFBP-2, -5 and -6) all have higher affinity for IGF-II than IGF-I. These changes suggest that the importance of IGF-II signalling relative to that of IGF-I will increase during sNEB, although the consequences of this are currently unknown. It was also of note that IGFBP-2 expression changed in all tissues examined but not in a consistent direction. A review by Hoeflich *et al.* (2001) concluded that 'the regulation of IGFBP-2 expression and abundance is highly complex and influenced by multiple hormones and growth factors'. Furthermore, IGFBP-2 can have both stimulatory and inhibitory effects on cell proliferation in different systems. While we do not currently have the evidence to say precisely how these measured changes in the IGF system affect fertility, it is tempting to speculate that they may be associated with the poor follicular development and the delayed repair to the post partum uterus which have been observed. At the present time, there is also uncertainty as to whether the elevated urea concentrations are directly responsible for decreasing conception rates, possibly due to an associated decrease in pH in the uterine lumen (Rhoads *et al.* 2004). Alternatively they may be just another marker of an underlying poor EB status which could influence fertility through other mechanisms (Laven *et al.* 2007).

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PROGESTERONE REGULATION OF PERI-IMPLANTATION CONCEPTUS DEVELOPMENT AND IMPLANTATION: GENES AND CONUNDRUMS

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INTRODUCTION

Establishment of pregnancy in domestic ruminants (sheep, cattle, goats) begins at the blastocyst stage and involves co-ordinate pregnancy recognition signalling and conceptus implantation. The phases of implantation can be designated as: 1) shedding of the zona pellucida; 2) pre-contact and blastocyst orientiation; 3) apposition; 4) adhesion; and 5) endometrial invasion (Guillomot 1995; Spencer *et al.* 2004a). After hatching from the zona pellucida on Day 8, sheep blastocysts develop into a tubular form by Day 11 and then begin to elongate on Day 12 to form a conceptus (embryo/fetus and associated extra embryonic membranes) of 25 cm or more in length by Day 17. Elongation of the blastocyst is critical for developmentally regulated production of interferon tau (IFNT), the pregnancy recognition signal, and for implantation (Farin *et al.* 1989; Guillomot *et al.* 1990; Gray *et al.* 2002). Between Days 9 and 14, close association between conceptus trophectoderm and endometrial luminal epithelium (LE) is achieved and followed first by adhesion and then interdigitation of cytoplasmic projections of trophectoderm cells and microvilli of the LE assures firm attachment in both the caruncular and intercaruncular areas by Day 16 of pregnancy. Hatched blastocysts and trophoblastic vesicles do not elongate *in vitro*, but do so when transferred into the uterus (Heyman *et al.* 1984; Flechon *et al.* 1986). Presumably, uterine secretions are required for post hatching blastocyst development.

FUNCTIONAL ROLE OF THE ENDOMETRIAL EPITHELIA IN BLASTOCYST GROWTH AND ELONGATION

All mammalian uteri contain endometrial epithelia that synthesise and secrete or transport a complex mixture of amino acids, ions, glucose, enzymes, growth factors, hormones, transport proteins and other substances termed histotroph (Bazer 1975). During the pre-attachment period, nutrition of the conceptus depends on uterine secretions. The epithelial cells of the uterine lumen are highly secretory during implantation, and the trophectoderm exhibits intense pinocytotic activity which increases as the conceptus develops (Guillomot 1995). Therefore, factors supporting growth of pre- and peri-implantation blastocysts and elongating conceptuses are thought to be obtained primarily from uterine histotroph. This hypothesis is supported by results from studies of asynchronous uterine transfer of embryos and trophoblast vesicles (Lawson *et al.* 1983; Flechon *et al.* 1986) and from studies of uterine gland knockout (UGKO) ewes (Gray *et al.* 2001; Gray *et al.* 2002). The cellular and molecular mechanism(s) regulating trophectoderm outgrowth during blastocyst elongation, although not well understood, are hypothesised to require progesterone-dependent uterine secretions as well as apposition and transient attachment of the trophoblast to the LE that is mediated by factors of endometrial origin.

As the hormone of pregnancy, progesterone stimulates and maintains endometrial functions necessary for conceptus growth, implantation, placentation and development to term (Bazer *et al.* 1979; Geisert *et al.* 1992; Spencer and Bazer 2002; Spencer *et al.* 2004b). Circulating concentrations of progesterone in early pregnancy affect blastocyst survival and growth during early pregnancy (Mann and Lamming, 1999). The mechanisms whereby progesterone stimulates blastocyst survival and growth are not known, but presumed to be mediated by histotroph (Geisert *et al.* 1992). Uterine-derived growth factors, including colony stimulating

factor 2 (CSF2), fibroblast growth factor 2 (FGF2), insulin-like growth factor one (IGF1), and IGF2, stimulate IFNT production by cultured conceptuses and isolated mononuclear trophectoderm cells (Ko *et al.* 1991; Imakawa *et al.* 1993; Michael *et al.* 2006); however, these effects could be either direct or indirect due to effects on cell proliferation. Progesterone acts on the endometrium to induce a number of epithelial genes, including CST3 (cystatin C), CTSL (cathepsin L), GLYCAM1 (glycosylated cell adhesion molecule one), LGALS15 (galectin 15), and SPP1 (secreted phosphoprotein one) in a stage-specific manner that are hypothesised to regulate conceptus development during the peri-implantation period of pregnancy. Paradoxically, progesterone induction of those genes appears to be via the loss of progesterone receptors (PGR) in the endometrial epithelia as discussed next.

Progesterone receptor (PGR) regulation and endometrial gene expression

In most mammalian uteri, PGR are expressed in endometrial epithelia and stroma during the early to mid-luteal phase, allowing direct regulation (induction or repression) of genes by progesterone. However, continuous exposure of the endometrium to progesterone negatively regulates PGR expression in the endometrial LE and glands. In the ovine uterus, PGR protein is not detectable in LE and glands after Days 11 and 13 of pregnancy, respectively, but can be detected in the uterine stroma and myometrium throughout gestation (Spencer *et al.* 2004c). The paradigm of loss of PGR in uterine epithelia immediately prior to implantation is common in domestic ruminants and across mammals (Carson *et al.* 2000). PGR loss in those epithelia is determined by timing of the post ovulatory rise in progesterone and requires continuous exposure to progesterone, which in sheep is at least 8 days. Thus, an earlier increase in circulating progesterone advances the timing of PGR loss from uterine epithelia. Indeed, PGR loss in the endometrial LE is strongly associated with a reduction in expression of the anti-adhesive MUC1 (mucin glycoprotein one) and induction of LGALS15, GLYCAM1, CST3 and CTSL, which are hypothesised to regulate conceptus implantation in sheep (Burghardt *et al.* 2002; Johnson *et al.* 2003; Spencer *et al.* 2004a) for review). In the endometrial glands, PGR loss is associated with induction of SPP1, STC1 (stanniocalcin 1) and SERPIN (serine proteinase inhibitors or uterine milk proteins). Indeed, inhibition of progesterone action by an anti-progestin prevents PGR down-regulation and, in turn, progesterone induction of GLYCAM1, LGALS15, CTSL, CST3, SPP1, STC1 and SERPINs. Thus, anti-progestins inhibit progesterone down-regulation of PGR that, in turn, inhibits inductions of adhesion molecules and other factors needed for conceptus implantation.

Secreted adhesion molecules of the ovine uterus

Galectin 15 (LGALS15)

Galectins are proteins with a conserved carbohydrate recognition domain that binds beta-galactosides, thereby cross-linking glycoproteins as well as glycolipid receptors on the surface of cells, such as integrins, and initiating biological responses (Yang and Liu 2003). Galectin 15 (LGALS15; alias ovgal11) was originally identified in ovine intestinal epithelium as being induced in response to infection by the nematode parasite *Haemonchus contortus* (Dunphy *et al.* 2000). Interestingly, LGALS15 is the 14K protein from sheep endometrium initially characterised as a progesterone-modulated protein associated with crystalline inclusion bodies in uterine epithelia and conceptus trophectoderm (Kazemi *et al.* 1990). LGALS15 is implicated in conceptus implantation (Spencer *et al.* 2004a), because functional studies of other galectins have implicated these proteins in cell growth, differentiation and apoptosis as well as in cell adhesion, chemoattraction and migration (Yang and Liu 2003). Indeed, some galectin family members are involved in both innate and adaptive immune responses and participate in the activation or differentiation of immune cells. Similar to CTSL and CST3, LGALS15 is induced by progesterone in the endometrial LE and superficial glands between Days 10 and 14 and is further increased by IFNT between Days 14 and 16 of pregnancy (Gray *et al.* 2004). Ovine trophectoderm cells attach to LGALS15 *in vitro* (unpublished results).

Secreted Phosphoprotein One (SPP1)

SPP1 (alias osteopontin or OPN) is a member of the Small Integrin-Binding Ligand, N-Linked Glycoprotein (SIBLING) family of related extracellular matrix proteins recognised as key players in a number of diverse processes such as bone mineralisation, cancer metastasis, cell-mediated immune responses, inflammation, angiogenesis, and cell survival (Sodek *et al.* 2000; Johnson *et al.* 2003). During the peri-implantation period of pregnancy in sheep, SPP1 mRNA is first detected in the endometrial glands of some ewes by Day 13 and is present in all glands by Day 19 (Johnson *et al.* 1999). In the uterine lumen, SPP1 protein appears on Day 15 and is found at the trophectoderm-LE interface throughout gestation, suggesting that it plays a key role in adhesion of the trophectoderm to LE via integrin receptors (Johnson *et al.* 2001). Ovine trophectoderm and LE cells show evidence of integrin receptor activation and cytoskeletal reorganisation in response to SPP1 binding *in vitro* (Johnson *et al.* 2001). Progesterone induces expression of SPP1 in endometrial glands, and this requires loss of PGR (Spencer *et al.* 1999; Johnson *et al.* 2000). SPP1 is hypothesised to serve as a bifunctional bridging ligand that mediates adhesion between LE and trophectoderm essential for implantation and placentation in sheep (Johnson *et al.* 2003).

Conclusions

During the past decade, knowledge of mechanisms and factors regulating fetal-maternal interactions during establishment of pregnancy has increased in domestic ruminants. Transcriptional profiling studies are now accelerating the pace of discovery; however, our knowledge of cellular and molecular mechanisms governing fetal-maternal interactions and, in particular, progesterone actions and trophectoderm growth and differentiation remain very limited. Results from studies of rodents strongly suggest that implantation involves a multiplicity of receptor-ligand interactions that are organised into a combinatorial cascade (Aplin 1997). Therefore, individual and integrative roles of adhesion factors must be mechanistically determined using *in vivo*, *ex vivo* and *in vitro* experimental models. Pregnancy loss in domestic animals is greatest during the period of pregnancy recognition and establishment (Mann and Lamming 2001). Therefore, a more complete understanding of key molecules and signal transduction pathways that regulate fetal-maternal interactions during establishment and maintenance of pregnancy can be used to diagnose and identify the cause(s) of recurrent pregnancy loss and improve pregnancy rates and reproductive efficiency in domestic animals and humans.

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FETAL GROWTH AND DEVELOPMENT

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Low birth weight lambs make up the greatest percent of mortality observed in the first few days following birth. Approximately 65% of lambs born less than 4 lbs die in the first few days, while lambs born only a pound heavier have a 50% greater survival rate (Shelton 1964). A greater understanding of fetal growth and development may result in an increase in weight of the low birth weight lambs, dramatically decreasing early post natal mortality. Furthermore, several researchers are trying to examine the effects of adverse uterine environment on fetal growth and development, which leads to low birth weight, and how these developmental alterations may influence the onset of adult diseases. This is a concept called 'fetal programming'.

Fetal programming is the term used to describe effects of an altered uterine environment during the development of key tissues and organs, which can lead to permanent alteration in organ function during adult life (Drake and Walker 2004). In particular type II diabetes, hypertension, glucose intolerance, insulin resistance, hyperlipidemia and renal failure have all been linked to low birth weight (Fowden and Forhead 2004). Several animal models have been used in an attempt to further our understanding of fetal programming. The current animal models examine fetal programming by applying a stressor directly to the dam by limiting nutrient intake (ie energy and protein restriction), hormone administration (ie glucocorticoid exposure) or heat stress (Fowden and Forhead 2004). As expected, these treatments are often detrimental to growth and development of the offspring, but results vary depending on the particular type and duration of treatment. In some fetal programming models, offspring exhibit lower birth weights when compared to controls while others report no difference in birth weight, but observed altered peripheral vascular resistance, metabolism and hormonal responses (Jensen *et al.* 2002; Bloomfield *et al.* 2003; Fowden and Forhead 2004).

Current animal paradigms for fetal programming induce stress, which alters the hypothalamic pituitary adrenal axis, as opposed to directly altering the growth hormone axis. In the hypothalamic pituitary adrenal axis, fetal glucocorticoids rise in response to stressors and it is these glucocorticoids that can alter development. High levels of glucocorticoids have been found to alter development of specific tissues depending on the time of gestation and the amount of glucocorticoids administered. Indirectly, high levels of glucocorticoids can block the effects of growth hormone leading to a decrease in insulin like growth factor-I (IGF-I) or glucocorticoids can directly block the effects of IGF-I (Jensen *et al.* 2002). In low nutrient intake paradigms the nutrients available for fetal development are already reduced and with low IGF-I, as a result of glucocorticoid administration, the stimulus for growth and development is highly reduced (Fowden and Forhead 2004).

Proper fetal development requires a fully functional growth hormone axis which appears to be fully functional before birth and be under similar regulation as the adult axis. Disruption of this axis during development may result in permanently abnormal growth patterns. Treatments that result in elevated concentrations of fetal growth hormone have been shown to increase body length at birth (Bauer *et al.* 2000). The opposite is true in subjects with an observed growth hormone deficiency during fetal development in that they are born with a decreased body length at birth, but no observable alteration

in body weight or bone development. Administration of IGF-I for 10 days to a normally developing fetus results in increased growth of the visceral organs (ie increase in abdominal size), but not of long bones or muscle (Bauer *et al.* 2003). Others have shown that IGF-I can not attenuate the effects caused by intra-uterine growth restriction (Bauer *et al.* 2003). Contrary to the current models for altering fetal programming few experimental paradigms have attempted to study the effects of a positive stimulator on intra-uterine growth and how it may alter post natal growth and development. Recently our laboratory has been working on developing an experimental paradigm that stimulates both growth and development of the fetus through treatment of the dam with growth hormone at breeding.

Growth hormone at breeding

A single injection of sustained release growth hormone given at breeding appears to positively alter fetal growth and development. A single injection of growth hormone at breeding increases both maternal serum IGF-I and the concentration of IGF-I in uterine flushings by Day 7 of gestation (Costine *et al.* 2005). By the seventh day post fertilisation the embryo has already reached the blastocyst stage which is made up of both an inner cell mass, which will become the embryo proper, and trophectoderm, which will become the fetal portion of the placenta. Trophoblast exposure to an altered uterine environment as early as Day 7 may alter placental function and efficiency later in gestation. Placental efficiency is increased by growth hormone treatment at Day 80 of gestation, as both growth hormone and control fetuses are similar in size, but placentae from treated ewes are approximately one third of the size of control placentae (Costine *et al.* 2005).

Growth hormone at breeding alters the overall composition of the lambs, in that lambs born to ewes treated with growth hormone at breeding are 20% heavier and have a greater abdominal girth compared to lambs born to control ewes, but there is no difference in crown rump length between control lambs and growth hormone lambs. Not only is the physical size of the lambs used to measure the effects of intra-uterine environment on growth and development, but organ size and function are important to consider when assessing permanent alterations. Increased heart weight or wall thickness are thought to reflect elevated mean arterial pressure *in utero*, which may lead to adult hypertension. Lambs born to ewes treated with growth hormone have a thinner left ventricular wall than those born to control ewes, which probably relates to a decreased peripheral vascular resistance *in utero*, giving some insight on how cardiovascular function is changed by growth hormone treatment. Treating ewes at breeding appears to manipulate the growth hormone and IGF-I axis as well. Lambs born to ewes treated with growth hormone have a reduced expression of hepatic growth hormone receptor and hepatic IGF-I compared to controls. Surprisingly even with a reduced expression of IGF-I there is no difference in the concentrations of IGF-I found in fetal serum between control and growth hormone groups on Days 80 and 140 of gestation or in lambs following birth (Costine *et al.* 2005). There is however a positive correlation between birth weight and serum IGF-I.

Placental transport of nutrients and amino acids from maternal to fetal circulation is essential for proper growth and development of the fetus. The limiting factor in the flux of nutrients across the placenta is transporters for specific molecules. L type amino acid transporters are sodium independent transporters that transfer aromatic amino acid or those that have large side changes. Excitatory amino acids transporters are sodium dependent and transport amino acids like glutamate and aspartate. It was observed that the expression of L-amino acid transporter-2 is greater on Days 80 and 140 of gestation in the placentae of the growth hormone group compared to controls. Expression of glucose transporters 1 and 3 increased from Day 80 to 140 in controls, however expression of these 2 glucose transporters did not change in the growth hormone group and was lower in the treated group on Day 140 compared to the control group. Expression of transporters is most likely increased because glucose is limiting to the fetus. Excitatory amino acid transporters 1 and 3 were not different between control and treated groups; however, expression of both transporters increased from Day 80 to 140 of gestation. Fetal weight and crown rump length are positively correlated to both glucose transporter 1 and 3. However IGF-I is negatively correlated with all 3 nutrient transporters.

Ameliorating the negative impacts of a detrimental uterine environment and reducing the

number of low birth weight and therefore high risk lambs requires that we understand more than what happens in response to a poor uterine environment. The use of growth hormone treatment at breeding appears to alter the uterine environment in a positive way increasing the birth weight of the lamb and altering its physiology in a manner opposite to that often described for fetuses exposed to a detrimental uterine environment. A greater understanding of endocrine and metabolic responses to the treatment with growth hormone at breeding should allow for rationale manipulation of nutrition and management to reduce lamb mortality and the detrimental consequences of exposure to poor environment *in utero*.

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NUTRITIONAL INSIGHTS INTO THE ORIGINS OF EMBRYONIC LOSS IN THE PIG

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INTRODUCTION

In pigs, approximately 30% of oocytes shed at the time of mating are not represented by piglets at birth. The majority of pre-natal death occurs during embryonic life, which coincides with the first few weeks of pregnancy. A range of factors including maternal maturity, nutrition, genotype, endocrine status and rate of embryo development are known to be associated with embryo mortality in the pig (reviewed by Ashworth and Pickard 1998). However, attempts to reduce embryo death through post mating modifications to maternal nutritional and/or endocrine status have achieved only limited success. New opportunities to improve prenatal survival in pigs arise from the growing body of evidence suggesting that oocyte quality is a major determinant of subsequent embryo survival. Hence, strategies that promote oocyte quality would be expected to improve pre-natal survival.

THE LINK BETWEEN OOCYTE MATURITY AND EMBRYO SURVIVAL

Several studies indicate that in the pig, as in other species, the period of oocyte maturation represents a nutritionally sensitive stage of development. Importantly, nutritionally induced changes in oocyte maturation may affect not only the oocyte itself, but can also impart a legacy for the development of the resultant embryo and fetus. For example, primiparous sows fed to appetite from Day 21–28 of lactation had a higher proportion of oocytes that developed to metaphase II of meiosis *in vitro* and greater pre-natal survival to Day 28 of pregnancy compared with sows in which feed intake was restricted during the same period (Zak *et al.* 1997). Similarly, gilts fed increased rations prior to mating, a regimen associated with increased embryo survival, had a higher proportion of oocytes that reached metaphase II *in vitro* than gilts receiving a maintenance diet during the same period (Ferguson *et al.* 2003). In addition to the amount, the composition of the diet consumed before mating also affects oocyte maturation and pre-natal survival. Studies involving alterations to the protein, starch or fibre content of the pre-mating diet showed that increasing the content of dietary fibre prior to mating improved embryo survival (Ferguson *et al.* 2006). A series of studies was therefore conducted examining the effects of a high fibre diet prior to mating on reproductive traits in the pig. More oocytes recovered on Day 19 from gilts receiving a high fibre diet were at metaphase II after 46 h of culture in medium containing 10% of their own follicular fluid, and gilts fed such diets had 18% higher embryo survival on Days 27–29 of pregnancy, compared to control fed gilts (Feguson *et al.* 2007). This provides the first evidence of a direct link between oocyte maturity and embryo survival in the gilt. This feeding regimen also achieved an average benefit of an extra 0.96 piglet per litter when tested in multiparous sows in a commercial environment (Ferguson *et al.* 2004).

While such results are compelling, it is important to determine whether such dietary regimens alter not only oocyte maturity, but also oocyte quality, as determined by their ability to form a blastocyst. The recent study described here assessed the ability of oocytes recovered from gilts fed either a control or a high fibre diet to form a viable blastocyst *in vitro* and estimated blastocyst cell number.

TABLE 1: Effect of a high fibre diet prior to ovulation on follicular fluid oestradiol and *in vitro* fertilisation

	Control	High Fibre	P
% Gilts producing blastocysts	55 (11/20)	27 (6/22)	0.042
Cleavage rate (%)	53 ± 1.8	41 ± 6.9	0.245
Blastocyst rate (%)	26 ± 1.71	34 ± 3.0	0.43
Blastocyst cell number on Days 6 and 7	31 ± 1.6	39 ± 2.4	0.012
Follicular fluid oestradiol (ng/ml)	92.8 ± 15	111.1 ± 14	0.361

These data provide preliminary evidence to support the hypothesis that a high fibre diet increases prenatal survival by improving oocyte quality.

Materials and methods

Large White x Landrace gilts were fed either a high fibre (50% unmolassed sugar beet pulp inclusion; n=22) or a control barley based (n=20) diet at 1.8 x maintenance during their third post pubertal oestrous cycle. A maximum of 16 oocytes and associated follicular fluids per gilt were obtained following slaughter on predicted Day 19. Oocytes were matured *in vitro* in TCM 199 supplemented with LH and FSH at 0.5 µg/ml and 10% of the animals own pooled follicular fluid, fertilised and the resultant embryos cultured in NCSU-23 medium for 6 or 7 days. Blastocyst cell numbers were determined by fixing the blastocysts and staining with Hoescht 33258. Follicular fluids were pooled within animals and oestradiol concentrations determined by radio-immunoassay.

Results

Fewer gilts fed the high fibre diet had oocytes that fertilised and produced blastocysts. Blastocysts formed from oocytes from gilts fed the high fibre diet had more cells than those recovered from control gilts (Table 1).

Discussion

Collectively, the data described in this paper highlight that the period of oocyte maturation within the ovarian follicle is sensitive to alterations in maternal nutrient supply, and that the changes induced have immediate effects on oocyte maturation and also impart longer-term effects on the embryo. The concept that embryo viability is a consequence of oocyte quality highlights the need to understand determinants of oocyte development. Central to the appropriate development of the oocyte is the follicular environment in which the oocyte matures (Hunter 2000). There is some evidence that altered nutrient intake affects the composition of follicular fluid. For example, sows fed a low lysine diet during lactation had follicular fluids with reduced oestradiol concentrations that were less able to support oocyte maturation *in vitro* (Yang *et al.* 2000a). Furthermore, increased feed intake prior to mating, which increases oocyte maturity and embryo survival, is also associated with increased follicular fluid oestradiol concentration (Ferguson *et al.* 2003). This is clearly not a universal mechanism by which altered nutrition affects oocyte development, as our studies have not consistently provided evidence of altered follicular fluid oestradiol concentrations in gilts fed the high fibre diet.

The mechanism(s) whereby altered feed intake modifies the preovulatory follicle clearly warrant further study. Changes to the quantity (Ferguson *et al.* 2003) and composition (Yang *et al.* 2000b) of the diet alter circulating levels of intermediary metabolites and reproductive hormones, which in turn would be expected to alter ovarian function. For example, in the studies of Ferguson *et al.* (2003), increased feed intake was not only associated with an increase in the proportion of oocytes at metaphase II, but also with an increased number of LH pulses, reduced circulating concentrations of oestradiol and progesterone and increased insulin-like growth factor 1 and leptin concentrations.

The increase in blastocyst cell number observed following *in vitro* fertilisation of oocytes recovered from gilts fed the high fibre diet is intriguing in that other studies in which pre-mating nutrition has increased embryo survival also report increased blastocyst cell numbers (Ashworth *et al.* 1999). Further studies are required to determine the importance of blastocyst cell number in nutritionally mediated improvements in embryo survival.

Greater awareness of the opportunities to reduce embryo loss through appropriate gilt and

sow nutrition during oocyte development could provide consumer acceptable means to improve reproductive outcome in the pig.

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SESSION III:

Chairman:
Sandra Wilsher

THE PERI-CONCEPTION AND PRE-IMPLANTATION ENVIRONMENT AND SUBSEQUENT PLACENTAL DEVELOPMENT

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The placenta has myriad functions, the best known of which are the transfer of oxygen and nutrients from the maternal to the fetal circulations and synthesis of a variety of steroid and peptide hormones that regulate placental growth and function, maternal adaptation to pregnancy and preparation for lactation (Gude *et al.* 2004). Hence, optimal placental function is key to pregnancy success. Poor placental development has been associated with a variety of pregnancy complications, most notably intra-uterine growth restriction (IUGR) and pre-eclampsia. Both epidemiological and animal studies have shown that disproportionately poor growth of the fetus programs the individual inducing a predisposition to a variety of adult onset diseases including cardiovascular disease and type 2 diabetes (Barker 1995). Maternal nutrition at critical times during pregnancy has been implicated.

Gene ablation studies have shown important roles for insulin-like growth factor (IGF) –II in placental growth and function. Global ablation of the IGF-2 gene restricts both placental and fetal growth while that of IGF-1 restricts fetal growth only (DeChiara *et al.* 1990; Baker *et al.* 1993; Liu *et al.* 1993). Placenta specific ablation of the IGF-2 gene induces similar effects to those observed in the global knockouts but the restriction in fetal growth follows that of the placenta by 2 days (Constancia *et al.* 2002, 2005; Sibley *et al.* 2004). In addition, IGF-II promotes extravillous cytotrophoblast invasion of the decidua and its vasculature (Irving and Lala 1995), a process that occurs in early to mid-gestation and is critical to subsequent placental function and pregnancy success.

Research over the last 10 years has shown that the pre-implantation environment profoundly affects subsequent fetal development. Maternal protein restriction in rats for the first 4 days of pregnancy altered cell allocation in the blastocyst, reduced birth weight, programmed post natal blood pressure, growth and body composition (Kwong *et al.* 2000). In addition, previous studies in mice have shown that embryo culture with the addition of fetal calf serum alters fetal imprinted gene methylation and transcription (Khosla *et al.* 2001). Of increasing concern is the observation that these deleterious effects may indeed also apply to human babies born following assisted reproduction (Thompson *et al.* 2002).

However, little research has focussed on the effect of perturbation of the peri-conceptional and/or pre-implantation environment on subsequent placental growth, gene expression and function. Over recent years the authors have been investigating the effect of various insults in the peri-conceptional and/or pre-implantation environment on placental differentiation and gene expression later in gestation.

Mice were undernourished (90 and 95% of ad libitum intake) from 14 days prior to mating and up to Day 5 of pregnancy and euthanised at Day 17 (term = 19 days). Of the mice that became pregnant, it took 3 times longer for food restricted females to mate after pairing with a male than ad libitum fed mice (100% 4.47±0.9 days, 95% 14.12±2.2 days, 90% 11.75±1.6 days). Some undernourished mice did not become pregnant until their food intake had been restored to ad libitum and they regained the weight lost during the period of under-nutrition. This suggests that even a very small reduction in food intake in mice has profound effects on oestrous cyclicity and may affect oocyte development. Although fetal weight was slightly lighter and placental weight slightly heavier with a reduced fetal weight : placental weight ratio at Day 17, these were not

significantly different between groups (n=5-7 dams in each group).

Food restriction in mice at 95% ad libitum intake from 2 weeks before and throughout pregnancy reduced fetal weight by about 20% but did not affect placental weight. However, placental architecture was altered with a 20% increase in the proportion of the placenta composed of the junctional zone ($P<0.05$) and a reduction in the ratio of the placental labyrinth (exchange region) to the junctional zone, which indicates delayed placental structural maturation and is consistent with the reduced fetal weight observed. It is also similar to previous observations in the undernourished pregnant guinea pig (Roberts *et al.* 2001).

When ewes were undernourished (70% maintenance diet) around conception (food restricted for 45 days before mating and up to Day 7 of pregnancy; PCUN), fetal and placental weights were normal at Day 55 of gestation (term = 147 days) (MacLaughlin *et al*. 2005). However, ponderal index in twin but not singleton fetuses was reduced by exposure to maternal food restriction during the peri-conceptional period. In addition, the relationships between maternal weight change at conception and fetal and placental weights were altered (MacLaughlin *et al.* 2005).

Peri-conceptional under-nutrition (PCUN as above and peri-implantation under-nutrition (PIUN) food restricted from mating to Day 7 of pregnancy) did not affect fetal and placental growth but altered placental gene expression at Day 138 gestation as quantified by reverse transcription qPCR. Placental igf2 transcription was reduced by 50% in PIUN in both singletons and twins and by PCUN in twins only ($P<0.05$). There was no difference in placental transcription between singletons and twins for any other gene analysed. Placental igf1 transcription was reduced by about 60% in PCUN while expression of igf2r and glucose transporter genes (Glut 1, 3 and 8) were unaffected (Fletcher *et al.* unpublished).

Recently, the authors have investigated the effect of embryo transfer only and *in vitro* embryo culture, with or without the presence of human serum, compared to naturally mated controls, on fetal and placental growth and placental gene expression. Fetal weight at Day 144 of gestation (term =147 days) was increased in singleton fetuses derived from embryos cultured in the presence of human serum compared to all other groups. However, in twins, fetal weight was reduced by embryo transfer and embryo culture but addition of human serum to the culture media restored fetal weight to normal. In twins, placental weights were reduced in each treatment group compared to those in naturally mated controls. Most interestingly, placental gene expression at Day 144 was similar in singletons and twins with reductions in igf2, igf2r and glucose transporters (glut1 and glut 3) in embryo transfer and culture groups compared to controls. Surprisingly little manipulation of the embryonic environment such as embryo transfer has the ability to alter the gene expression of the placenta nearly 140 days later near term.

It was shown recently that there is a profound effect of embryo culture from the 2 cell to blastocyst stages on murine placental morphogenesis, birth weight and post natal growth trajectory and body composition compared to *in vivo* derived controls (Sjoblom *et al.* 2005). Specifically, embryo culture reduced fetal growth and delayed structural and functional maturation of the placenta and programmed the fetus for adult obesity. The addition of GM-CSF to the culture media largely alleviated these effects, particularly those on the placenta.

Research in one of our laboratories first led to the discovery of the fetal overgrowth syndrome at term following the transfer of cultured embryos in sheep (Walker *et al.* 1992; Walker *et al.* 1996). Overgrowth was associated with heat inactivated human serum in the culture media. Further work with progesterone administered to ewes during the first three days of pregnancy perturbed fetal growth at Day 74 of gestation (Kleeman *et al.* 1994). Disproportionate growth of the fetus with increased relative weights of brain, heart and M. tibialis were observed (Kleeman et al. 2001). Most interestingly, although placental weight was not altered in these sheep, the volume and surface area of the trophoblast and feto-maternal syncytium (maternal epithelium) were increased by progesterone supplementation (Kleeman *et al.* 2001) and these were correlated with the increases in fetal relative organ weights.

These data and those of others show that the peri-conception and pre-implantation environment affect the individual from the blastocyst stage and program adult health. Early perturbations appear to have an impact on critical epigenetic events before implantation. Simple perturbations of the peri-conceptional and pre-implantation environment

have subtle effects on the placenta. These effects may be mediated by epigenetic changes in the pre-implantation conceptus that affect both the placenta and embryo and program post natal health and well being.

These have profound consequences for both animal and human reproduction. That all these effects are induced before pregnancy is detected, points to the importance of nutrition before pregnancy in all species of economic worth. In the human population, the health of women of child bearing age must become a major public health issue. In addition, assisted reproductive techniques require additional research to minimise the effect of *in vitro* embryo culture on health of the conceptus, infant and adult. The addition of specific growth factors to which the embryo is exposed in the oviduct may be beneficial.

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THE EQUINE ENDOMETRIAL GLAND: A BOUNTIFUL MILCH-COW OF PREGNANCY IN THE MARE

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The equine embryo can reasonably be said to go from one extreme to the other during its 11 month existence *in utero*. For the first month it retains a spherical outline due to the persistence of the enveloping glycoprotein blastocyst capsule until Day 23; it moves continually throughout the uterine lumen until Day 16 to announce its presence to the maternal organism and maintain the supply of luteal progesterone to support the 'pregnancy state', and thereby control the secretory functions and products of the endometrial glands; it relies entirely upon the imbibition of these histotrophic, endometrial gland secretions ('uterine milk') as its source of nutrients to sustain the differentiation and development of its membranes and vital organs; it secretes copious amounts of oestrogens onto the endometrium to increase local vascularity and promote 'uterine lactogenesis'; and it shows no inclination to form a stable attachment to the endometrium for the purposes of placentation until it has actively 'injected' its specialised invasive trophoblast cells of the chorionic girdle into the endometrium around Day 36–38 to form the equine-unique gonadotrophin (eCG)-secreting endometrial cups.

In contrast, by the final month of gestation, the outermost trophoblast layer of the non-invasive allantochorion has developed an incredibly complex, multi-branched interdigitation with the endometrial epithelium to form the haemotrophic gaseous and nutrient exchange microplacentomes which unite endometrium and allantochorion over the entire surface of both organs; the total

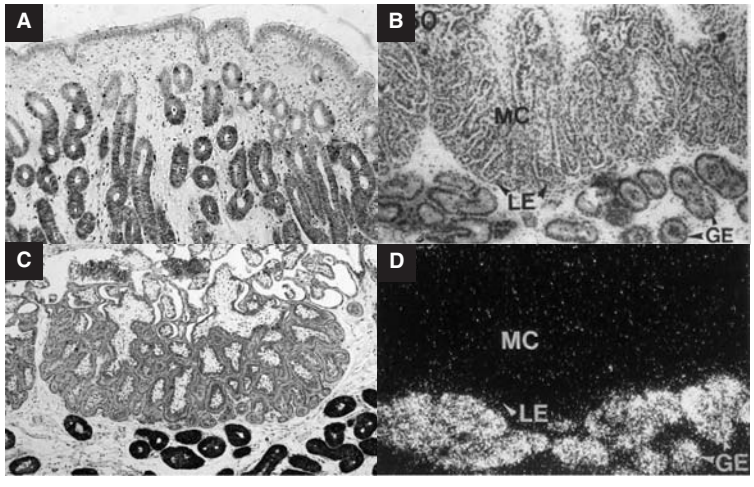

Fig 1 (A and C): Sections of endometrium and/or the placental interface labelled immunocytochemically with antibodies specific for; A) uterocalin at 18 days of gestation; C) VEGF at day 309 of gestation. B = H & E stained section of the microcotyledons (MC) and underlying endometrial glands (GE) in a pregnant mare at day 154 of gestation; LE = lumenal epithelium of the endometrium. D = in situ hybridisation labelling of the same sectionshown in B for RGFmRNA. Note the dense labelling of only the endometrial glands.

microscopic area of feto-maternal haemotrophic exchange contact reaches as high as 50–55 m^2; each microplacentome is literally crammed full of long, straight fetal and maternal capillaries to achieve an interhaemal distance as low as 4 μm; placental progestagen production is increasing with the aid of fetal adrenal sources of pregnenelone, while placental oestrogen production is declining as the enlarged fetal gonads regress; and the production of 'uterine milk' continues as an important source of fetal nutrients which are absorbed by groups of specialised pseudostratified trophoblast cells (areolae) situated between the primary stems of adjacent microplacentomes.

The epithelial cells that line the necks and apical portions of the endometrial glands are amazingly catholic and multi-tasking in their productivity of both nutrients and growth factors. For example, during the first 40 days of gestation they possess the capacity to secrete large quantities of the carrier proteins uteroferrin, uteroglobin and uterocalin (Fig 1a), which are presumed to be vital to transport essential vitamins, minerals and heavy metals across to the developing embryo. The gland epithelium also synthesises the 2 major prostaglandins, $F_{2\alpha}$ and E_2, which are closely involved in stimulating the marked increase in myometrial tonicity that 'fixes' and holds the conceptus at the base of one of the uterine horns from Day 17 until stable attachment and placentation commences around Day 40. They also synthesise an array of mitogenic growth factors, including insulin-like growth factor I (IGF-I), transforming growth factor βI (TGFβI) and epidermal growth factor (EGF) (Fig 1 b and d), all of which are almost certainly vital to stimulate growth, differentiation and vasularisation of the fetal membranes and the fetus itself. Furthermore, glandular production of EGF and VEGF (Fig 1 c and d) persists throughout gestation, indicating the continuing importance of EGF to stimulate further growth and modification of the microplacentomes until term and VEGF (Fig 1 c and d) to promote the capillary enlargement and elongation to keep pace with the placental modifications on both sides of the feto-maternal interface.

The ubiquitous endometrial gland in the equine uterus is truly a mini-organ for 'all seasons' of both the oestrous cycle and pregnancy.

NUTRITION, GENOTYPE AND EWE PROLIFICACY

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INTRODUCTION

Ewe prolificacy refers to the number of offspring actually produced and is a function of ovulation incidence, egg fertilisation and subsequent embryo/fetal survival. Prolific ewes are those with litter sizes 'greater than 2' and ewe prolificacy in its most extreme naturally occurring forms usually is influenced by one or more major genes in the TGFβ superfamily which owe their effects to point mutations in BMP-15/GDF-9 ligand or BMPR-1B receptor gene coding sequences. Such effects can be dramatic and sometimes are extreme: for example, in reporting that one ewe of the composite Belclare breed was found to have 18 corpora lutea, Hanrahan (1989) noted that this represented probably the highest natural ovulation rate ever reported for sheep. Prolificacy in some breeds (eg Finnish Landrace and Romanov) is the product of multiple gene (polygenic) effects whereby each gene exerts a small effect. Prolificacy also, of course, can be stimulated by superovulatory or immunisation regimens but neither of those scenarios is considered here. Instead, the focus is on nutritional modulation of key facets of natural prolificacy, from its prevalence within populations to its modification either by manipulating maternal diets while prolific genotypes are developing *in utero* or, post natally, by altering their own diets or intakes during reproductive life.

There is widespread appreciation of the fact that nutrition can influence ovulation rate in flocks of non-prolific sheep (Scottish Blackface breed: Gunn 1983; Merino: Nottle *et al.* 1997) and consequently 'flushing' regimens at tupping have long been used in commercial agriculture to increase average litter size among such animals. In contrast, the impact of various nutritional regimens on prolific ewe genotypes is less well understood and remains to be characterised. Here some of the work at Scottish Agricultural College and elsewhere that has begun to explore the responsiveness and sensitivity of genotypically 'prolific' sheep to nutrition *in utero* and in later life is reviewed.

PREVALENCE OF PROLIFICACY GENES

Nutrition can influence the expression and, over time, the prevalence of prolificacy genes in sheep populations. A frequent consequence of prolificacy is that carriers of such genes have more lambs per litter and these individually weigh less than offspring from non-carrier dams. Low birth weight tends to be associated with increased mortality unless enhanced nutrition intervenes to rescue 'at risk' neonates. In flocks of Indonesian thin tail sheep the frequency of the Javanese prolificacy gene, estimated to be 0.35 in lambs at weaning in lean years, was found to rise to 0.47 over just 3 years in more favourable conditions (Roberts 2000). In litters born to prolific ewes, individual lamb birth weight inevitably reflects competition *in utero* and the extent of this competition can be greater than full-term litter-size suggests. This is because embryonic loss also plays a part and consequently twin birth weights frequently fail to match those of twins borne by ewes with limited capacity for ovulation. Studies of Booroola (FecB) ewes, for example, have indicated that mean live weights of twins born to ewes that experienced no embryonic loss were >3.5 kg at birth whereas twins from ewes that had 6 ovulations weighed scarcely 2.5 kg each (Hinch *et al.* 1985). Such low birth weights, consequent on the restricted occupancy of cotyledonary

attachment sites associated with embryonic losses post implantation, mean that, as with higher order multiples, prompt provision of colostrum is essential for survival because of the undersized lamb's limited lipid reserves (Robinson and McDonald 1989). Recent data from studies of offspring born to crossbred ewes carrying a single copy of the Inverdale fecundity gene (FecXI) likewise indicated that birth weight is a critical determinant of neonatal viability, with 3.0 kg deemed a preferred target minimum live weight at term (Alink *et al.* 2006a). Intriguingly, a key to achieving this target is to devise appropriate nutritional management regimens for prolific ewes both immediately before and in the 2 weeks immediately following conception. Evidence relating to the Inverdale genotype suggests, for example, that the required management is distinctly different from that used to 'flush' conventional genotypes.

Management at mating – key to optimal birth weights?

Investigations into effects of pre-ovulatory over-feeding on quality of embryos from superovulated ewes (McEvoy *et al.* 1995) and studies relating to factors affecting birth weights among offspring of non-prolific adolescent ewes (Wallace *et al.* 2003) are among those that have highlighted the influence of progesterone during peri-ovulatory phases on optimising the developmental competence of oocytes and embryos, the establishment of adequate placentae and ultimately the delivery of viable offspring. Despite the fact that multiple ovulating prolific ewe genotypes establish more corpora lutea there tends not to be any associated increase in peripheral concentrations of progesterone (McNatty *et al.* 2005). Data from elsewhere indicate that corpora lutea of prolific ewes are smaller and less productive sources of progesterone than those of non-prolific ewes (Bartlewski *et al.* 1999). A study at our laboratory based on twice-weekly sampling indicated that, during a 7 week period immediately preceding the winter solstice, pubertal Inverdale-carrier crossbred ewe lambs had lower mean plasma progesterone concentrations in their peripheral circulation than non-carrier counterparts (Alink *et al.* 2006b). Consequently, progesterone could quickly become sub-optimal in conditions where nutrition tends to stimulate its hepatic clearance. This scenario also could explain the New Zealand experience whereby so-called 'negative flushing' has evolved as a management strategy for Inverdale ewes at mating, with an additional benefit in terms of avoiding over-stimulation of the ovary and thus limiting litter size. Failure to provide the conditions whereby progesterone concentrations are sufficient to ensure adequate placental development predisposes ewes of a prolific genotype to deliver undersized lambs reminiscent of those from overfed adolescent ewes except when the latter received supplementary exogenous progesterone (necessary before Day 11) which ensured birth weights were improved (Wallace *et al.* 2003).

Is lifetime prolificacy programmed *in utero*?

One of the factors that raises concerns regarding the value of prolific ewe genotypes in commercial agriculture is that moderate rather than dramatic increases in litter size are desirable. Consequently, management regimens that could curb but not nullify their effects might facilitate their exploitation. Awareness of the fact that ovarian function in conventional breeds can be influenced *in utero* by restriction of maternal feed intakes (Borwick *et al.* 1997) prompted an investigation into the extent to which similar restriction at the fetal stage of development does or does not curtail the capacity of prolific genotypes to produce multiple offspring later in life. Preliminary findings demonstrated that nutrition during embryonic and early fetal development (0.5 vs 1.0 Maintenance ME requirements) did influence the magnitude of expression of a single copy of the X-linked Inverdale prolificacy gene in ewe lambs. Offspring of dams that had been feed-restricted during pregnancy had a lower incidence of multiple ovulations than counterparts from unrestricted dams (F.M. Alink, unpublished). Ongoing studies are investigating whether the observed *in utero* nutritional influences on expression of ovarian function in juveniles persist throughout adult life.

Conclusion

It is becoming clear from our work that nutrition in the peri-ovulatory phase and during early fetal

development can have profound effects on the expression of those genes that control reproductive function in sheep of prolific genotype.

ACKNOWLEDGEMENTS

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SESSION IV:

Chairman:
Tom Spencer

PLACENTAL DEVELOPMENT AND ENDOCRINE FUNCTION IN GROWTH RESTRICTED PLACENTAE FROM OVER-NOURISHED ADOLESCENT SHEEP

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Overnourishing pregnant adolescent sheep promotes maternal growth but restricts placental development and mass at term resulting in reduced lamb birth weight. Circulating concentrations of progesterone and oPL are also reduced but this occurs as early as Day 50 and prior to changes in placental and fetal weight. Although this suggests a possible causative role, the mechanisms by which hormone concentrations are reduced and by which placental growth is altered are unknown. The hypothesised that: 1) reduced maternal oPL concentrations reflect decreased placental oPL synthesis and/or altered placental differentiation affecting release; and 2) reduced circulating progesterone may be due to altered placental biosynthesis. To investigate this, adolescent ewes with singleton pregnancies were offered high (H) or moderate (M) nutrient intake diets designed to restrict or support normal placental growth, respectively. Pregnancies progressed to mid- (Day 81. M: n=11, H: n=13) or late gestation (Day 130. M: n=21, H: n=22). Placental oPL, StAR (cholesterol transporter) and steroidogenic enzyme mRNA were measured by qPCR, oPL protein by immunohistochemistry, and progesterone by RIA. Mid-gestation trophoblast proliferation (brd-U) and bax protein were also assessed. Mid-gestation H pregnancies were characterised by reduced peripheral oPL and progesterone levels but no nutritional effect on placental oPL mRNA or the expression of StAR, 3BHSD, CytP450scc and CytP450c17. Trophoblast proliferative activity was reduced and bax protein levels increased. Day 130 H pregnancies were similarly characterised by reduced peripheral oPL and progesterone levels ($P<0.001$) but no change in placental oPL mRNA. In the most perturbed H animals with IUGR and thus the smallest placentae, only cytochrome P450scc mRNA ($P<0.05$) levels were reduced whereas StAR, 3BHSD and CytP450c17 were unaltered. Day 130, progesterone positively associated with placental weight ($P<0.0001$, $R2=0.42$, $n=43$), fetal weight ($P<0.0001$, $R2=0.50$, $n=43$), cytochrome P450scc ($P<0.05$, $R2=0.18$, $n=31$) and 3BHSD ($P<0.05$, $R2=0.17$, $n=31$). In conclusion, because oPL release depends on the fusion of trophoblast bi-nuclear cells to maternal syncytium, reduced mid-gestation peripheral oPL in the absence of altered placental mRNA indicates that this may be due to altered placental differentiation. Reduced trophoblast proliferation supports this theory. Placental progesterone is released by diffusion and reduced peripheral levels mid-gestation probably reflect increased clearance by the liver. At late gestation, small placental size and reduced biosynthesis may account for reduced progesterone in the most perturbed H ewes with IUGR fetuses.

EFFECT OF A HYPERLIPIDIC HYPERCHOLESTEROLEMIC DIET IN PUBERTAL DOES ON FETAL AND POST-NATAL DEVELOPMENT: PRELIMINARY RESULTS FOR THE VALIDATION OF A RABBIT MODEL

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INTRODUCTION

It has been suggested in recent studies that adolescent pregnant women are more likely to become obese at the end of pregnancy than adult women. The major hypothesis to explain this trend is a competition for nutrients between needs for maternal versus fetal growth, which leads to intra-uterine growth retardation (IUGR). Children born from these mothers have a low Body Mass Index (BMI) which can predispose them to diabetes, cardiovascular desease (including atherosclerosis) and obesity (Barker and Clark 1997). A sheep model of adolescent and obese pregnant dam has been developed in order to understand the physiological and pathological processes involved in this phenomenon (Wallace *et al.* 1999; Wallace *et al.* 2004). The rabbit model was chosen in the present study as its lipid metabolism is close to that of humans, therefore allowing the study of the effect of hyperlipidic nutrition. Moreover, placentation is haemochorial and the relatively large size compared to other rodents allows fetal monitoring by ultrasound.

OBJECTIVES

The objective of this study was to investigate the maternal transmission of predisposition to obesity and atherosclerosis from juvenile dams fed with a hyperlipidic hypercholesterolemic diet to their offspring.

MATERIALS AND METHODS

Sixty does (New-Zealand x Californian cross) were used. The protocol used is described in Figure 1.

Experiment 1

Twenty does (L1) were fed with a hyperlipidic (9% lipids, ie 7% digestible fatty acids) and hypercholesterolemic (0.2%) diet (HH diet) from 10 weeks of age for 15 weeks whereas the control group (T1, N=20) was fed a diet with 3% lipids (1.2% digestible fatty acids).

Experiment 2

Treated does (L2, N=10) were fed the HH diet and controls were fed the control diet from the time of breeding at 17 weeks of age.

In both experiments, food intake and body weight was monitored every week from the beginning of the experiment. Blood samples were drawn and arterial pressure was measured repeatedly (N=3). All does were bred at 17 weeks and fetal and placental growth were monitored by ultrasound (N=4 throughout pregnancy at 9, 14, 21 and 28 days) (Chavatte-Palmer *et al.* 2005). At birth, pups were cross-fostered between the 2 groups and growth was evaluated weekly. Dams and offspring were euthanasied at the time of weaning (5 weeks). Plasma and samples of adipose tissue, liver, kidneys and aorta were collected at that time.

RESULTS

Experiment 1

L1 does reduced their total food intake from the 6th week of treatment ($P<0.05$) but the amount of lipid ingested was statistically increased (771.8 ±183.5 g per week vs 223.9 ± 55.12 g in the first 7 weeks in L1 vs T1, respectively, $P<0.0001$). There was no

Experiment1: 20 does under hyperlipidic hypercholesterolemic diet L1, 20 does under control diet T1.
Experiment 2: 10 does with lipids L2, 10 controls T2.

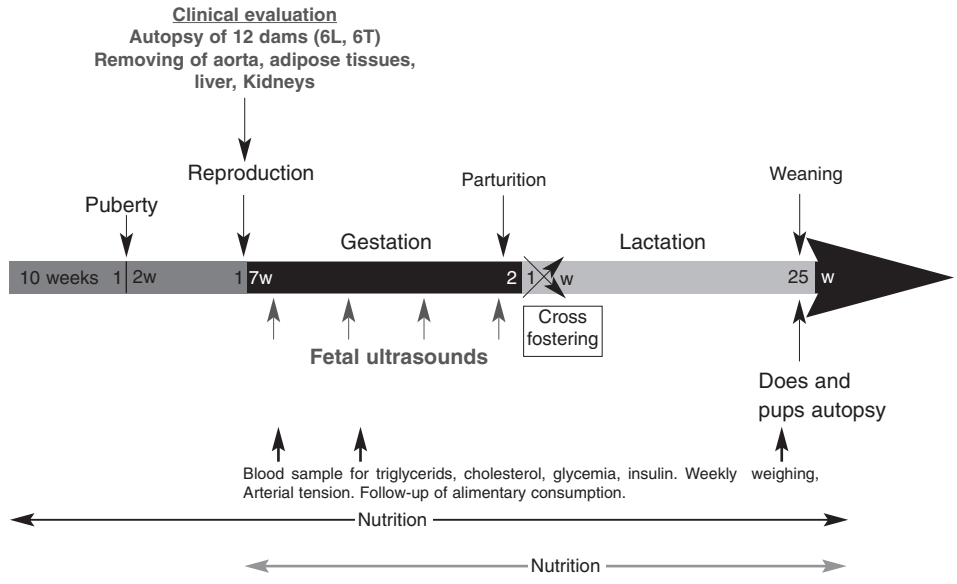

Fig 1: Diagrammatic representation of the protocol used.

difference for weight gain between groups, nor for organ weight and arterial pressure. Atherosclerotic plaques, however, were observed on the aorta of all L1 does (Fig 2). Liver steatosis was observed in L1 animals. Moreover, increased fasting insulin concentrations were observed despite normal glycaemia. Fertility and prolificity were significantly reduced and fetal IUGR was observed from 9 days of pregnancy. Abortion occurred in one third of the does and another third were agalactic after birth. L1 pups were statistically lighter at birth. There was no difference between groups for post natal growth. Moreover, no atherosclerotic lesions were found in any of the pups although liver steatosis was observed and all plasma concentrations of lipid metabolism markers were statistically increased in pups fed by L1 does.

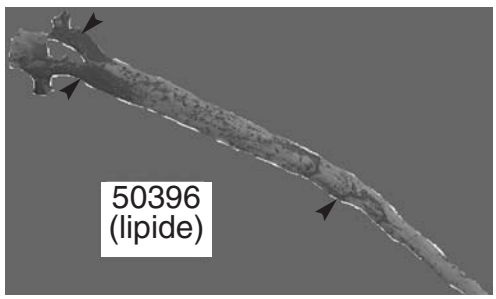

Fig 2: Aorta from a L1 doe stained with red oil to show aortic plaques (arrows heads).

Experiment 2

The same observations were made as in Experiment 1 for food, fat intake, weight gain and organ weight in does. Atherosclerotic lesions were observed only in half of the L2 does but liver steatosis was observed. Plasma concentrations of total cholesterol, HDL and LDL-cholesterol, triglycerids and β-hydroxy-butyrate were not increased in L2 does. No abortions nor agalactia were observed and fertility was normal. Fetal and post natal growth, as well as plasma markers were not statistically different between groups. In 4 out of 14 pups both born from L2 dams and fed by a L2 dam, fatty streaks were noted on the aorta.

Discussion and conclusion

These results are a first step in the validation of the rabbit model for studying the aetiology of

atherosclerosis and predisposition to the metabolic syndrome in human. In this experiment, atherosclerosis and liver steatosis were induced in adolescent does and this diet also affected fertility and prolificity. IUGR was shown to be a consequence of the diet in L1 but pups caught up with controls for post natal growth and no aortic lesions could be demonstrated at the time of weaning. The pups, however, were still growing at that time and it might have been too early to demonstrate a negative outcome. Nevertheless, the differences observed between the 2 experiments show that the length of exposure to the hyperlipidic and hypercholesterolemic diet before conception plays an important role. Further studies are currently being conducted where pups from L1 does are kept under normal conditions until adulthood to evaluate later outcome.

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EFFECTS OF A *STREPTOCOCCUS EQUI* INFECTION-MEDIATED NUTRITIONAL INSULT DURING MID GESTATION ON PLACENTAL AND FETAL DEVELOPMENT IN PRIMIPAROUS THOROUGHBRED FILLIES

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It has been demonstrated previously that both age and parity have profound effects on the development of the microcotyledons on the surface of the diffuse, epitheliochorial equine placenta (Bracher *et al.* 1996; Wilsher and Allen 2003). A reduction in the surface, or plexiform nature, of the microcotyledons occurs in older mares suffering age-related degenerative changes (endometrosis) in the opposing endometrium and also in young, primigravid mares. This results in lower birth weight foals from both maiden and aged mares, compared to young and middle-aged multiparous mares. Surprisingly, however, assessment of placental efficiency in terms of kg foal birth weight/m^2 microscopic feto-maternal contact shows that the allantochorion of both primiparous and aged multiparous mares is more 'productive' than that of the other groups of mares. Indeed, regardless of mare age and parity, microscopic surface area of the allantochorion is correlated negatively with placental efficiency (Wilsher and Allen 2003). Similar correlations matching increases in placental weight with decreases in efficiency have been reported in the pig (Wilson *et al.* 1999; Wilson and Ford 2001).

It is clear from work in many species that placental development can be enhanced or compromised by a variety of external influences. For example, a critical period of placental sensitivity occurs in the ewe between 40 and 80 days of gestation, which coincides with its phase of rapid proliferative growth (Ehrhardt and Bell 1995). During this period, nutritional modulation of placental growth may be influenced by the size (Russell *et al.* 1981; McGrabb *et al.* 1992), body condition (Clarke *et al.* 1998; Osgerby *et al.* 2003) and maturity of the ewe (Wallace *et al.* 1996 and 1997). Short-term nutritional insults can also suppress placental and fetal growth in the ewe, which may result in long-term changes in physiology and metabolism of the offspring (Barker and Clark 1997; Heasman *et al.* 2000; McMullen *et al.* 2005).

There is a paucity of experimental data relating nutrition with placental development and function in the mare. During an experiment to examine the effects of moderate versus excessive nutrition during pregnancy on microcotyledonary development of the placenta in primigravid Thoroughbred fillies, all the fillies became infected with *Streptococcus equi* ('Strangles') which causes pyrexia and inappetence for 7–10 days. This resulted in dramatic weight loss in all the affected fillies when they were between 90 and 150 days of gestation and, hence, still in the proliferative growth phase of placentation. Thus, it was possible to study the effects of a disease-mediated nutritional insult before mid-gestation on placental and fetal growth.

Gains in body weight during pregnancy, judged either in kilograms or as percentage increase, were significantly higher in the High versus the Moderate nutrition groups. Figure 1a depicts the changes in maternal body weight for both planes of nutrition and Figure 1b shows the same values adjusted for the stage of gestation. The percentage of weight lost as a consequence of the *S.equi* infection ranged from 0 to 19.5% (mean 9.1 ± 1%). Although the plane of nutrition per se did not influence placental and fetal growth parameters, the transient weight loss mediated by the *S. equi* infection did result in morphological changes to the allantochorion and decreases in foal birth weights when compared to an historic control group of non-infected pregnant maiden Thoroughbred fillies (Table 1). With increasing maternal weight loss the mass, volume (total and chorionic), depth and total surface area of the

TABLE 1: Mean ± s.e. fetal and placental parameters measured in primiparous Thoroughbred fillies, infected or not infected with *S.equi*, during gestation

	Primiparous Thoroughbred fillies aged ≤ 6 years	
	Infected with *S.equi* during gestation (n = 20)	Not infected with *S.equi* during gestation (n = 24)
Foal birth weight (kg)	44.9 ± 0.9[a]	47.3 ± 0.8[b]
Mass of allantochorion (kg)	3.4 ± 0.1	3.5 ± 0.1
Gross area of the allantochorion ($cm^2 \times 10^3$)	10.6 ± 0.3[a]	11.7 ± 0.3[b]
Volume of the allantochorion (l)	3.2 ± 0.1	3.3 ± 0.1
Volume of the chorion (Vc;l)	1.3 ± 0.1[a]	1.1 ± 0.4[b]
Depth of the chorion (mm)	1.20 ± 0.07	1.10 ± 0.05
Microcotyledon surface density (S_v; μm^{-1})	0.034 ± 0.001	0.034 ± 0.001
Total microscopic area of feto-maternal contact ($S_v \times V_c$; m^2)	43.8 ± 2.5[a]	37.6 ± 1.4[b]
R_v ($S_v \times V_c$/ gross area; m^2)	40.8 ± 2.7[a]	32.1 ± 1.0[b]
Placental efficiency: foal wt per m^2 allantochorion (kg/m^2)	1.08 ± 0.06[a]	1.29 ± 0.04[b]

Rv = ratio of total microscopic to gross area of the allantochorion. Different superscripts within rows indicate significant differences (P<0.05). Wilsher and Allen (2006).

allantochorion all decreased (Figs 1a, b and c). However, placental efficiency (kg of foal birth weight per square metre of microscopic contact of allantochorion) actually showed a positive correlation with the percentage of weight lost during the infection period (Fig 1d). In addition, the crown-rump lengths and ponderal indices of the foals at birth and at weaning, plus their serum IGF-1 concentrations at birth, were all correlated to the percentage of weight lost as a result of the *S. equi* infection during gestation.

Comparison of morphology of placentae from the infected versus the control fillies revealed that the greater microscopic area of feto-maternal contact in the former originated from an increase in the volume of the chorion rather than from any increase in microcotyledonary Sv. However, despite this increase in placental contact with the endometrium resulting in a greater area for feto-maternal exchange in the infected fillies, the mean foal birth weight for this group was lower than the non-infected control fillies. This can be explained by the lower placental efficiency in the infected fillies. Larger Rv values (ratio of total microscopic to gross area of the allantochorion) in the infected fillies were likely to have arisen from an increase in the length of the fetal villi, rather than any increase in their branching patterns. Allen *et al.* (2002) reported the same morphological adaptation in an experimental model of intra-uterine growth restriction, created by transferring Thoroughbred embryos to the uteri of smaller Pony mares.

Development of the microcotyledons, as judged by Sv values, was not altered by either the pre- or post infection level of maternal nutrition or the nutritional insult that accompanied the *S. equi* infection. Furthermore, there was no correlation between the degree of weight loss and the Sv of the microcotyledons. Allen *et al.* (2002) also failed to show any remodelling of the microcotyledons, in terms of Sv, as a consequence of either intra-uterine growth restriction, or intra-uterine excess, occasioned by reciprocal transfer of embryos between Pony and Thoroughbred mares. In women, stereological measurement of placental villi has demonstrated that the Sv can be modified by intra-uterine growth restriction (Mayhew *et al.* 2003), pre-eclampsia (Boyd and Scott 1985; Teasdale 1985; Burton *et al.* 1996), hypoxia at high altitude (Mayhew 2003) and exercise (Jackson *et al.* 1995). By contrast, to date the only factors that appear able to influence microcotyledonary Sv values in the mare are maternal age, parity and genotype (Allen *et al.* 2002; Wilsher and Allen 2003). This suggests that the maternal endometrium is the over-riding influence on these measures of microcotyledon development in the mare. Furthermore, the epitheliochorial architecture of the equine placenta, compared to the discoid haemochorial stucture of the human placenta with its associated breakdown of maternal tissues (Steven 1975), no

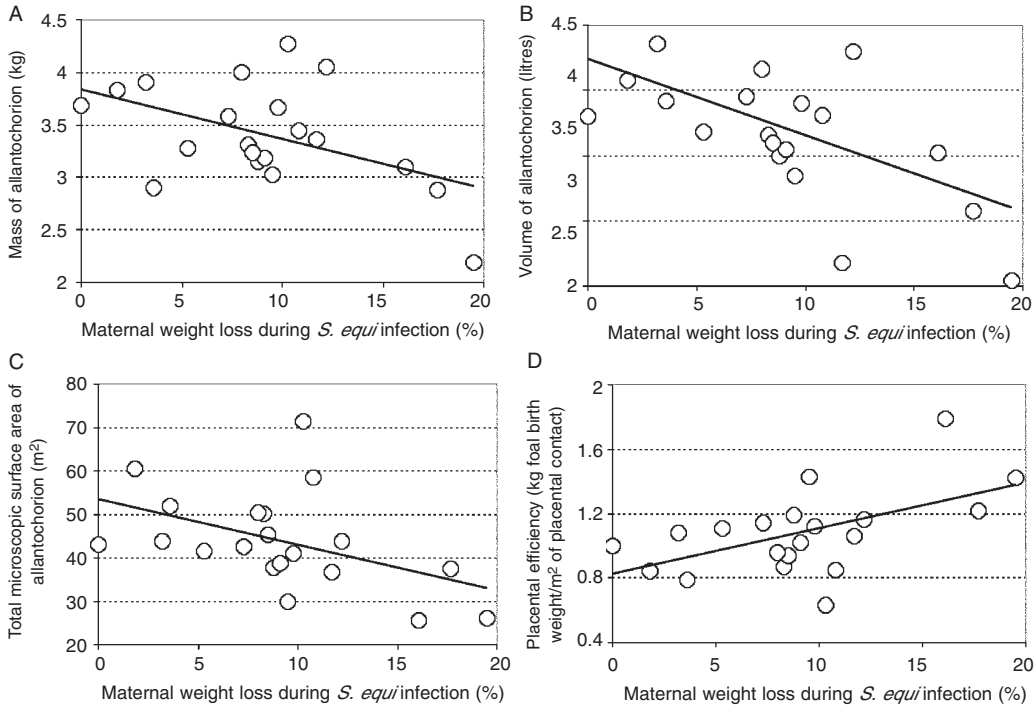

Fig 1: Relationship between percentage weight loss during S.equi infection and, a) mass of the allantochorion (y = -0.0472x + 3.8298; r = 0.48; n = 20; P = 0.03); b) volume of the allantochorion (y = -0.0585x + 3.7356; r = 0.56; n = 20; P = 0.01); c) total microscopic surface area of the allantochorion (y = -1.0462x + 53.3; r = 0.46; n = 20; P = 0.04); and, d) placental efficiency (kg of foal birth weight per m^2 of microscopic contact at the placental interface (y = 0.0282x + 0.8252; r = 0.54; n = 20; P = 0.01) (Wilsher and Allen 2006).

doubt influences the ability of the chorionic villi to modify themselves under different circumstances. Thus, while the original aim to determine if the rapid weight gain exhibited by maiden fillies when they retire to stud from the training yard might modify placental development, failed to yield fruit, the protracted period of fever and inappetance induced by the *S. equi* infection did exert measurable changes on placental development. The results also indicated that 3- and 4-year old Thoroughbred fillies do not exhibit the same degree of biological immaturity as the adolescent sheep model studied by Wallace *et al.* (1996, 1997 and 2001).

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SESSION V:

Chairman:
Claire Wathes

REGULATION OF PLACENTAL NUTRIENT TRANSFER CAPACITY

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INTRODUCTION

Many animal studies and human epidemiological findings have shown that impaired growth *in utero* is associated with physiological abnormalities in later life and have linked this to tissue programming during suboptimal intra-uterine conditions (Fowden *et al.* 2006a). However, few of these studies have considered the placental contribution to the ensuing phenotype. In mammals, the major determinant of intra-uterine growth is the placental supply of nutrients to the fetus (Harding and Johnston 1995). In turn, this depends upon the size, morphology, blood supply and transporter abundance of the placenta and on the synthesis and metabolism of nutrients and hormones by the placenta itself (Fig 1). These factors are all influenced by environmental conditions, such as nutrition, during pregnancy (Fig 1).

NUTRITIONAL REGULATION OF PLACENTAL NUTRIENT TRANSFER CAPACITY

Both under- and over-nutrition during pregnancy affect placental size, although the specific effects depend on the severity, duration and gestational age at the onset of nutritional perturbation (Heasman *et al.* 1999). In sheep, moderate under-nutrition during the peri-conceptual period alone has no effect on placental and fetal weights in late gestation (Oliver *et al.* 2005) but, when the period of under-nutrition is extended to cover the period of rapid placental growth, placental weight is frequently increased near term (Fowden *et al.* 2006c). This overgrowth appears to compensate for the reduced nutrient availability early in gestation as fetal weight is normal, or even enhanced, in late gestation after restoration of normal nutrition (Kelly 1992). Similar compensatory increases in placental weight have been observed in response to undernutrition in pregnant pigs, rats and humans (Fowden *et al.* 2006c). By contrast, moderate under-nutrition during mid to late gestation when the placenta has formed tends to reduce placental weight near term (Heasman *et al.* 1999). When nutrient deprivation occurs throughout pregnancy, fetal and placental weights both decrease but, generally, more fetus is produced per gram of placenta than in normally nourished animals (Woodall *et al.* 1996; Osgerby *et al.* 2002). Exposure to poor nutrition at critical stages of placental development, therefore, appears to increase the efficiency with which the small placenta transfers nutrients to the fetus.

Variations in nutrient availability induce a range of gross morphological and ultrastructural changes in the placenta, including alterations in the surface area, vascularity, barrier thickness and cell composition, all of which influence the transport characteristics of the placenta (Sibley *et al.* 1997). In sheep, the proportion of the more everted C/D type placentomes increases in late gestation after under-nutrition earlier in gestation (Heasman *et al.* 1999), although this may only affect transplacental nutrient transfer when fetal cortisol levels are high (Ward *et al.* 2006). In guinea pigs, under-nutrition throughout pregnancy reduces placental surface area by 60–70% and increases barrier thickness by 40% in late gestation (Roberts *et al.* 2001). The proportion of the placenta occupied by the labyrinthine zone involved in nutrient transfer is also reduced in these circumstances. When ovine placental growth is compromised by either under- or over-nutrition, placental vascularity decreases in association with reduced placental expression of the angiogenic

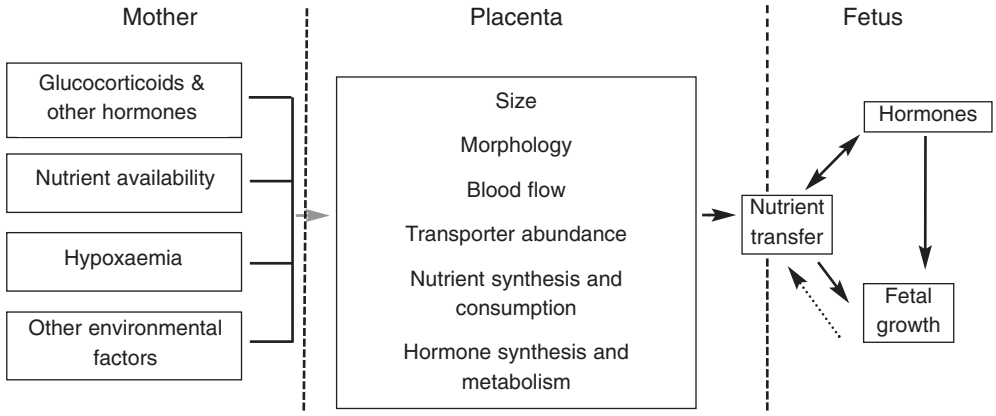

Fig 1: Schematic representation of the factors influencing the placental supply of nutrients to the fetus.

factor, VEGF, and its receptor (Reynolds *et al.* 2005). However, weight specific rates of umbilical and uterine blood flow in late gestation are unaffected by acute fasting or prolonged nutritional manipulations earlier in gestation (Hay 1995).

During the second half of gestation in rats, sheep and humans, placental abundance of the 2 glucose transporters (GLUT) involved in transplacental glucose flux is altered in an isoform-specific manner by variations in nutrient availability induced by fasting, restriction of dietary intake, diabetes and direct maternal infusions of glucose and insulin (Fowden *et al.* 2006c). Both increases and decreases in GLUT protein abundance are observed in response to these manipulations depending on their timing and duration (Jansson and Powell 2006). Placental GLUT protein expression is, therefore, responsive to nutrition and/or the concomitant changes in fetal growth (Fig 1).

Nutrition also influences the production and utilisation of carbohydrates and amino acids by the placenta per se. In turn, this affects the quantity and specific composition of the nutrients supplied to the fetus. Fasting and moderate maternal under-nutrition for short periods reduces placental glucose consumption but has no effect on the partitioning of glucose between ovine placental and fetal tissues (Harding and Johnston 1995; Hay 1995). However, when maternal hypoglycaemia is prolonged, the placental tissues conserve glucose for their own use and transfer proportionately less glucose to the fetus (Hay 1995). Placental lactate production and delivery to the sheep fetus also decreases in response to under-nurition but increases above normal upon re-feeding (Harding and Johnston 1995). Similarly, there are nutritionally-induced changes in the placental synthesis and metabolism of hormones, such as progesterone, placental lactogen and the prostaglandins, that affect placental nutrient transfer to the fetus indirectly (Fowden *et al.* 1994; Whittle *et al.* 2001).

Mechanisms by which nutrition acts on the placenta

The mechanisms by which nutrient availability controls placental development and nutrient transport efficiency remain unknown but may involve the *Igf2* gene and/or glucocorticoid over-exposure.

Glucocorticoids

In late gestation, glucocorticoid concentrations in both the fetal and maternal circulations are raised by under-nutrition and other dietary manipulations (Whittle *et al.* 2001). Bioavailability of these hormones to the placenta also increases during under-nutrition due to reduced placental activity of 11β-hydroxysteroid dehydrogenase type 2, the enzyme responsible for inactivating glucocorticoids (Seckl and Meaney 2004; Fowden *et al.* 1998). In rats, sheep and monkeys, maternal glucocorticoid administration during late gestation reduces placental growth and increases placental efficiency measured as fetal to placental weight (see Fowden *et al.* 2006). These changes are accompanied by both increases and decreases in placental GLUT abundance depending on the

TABLE 1: The effects of deletion or disruption of genes relating IGF-II bioavailability on the growth of the placenta and fetus and the efficiency of the placenta measured as the fetal to placental weight ratio

Gene	Knockout % wildtype		
	Placental weight	Fetal weight	F:P weight ratio
Igf2	75%	60%	85%
Placental specific *Igf2P0*	65%	75%	115–30%
Igf-2R	140%	140%	100%
H19	140%	130%	90%
Igf-2R and *H19*	230%	200%	80%

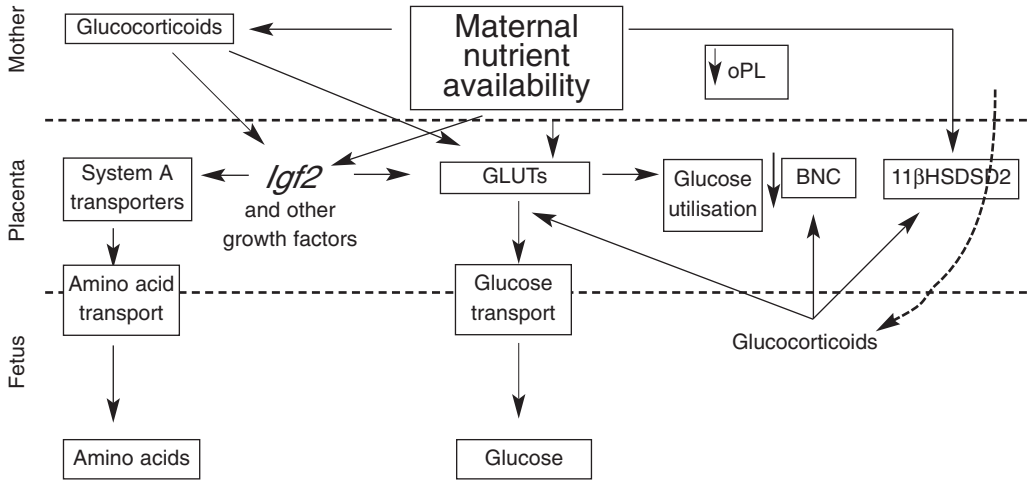

Fig 2: Schematic diagram showing the effects of varying nutrient availability on the placental capacity for nutrient transfer. Composite diagram from sheep and mice. BNC, binucleat cells; GLUT, glucose transporters; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2; oPL placental lactogen. Adapted from Fowden et al. 2006c.

duration and gestational age at onset of glucocorticoid over-exposure (Fowden *et al.* 2006c; Jansson and Powell 2006). In sheep, glucocorticoids also alter cell composition, nutrient utilisation, and hormone secretory activity of the placenta (Fowden *et al.* 1994; Ward *et al.* 2002; Ward *et al.* 2004).

The Igf2 gene

This gene controls the growth, morphology and nutrient transfer capacity of the mouse placenta (see Fowden *et al.* 2006b). Its expression in rodent placenta is also down-regulated by under-nutrition and glucocorticoid administration in late gestation (Fowden 2003; Ain *et al.* 2005). Disruption or deletion of the *Igf2* gene causes placental growth retardation while, conversely, *Igf2* over-expression leads to placentomegaly (Table 1). Both up- and down-regulation of *Igf2* gene expression alter placental phenotype and efficiency (Table 1, Fowden *et al.* 2006b). In the complete *Igf2* null, placental growth retardation is accompanied by general hypoplasia and reduced placental efficiency associated with a disproportionate decrease in the labyrinthine area (Constancia *et al.* 2005). When *Igf2* deficiency is induced solely in the nutrient exchange area by deletion of the labyrinth-specific *Igf2P0* promoter, all placental layers are proportionately smaller and there is a 50% reduction in surface area and a 28% increase in barrier thickness (Sibley *et al.* 2004). However, despite its small size and reduced diffusion capacity, the *Igf2P0* null placenta is more efficient. It transfers more glucose and amino acids per gram than the wild type placenta due to up-regulation of the genes encoding specific isoforms of the glucose and System A amino acid transporters (Constancia *et al.* 2005). In contrast, there is no up-regulation of these

transporters or of transplacental glucose and amino acid transfer in the small, complete *Igf2* null placenta (Constancia *et al.* 2005). Placental nutrient transfer capacity, therefore, appears to be responsive to fetal nutrient demands and is regulated by the inter-play between placental and fetal *Igf2* in mice (see Fowden 2003).

Conclusions

Placental nutrient transfer capacity is responsive to nutritional stimuli and can adapt to help maintain the fetal nutrient supply, particularly when the placenta is small. Nutrition alters placental phenotype, in part, via changes in glucocorticoid exposure and/or in the expression of *Igf2* and other growth factors (Fig 2). Nutritional signals also alter the synthesis and metabolism of both nutrients and hormones by the placenta per se which, directly and indirectly, influence the balance and absolute amounts of specific nutrients supplied to the fetus (Fig 2). Nutritionally-induced reductions in placental enzymes, such as 11βHSD2, will exacerbate the placental actions of the glucocorticoids (Fig 2) and increase fetal glucocorticoid exposure with consequences for fetal development more generally (Fowden *et al.* 1998). Placental nutrient transfer capacity is also regulated by the genetic drive for fetal growth, although the specific nature of the fetal nutrient demand signals has yet to be determined (Constancia *et al.* 2005). Indeed, the epigenetic mechanisms by which nutrition controls the placental capacity for nutrient transfer at the molecular level remain largely unknown.

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NUTRITION DURING PREGNANCY: EFFECTS ON RENAL DEVELOPMENT AND CONSEQUENCES FOR SUBSEQUENT FUNCTION

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INTRODUCTION

It is generally accepted that a range of factors during early and adult life interact with the prevailing genotype to determine an individual's risk of developing hypertension. Most evidence is consistent with Guyton's hypothesis that a long-term increase in blood pressure involves a physiological defect in the efficiency of renal sodium excretion (Guyton 1990). A variety of hypotheses have been proposed to underlie this defect (Johnson *et al.* 2005), including improper stimulation of the renal sympathetic nerves, disturbance of the renin-angiotensin system (RAS) and nephron insufficiency. Importantly, a wide range of animal models have demonstrated that these renal defects can originate from the developmental environment, when critical windows exist for physiological factors to have permanent effects on organ structure and function. A wide range of animal models are now established which demonstrate significant deficits in nephron number, disturbance of the RAS, and progressive renal dysfunction and hypertension in offspring exposed to mild nutrient restriction during fetal life (McMullen and Langley-Evans 2005a). Such animal models give biological plausibility to the large body of epidemiological evidence indicating that risk of hypertension is related to factors that impair fetal growth.

NUTRITION AND RENAL DEVELOPMENT

The developing kidney has proved to be particularly sensitive to manipulation of the maternal diet. In animal models, a reduction in the number of nephrons present in the kidney at birth is a consistent response to nutrient restriction in the developmental period. This has led to the suggestion that a reduced nephron complement may limit the functional capacity of the kidney, leading to hyperperfusion of the remaining nephrons and a progressive deterioration of renal function (Mackenzie and Brenner 1995). This hypothesis is supported by animal studies demonstrating that surgical disruption of nephrogenesis results in hypertension in later life (Woods *et al.* 2001). However, support for the hypothesis that a nephron deficit per se is sufficient to cause hypertension is equivocal. Reduction of renal mass during adult life, as in the case of adult kidney donation, does not necessarily result in hypertension (Narkun-Burgess *et al.* 1993), indicating that there is a certain amount of functional redundancy within the organ. Additionally, supplementing glycine to low-protein fed rat dams prevented the reduction in nephron number in their offspring, whilst the programmed hypertension persisted (Jackson *et al.* 2002), demonstrating that the nutritional programming of nephron deficit and hypertension can be dissociated. It therefore appears that a congenital nephron deficit is neither necessary nor sufficient for the development of hypertension. Instead, it may contribute to the faster progression towards hypertension, once deterioration has been initiated by an additional permissive factor (Fig 1).

THE RENIN-ANGIOTENSIN SYSTEM

The RAS is a primary regulator of blood pressure and disturbance of this system has been strongly implicated in the nutritional programming of hypertension. The RAS regulates blood pressure directly, through its effects on vascular tone and fluid homeostasis, but is also critical to normal renal development and tissue remodelling. We and

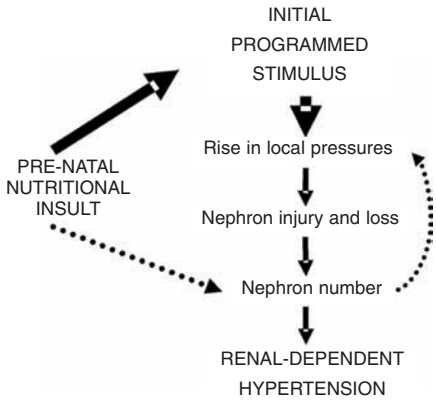

Fig 1: Schematic representation of current hypotheses regarding the contribution of a reduction in nephron number to programmed hypertension. Current evidence suggests that a nephron deficit is neither necessary nor sufficient for the onset of hypertension. However, a nutritionally programmed nephron deficit may exacerbate the progression of renal dysfunction (dashed line) once rising local pressures are stimulated by an alternative programming stimulus (solid line).

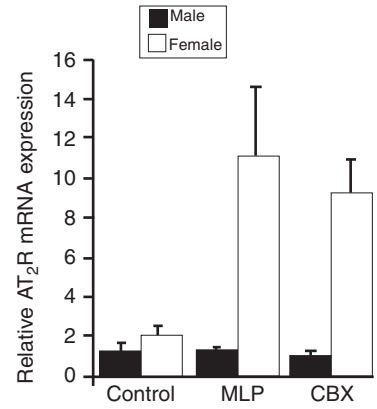

Fig 2: Relative expression of AT_2R mRNA in kidneys from 20 week old rats exposed to a low protein diet (MLP) or carbenoxolone (CBX) in utero (adapted from McMullen and Langley-Evans 2005c). Data is presented as mean ± s.e.m. Analysis of variance demonstrated a significant interaction between treatment and sex ($P<0.05$).

others have observed age and sex-specific changes in the expression of the angiotensin receptors in kidneys from rats exposed to a pre-natal low-protein diet (Sahajpal and Ashton 2003; McMullen and Langley-Evans 2005b; McMullen and Langley-Evans 2005c). In early post natal life widespread up-regulation of the RAS is observed, with low-protein offspring exhibiting increased expression of AT_1R (Sahajpal and Ashton 2003) and decreased expression of AT_2R in the kidney (McMullen and Langley-Evans 2005b; McMullen and Langley-Evans 2005c). Whilst AT_1R mediates the classic pressor responses to angiotensin II, AT_2R is believed to oppose actions at AT1R, promoting vasodilation via cGMP and bradykinin pathways. The shift in the balance of these 2 receptor subtypes observed is therefore consistent with the increased pressor responses to angiotensin II observed in low-protein offspring at 9 weeks of age (McMullen *et al.* 2004), and may provide the initial stimulus for a rise in blood pressure.

Our preliminary data suggests that these low-protein induced changes in kidney receptor expression are already apparent at birth (McMullen and Langley-Evans 2005c). During fetal life, AT_2R appears to be the dominant subtype expressed and has an antiproliferative effect critical to organogenesis and tissue differentiation. In contrast, AT_1R permits the mitogenic actions of angiotensin II and promotes the deposition of matrix proteins. A shift in the balance of the 2 receptor subtypes at this time may therefore affect angiotensin II mediated developmental processes. A study is currently underway in our laboratory to examine the impact of maternal nutrition on angiotensin receptor expression during the critical stages of renal development.

Interestingly, in contrast to the observations in early post natal life, considerable up-regulation of AT_2R was observed in female low-protein offspring at 20 weeks of age, secondary to the onset of hypertension (Fig 2) (McMullen and Langley-Evans 2005c). Recent work has shown AT_2R to be up-regulated in response to renal injury, acting to promote tissue remodelling via proliferative and apoptotic pathways (Bautista *et al.* 2001; Ruiz-Ortega *et al.* 2003). This may therefore constitute a compensatory mechanism acting to protect the kidney from the ongoing pathology. The attenuation and delay of renal disease and hypertension is observed in females in a number of rodent models of hypertension. The changes observed in AT2R expression in females only warrant further investigation as a potential sex-specific protective mechanism.

THE ROLE OF GLUCOCORTICOIDS

Ordinarily, maternal steroids reaching the placenta are metabolised to inactive forms by the enzyme

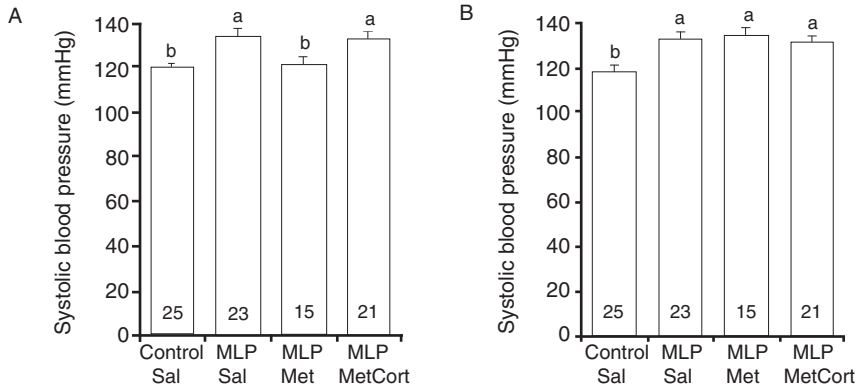

Fig 3: Systolic blood pressure in male (A) and female (B) 4 week old rats exposed to a prenatal control or low-protein (MLP) diet, with saline (Sal), metyrapone (Met) or metyrapone and corticosterone (MetCort) treatments during the first 14 days of pregnancy [Adapted from McMullen and Langley-Evans 2005b]. Data is presented as mean ± sem (a>b, P<0.05). Group size is displayed within the bar.

11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), thus protecting the fetus from maternal circulating concentrations. The spectrum of phenotypic characteristics associated with nutrient restriction during pregnancy can be mimicked by exposure of the fetus to dexamethasone (Ortiz *et al.* 2003), a synthetic glucocorticoid only partially metabolised by 11β-HSD2, and carbenoxolone (Lindsay *et al.* 1996), an inhibitor of 11β-HSD2. On the basis of this evidence, it was hypothesised that the programming effects of maternal nutrient restriction are mediated by over-exposure of the fetus to maternal glucocorticoids. Indeed the expression and activity of placental 11β-HSD2 have been shown to be decreased in response to nutrient restriction in the rat and sheep (Langley-Evans *et al.* 1996; McMullen *et al.* 2004) and increased expression of glucocorticoid-inducible genes has been observed in the fetal tissues of low protein exposed rats (Langley-Evans and Nwagwu 1998). It is now widely reported that the effects of maternal nutrient restriction on the developing fetus are mediated by glucocorticoids. In support of this, pharmacological blockade of maternal glucocorticoid synthesis with metyrapone prevented the onset of hypertension in low protein offspring, which were then restored by corticosterone replacement (Langley-Evans 1997). However, recent work in our laboratory suggests that not all of the characteristics of the programmed offspring are mediated in this way. Whilst the reduction in nephron number proved dependent upon glucocorticoid exposure, the observed changes in type 2 receptor expression proved glucocorticoid-independent (McMullen and Langley-Evans 2005b). Further investigation showed pre-natal low-protein and glucocorticoid exposures to have directly opposite effects on post natal receptor expression in early life (McMullen and Langley-Evans 2005c). Importantly, the onset of hypertension in female offspring was not prevented by metyrapone treatment (Fig 3), despite normalisation of nephron number in this group (McMullen and Langley-Evans 2005b). This suggests that over-exposure to glucocorticoids is not responsible for the initial rise in blood pressure in female offspring. In contrast, glucocorticoid-dependent hypertension has been observed in female low-protein offspring later in life (Langley-Evans 1997), perhaps reflecting the emerging contribution of the glucocorticoid-dependent nephron deficit to the programmed hypertension as renal dysfunction progresses.

Summary

It is clear that the factors important in mediating the pre-natal origins of adult hypertension may differ from those important in regulating the severity and progression of subsequent disease. Whilst the evidence does not fully support a reduction in nephron number per se as a cause of hypertension, it is likely to exacerbate the progression of renal dysfunction. The RAS has the potential to mediate both the initiation and progression of renal dysfunction in programmed hypertension, via regulation of renal development,

remodelling and function. However, the complex interactions of the RAS with glucocorticoid exposure, age and gender remain to be fully understood. The evidence of the sex-specificity of these interactions highlights the need to assess the impact of gender in future studies examining the mechanistic aspects of programming. The substantial up-regulation of AT_2R mRNA expression secondary to the programmed phenotype in female offspring is of particular interest. Further work is required to ascertain the role of this receptor in mediating the effects of angiotensin II in the adult kidney and thus the functional significance of such substantial up-regulation.

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ROLE OF THYROID HORMONES IN THE CONTROL OF FETAL GROWTH AND MATURATION

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Growth and development before birth depends upon the activity of endocrine systems in the offspring (Fowden 1995). In particular, the thyroid hormones have a key role in matching the growth, metabolism and development of the fetus to the nutrients and oxygen available. There are several aspects to the control of thyroid hormone activity, or bioavailability, which may be influenced during development and in response to changes in nutrition *in utero*.

THYROID HORMONE BIOAVAILABILITY IN THE FETUS

The bioavailability of the thyroid hormones is dependent upon several factors including: 1) the activity of the hypothalamic-pituitary-thyroid axis and production of thyroxine (T_4) and triiodothyronine (T_3); 2) the metabolism of T_4 to more biologically-active T_3 or to inactive metabolites to vary circulating and tissue-specific concentrations; and 3) the uptake of thyroid hormones into target tissues and activation of cellular processes by binding to thyroid hormone receptors. Before birth, all of these factors contribute to the bioavailability of thyroid hormones in the fetus, and each shows developmental, and often tissue-specific, regulation.

From mid-gestation in human and ovine fetuses, the thyroid gland secretes T_4 under the influence of the developing hypothalamic-pituitary axis (Polk 1995). In the fetus, T_4 is primarily metabolised to reverse-triiodothyronine (rT_3) and a variety of sulphated thyroid hormones, all of which are biologically inactive. Therefore, for most of gestation, plasma T_3 concentrations are relatively low as placental enzymes also maintain a high rate of T_3 clearance. Close to term, however, thyroid hormone metabolism changes such that T_4 is preferentially deiodinated to T_3 instead of rT_3, and consequently, a rise in plasma T_3 concentration occurs in the fetus near term. Deiodination is controlled by three deiodinase enzymes that convert T_4 to T_3 (D1, D2) and T_4 to rT_3 (D3, Fig 1). These enzymes are regulated developmentally by glucocorticoids in a tissue-specific manner to promote T_3 production and reduce T_3 clearance towards term (Forhead *et al.* 2006).

Thyroid hormone bioavailability also depends on the expression of transporters and intracellular receptors in the target tissues. Although thyroid hormones are lipophilic, several thyroid hormone transporters have been identified which are necessary to allow the hormones access to target tissues (Friesema *et al.* 2005). These transport proteins are likely to have an important role in determining tissue-specific bioavailability of the thyroid hormones in fetal as well as in adult life. However, to date, the developmental expression and regulation of these transporters in fetal tissues of any species is unknown. In fetal sheep, thyroid hormone binding in the liver and brain is evident from relatively early in gestation (Polk *et al.* 1989). In the fetal liver, T_3 binding increases progressively with gestational age, while changes in T_3 binding in the brain are seen in early neonatal life (Polk *et al.* 1989). Therefore, there are developmental changes in tissue sensitivity to thyroid hormones.

Another important aspect of thyroid hormone bioavailability *in utero* is the placental transfer of thyroid hormones, and the relative contribution of maternal thyroid hormones in the fetal circulation. The transfer of thyroid hormones from the mother to fetus varies between animal species and type of

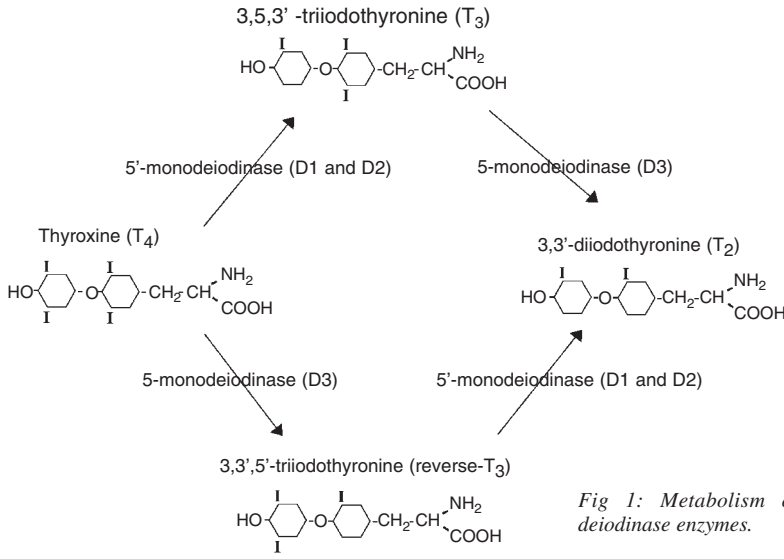

Fig 1: Metabolism of thyroid hormones by deiodinase enzymes.

placenta. The haemochorial placenta in human and rodent species is relatively permeable to T_4 and TRH, but not T_3 nor TSH to any significant extent (Fisher 1997). In contrast, the epitheliochorial placenta of the sheep appears impermeable to maternal thyroid hormones (Hopkins and Thorburn 1971). Therefore, in the human and rodent fetus, maternal thyroid hormones are likely to have an important role in fetal development, especially during early gestation. However, the effectiveness of the ovine placenta as a barrier to the transfer of maternal thyroid hormones means that the sheep fetus is highly dependent upon the normal development of the thyroid hormone axis *in utero*. Therefore, fetal hypothyroidism has marked effects on growth and development in this species (Hopkins and Thorburn 1972).

NUTRITIONAL CONTROL OF THYROID HORMONE BIOAVAILABILITY *IN UTERO*

Thyroid hormone activity *in utero* is sensitive to nutrient and oxygen availability and, with other hormones, thyroid hormones act to match the nutrient supply to the growth, metabolism and development of the fetus (Fowden 1995). In the relatively few studies that have investigated

TABLE 1: Changes in maternal and fetal thyroid activity in response to various nutritional challenges

Nutritional challenge	Species	Gestational age studied	Mother	Fetus	Reference
50% ME from mating to 119 days gestation (term 145 days)	Sheep	113-119 days	↓ plasma T_3	↓ plasma T_3	Rae *et al.* 2002
40% reduction in food intake from 25 days gestation to birth (term 70 days)	Guinea Pig	Term	No change in plasma T_4 or T_3	↓ plasma T_4 and T_3	Dwyer and Stickland 1992
48h food deprivation from 50–51 days gestation (term 70 days)	Guinea Pig	52 days	No change in plasma T4	↓ plasma T_4 ↑D2 mRNA in brain. Sex-specific changes in thyroid hormone receptor mRNA in brain	Lingas *et al.* 1999 Chan *et al.* 2005

thyroid function during nutritional challenges, poor intra-uterine nutrition is associated with reduced circulating concentrations of thyroid hormones in both the mother and the fetus (Table 1). Furthermore, specific micronutrients are required for the normal production of thyroid hormones (iodine) and the deiodinase enzymes (selenium). In guinea pigs, maternal food deprivation for 48 h has been shown to increase gene expression of D2, and induce sex-specific changes in thyroid hormone receptor mRNA, in the fetal brain (Chan *et al.* 2005). These responses may be important in maintaining tissue-specific thyroid hormone production and activity during brain development. However, the effects of under-nutrition on other aspects of thyroid hormone bioavailability *in utero* are unknown.

Prematurity and intra-uterine growth retardation (IUGR) in response to a variety of challenges are associated with low circulating levels of thyroid hormones in the fetus (Williams *et al.* 2004; Table 2). In an experimental model of IUGR induced by ligation of the uterine artery and vein in pregnant rats, an association between bodyweight and plasma T_4 concentrations has been identified in fetuses at 20–21 days of gestation and at birth (Wrutniak and Cabello 1983). However, there is only limited information on tissue metabolism of, and sensitivity to, thyroid hormones in offspring with IUGR. Thyroid hormone receptor binding in skeletal muscle is suppressed in newborn runt compared to normal-sized piglets (Dauncey and Geers 1990). Furthermore, increases in MCT-8 transporter mRNA and protein levels are present in the placentae of growth-retarded human infants at 32–36, but not 37–38, weeks of gestation (Chan *et al.* 2006), which may promote placental transfer of maternal thyroid hormones over this period.

Thyroid hormones and growth of the fetus

Experimental and clinical hypothyroidism *in utero* leads to abnormalities in the growth and development of the fetus. The thyroidectomised sheep fetus is growth-retarded, especially in the skeleto-muscular system. Body weight, and crown-rump and limb lengths, are reduced by fetal thyroidectomy (Hopkins and Thorburn 1972; Fowden and Silver 1995). Growth retardation may be due to several factors, including changes in oxygen consumption and metabolism, and in the development of the somatotrophic axis in the liver and skeletal muscle of the fetus. In thyroidectomised fetal sheep, there are reductions in umbilical oxygen uptake and the amount of oxygen used in glucose oxidation, both of which are restored by a replacement infusion of T_4 (Fowden and Silver 1995). Overall, when control and experimental fetuses were considered, a significant positive correlation was observed between plasma T_4 and oxygen consumption (Fowden and Silver 1995). Furthermore, mRNA abundance of the growth hormone receptor (GHR) and insulin-like growth factor-I (IGF-I) in skeletal muscle is decreased, and normal developmental

TABLE 2: Changes in thyroid hormone activity in intrauterine growth retardation

Cause of IUGR	Species	Change to thyroid hormone activity	Reference
Spontaneous	Human	↓ plasma T_4 and T_3 at 24-35 weeks gestation No change in plasma TSH ↑ placental thyroid hormone receptor protein (TRα and β) ↑ placental MCT-8 mRNA and protein No change in placental D2 or D3 mRNA	Kilby *et al.* 1998 Chan *et al.* 2003; 2006
	Sheep	↓ plasma T_4 and T_3 at birth No change in plasma rT_3 or TSH ↑ relative thyroid size	Wrutniak *et al.* 1990
	Pig	↓ T_3 receptor binding in skeletal muscle at birth	Dauncey and Geers1990
Malnourishment and/or Anaemia	Human	↑ plasma T_4, ↓plasma rT3 and T3 in at birth	Mahajan *et al.* 2005
Carunclectomy	Sheep	↓ plasma T_4, T_3 and rT_3 at 120 days gestation (term 145 days)	Harding *et al.* 1985
Ligation of maternal uterine artery and vein	Rat	↓ plasma T_4 at 20 days gestation to term (21 days)	Wrutniak and Cabello 1983

changes in hepatic GHR, IGF-I and IGF-II gene expression are impaired, by fetal thyroidectomy (Forhead *et al.* 1998; 2000; 2002). Therefore, there appear to be both direct and indirect mechanisms by which thyroid hormones regulate fetal growth, metabolism and development.

THYROID HORMONES AND MATURATION OF THE FETUS

Towards term, the rise in circulating T_3 *in utero* contributes to fetal maturation and the preparation for extra-uterine life. For instance, in the fetal liver, glycogen deposition and gluconeogenic enzyme activity are stimulated in order to maintain euglycaemia over the immediate neonatal period. These maturational events depend upon the prepartum cortisol surge, and are mediated, in part, by glucocorticoid-induced changes in T_3. The normal increments in hepatic glycogen content and activities of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase seen in mature fetuses near term are attenuated in thyroidectomised sheep fetuses (Forhead *et al.* 2003; Matthews *et al.* 2006). Furthermore, an exogenous infusion of T_3 in preterm fetuses accelerates these processes prematurely (Forhead *et al.* 2003; Matthews *et al.* 2006). Therefore, the prepartum rise in plasma T_3 has an important role in the maturation of carbohydrate metabolism in preparation for the nutritional changes that arise at birth. Consequently, the hypothyroid fetus, due to IUGR, prematurity or an endocrine disorder, is unlikely to be able to maintain glucose concentrations in the immediate neonatal period.

Therefore, thyroid hormones have an important role in the control of fetal growth, metabolism and development appropriate for the level of nutrition. Changes in nutrition may have tissue-specific effects on thyroid hormone bioavailability which may act to reduce overall metabolism and growth, whilst preserving thyroid-dependent brain development. Near term, thyroid hormones may also mediate some of the maturational changes that occur in fetal tissues in preparation for the nutritional transition at birth.

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SESSION VI:

Chairman:
Tom McEvoy

CHANGES IN PLACENTAL MORPHOLOGY AND THE SUBSEQUENT EFFECT ON MATERNAL–FETAL TRANSFER

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Growth and development the fetus differs considerably from that of the young neonate. Initially during the embryonic period, growth is under genetic control via individual cells and nutrient supply is governed by histiotrophic mechanisms. Later, as the placenta develops and begins to function, fetal growth becomes substrate dependent, the fetus is now entirely dependent upon the placenta for oxygen, nutrients, growth factors and hormones, some of which will be transported across the maternal-fetal interface and some will be produced directly by the placenta. It is during this substrate dependent period that 98% of fetal growth occurs. Since the fetus is reliant upon the placenta for a considerable period *in utero*, it has lead to the speculation of the possible role of placental growth and function in the programming of the fetus and also that under-nutrition *in utero* has the potential for long-term consequences post natally (Barker 1998).

Birth weight is a major determinant of fetal growth and is routinely recorded at birth. At the lower end of the birth weight spectrum an infant may be viable but reduced birth weight may point towards an underlying dysmaturity or fetal growth restriction placing the infant at increased risk of mortality and morbidity in later life. Intra-uterine growth restriction (IUGR) affects between 4–6% of all pregnancies and places a significant burden on the health care system. Some, but not all, IUGR infants are born to mothers who have pre-eclampsia (PET) and vice versa. Although both PET and IUGR are thought to be associated with fetal hypoxia (Kingdom and Kaufmann 1997) resulting from disrupted placental morphology, the potential impact on oxygen and nutrient transfer resulting from altered placental villous and vasculature architecture has yet to be investigated.

Changes in placental morphology and cellular architecture have the potential to impede nutrient and oxygen delivery from the mother to the fetus. The oxygen diffusive conductance (Dp) (Mayhew *et al.* 1984) is a morphometric model for assessing the transfer of oxygen across the placental–fetal interface and a potential indicator of function. Using the Dp a combination of morphological and physio-chemical constants, it is possible to assess how changes in placental morphology may contribute to oxygen delivery and ultimately fetal growth.

Gestational age is the most important clinical variable affecting maternal and perinatal outcome (von Dadelszen *et al.* 2003). PET and IUGR are heterogeneous disorders which may be better understood by classification into early-onset (<34 + 0 weeks) and late-onset (>34 weeks + 0 weeks) (von Dadelszen *et al.* 2003).

A total of 69 placentae identified as controls, IUGR, PET or PET-IUGR were subdivided into either early (<34 weeks) or late (>34 weeks) disease onset. Each placenta was uniform randomly sampled and chosen samples processed to wax embedding. Combinations of stereological and physio-chemical estimators were used to estimate basic volumetric and exchange surface areas of the peripheral villi and villous membrane harmonic thickness. These estimates were then used to estimate the partial and total resistances and total conductances across the maternal-fetal interface.

In the early-onset cases both IUGR and PET factors were instrumental in bringing about a reduction in fetal and placental weight. In late onset cases IUGR was responsible for a reduction in placental and fetal weight, however, late-onset PET had no effect on birth weight. There was an expected decrease in placental: fetal birth weight

ratio with increasing gestational age. IUGR placentae, regardless of age of onset, had significant reductions in peripheral villi volume and surface areas; partial resistances were in turn significantly increased contributing towards significantly decreased total Dp. However, the mass specific Dp (which takes into account fetal weight) was not significantly reduced in IUGR. Early-onset PET had a far greater effect on placental morphology than late-onset PET, with significant reductions in both volumetric and exchange surface areas contributing to reductions in partial and total resistances and ultimately in significant reductions in both total and mass-specific Dp. Villous membrane harmonic distances were significantly reduced only in late-onset PET; concomitant with increased villous membrane resistance. There was little evidence to suggest that there is an interaction effect of IUGR and PET on placental morphology.

This data suggests that perturbations observed in placental villous and membrane morphology in both early-onset PET and IUGR were not sufficiently compensated for in order to maintain total Dp; evident by reduced fetal growth. This suggests that fetal growth may have been sacrificed in order to maintain placental function. The reduced mass specific Dp observed in early–onset PET suggests that there may be an imbalance between the supply and demand of oxygen which may have further contributed to the reduced fetal growth. Placental morphology in late onset-PET was comparable to that of age matched controls, except for villous membrane harmonic thickness which was reduced. Although the reduced diffusion distance would improve overall transfer capability of the placenta not only for oxygen but also for nutrients, it may point to a discrepancy in trophoblast regulation. There is considerable evidence highlighting the increased amount of apoptosis observed in PET placentae; specifically within the syncytial layer.

A recent publication investigating Dp (Mayhew *et al.* 2006) in PET and IUGR found that PET had no effect on placental morphology unlike IUGR, specifically the authors reported that villous membrane thickness was not significantly affected in either PET or IUGR placentae. Nor did the study find any changes in total Dp or mass-specific Dp. The latter study did not subdivide cases into early or late onset, making comparison between the 2 studies difficult.

Although Dp is a measure of oxygen transfer, the pathway used is not exclusive to oxygen and reduced villous surface area and villous membrane thickness has also the potential to limit nutrient transfer.

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ALTERATIONS OF ORGANOGENESIS IN SIDS AND IUGR: SEQUELAE OF INAPPROPRIATE GESTATIONAL NUTRITION

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Traditional microscopical assessment of sudden infant death syndrome (SIDS, Cot/Crib Death) and intra-uterine growth restriction (IUGR) have historically shown no differences between these conditions and control cases. A comprehensive raft of other investigations has also failed to identify specific pathogeneses for SIDS or IUGR. However, using recently developed micro-morphometric techniques, it has been revealed that many of the organs in SIDS and IUGR cases have numerical sub-organ component deficiencies. All of the organs, so far studied, which show these deficiencies, complete their organogenesis before birth and do not have post natal compensatory ability. These organs do not all complete organogenesis at the same time in gestation suggesting that there needs to be a long standing gestational compromise or a series of acute episodes coinciding with specific organogenic activity. It is possible that these compromises are the result of defined minor nutritional deficiencies affecting placental physiology and thus fetal progression or affecting the fetus directly.

INDUCTION OF LONG-TERM EFFECTS IN SOMATIC CLONES: NUCLEAR REPROGRAMMATION OR FETAL PROGRAMMING?

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It is now established that adult somatic clones can be obtained in most domestic or laboratory species. The toll to these is, however, a very high incidence of early fetal and perinatal loss, with increased fetal and placental weight. Moreover, the lifespan of most cloned species has not allowed many studies on the long-term effects of the procedure.

In cattle and in mice, descriptive studies have shown that gestational perturbations inducing fetal loss took place in the early period after implantation and in the late fetal period. Recent data obtained in cattle in our laboratory shows that although birth weight is increased in clones compared to controls, fetal development is retarded in early gestation (30–60 days out of a 280 day pregnancy), apparently due to abnormal placental development, although the timing of the delay appears to differ between laboratories (Constant *et al.* 2006; Chavatte-Palmer *et al.* 2006a; Hiendleder *et al.* 2004a; Lee *et al.* 2004). Moreover, placental growth appears disconnected from fetal growth, with continuous placental growth in late gestation at a time when placental growth has reached a plateau in normal pregnancies (Constant *et al.* 2006). We have extended these observations in mice where enlarged placentae can be recovered at the end of pregnancy despite the absence of a fetus, a situation barely observed even with transgenic models (Jouneau *et al.* 2006).

Despite these disturbed conditions of development during pregnancy, work currently performed at INRA indicates that biological parameters in apparently healthy adult clones are within normal limits (Chavatte-Palmer *et al.* 2004). It must be noted, however, that occasionally adult clones of normal physiological appearance and of proven fertility can exhibit some differences on specific parameters and often appear slightly different from controls (for example, lower blood haemoglobin contents and decreased cellular immune response to new antigens were observed) (Chavatte-Palmer *et al.* 2006b). Relevant of these observations is the increased death rate in adult clones compared to controls kept in the same conditions (Wells *et al.* 2004). Similarly in mice, some laboratories have described an increased incidence of obesity and decreased longevity in clones (Tamashiro *et al.* 2000; Tamashiro *et al.* 2002), but this has not been described elsewhere.

It is believed currently that long-term effects of cloning are due to abnormal reprogramming of nuclear activities part of which being related to abnormal epigenetic regulations. Imprinted genes appeared as obvious candidates for epigenetic abnormalities as many of those genes are involved in the control of growth and development during pregnancy and because their expression is dependent on methylation processes. Imprinted genes such as IGF-2, IGFR-II or H19 have been studied particularly. It has been shown that IGFR-II expression pattern was disturbed in fetal sheep where the Large Offspring Syndrome had been induced by addition of human serum in the culture medium of embryos (Young *et al.* 2001). In man, mutations of the IGF-II gene are associated with the Beckwith-Wiedemann syndrome, with placentomegaly and fetal macrosomy as major symptoms. Although some global studies show an hypermethylation of parts of the genome in the trophoblast of clones (Dindot *et al.* 2004), others have found that methylation patterns were normal in the fetal part of the placenta but that the liver was hyper-methylated (Hiendleder *et al.* 2004b).

In the authors' opinion, it is unlikely that long-term effects of cloning are only due directly

to abnormal methylation patterns due to nuclear reprogrammation. They are most certainly, at least with the advanced pregnancies, the result of a combination of early epigenetic modifications inducing abnormal placentation and adaptation of the developing fetus to a deregulated environment affecting its metabolic activities (fetal programming). The very varied pathologies observed in the neonatal period in bovine clones and the increased leptin concentrations observed post-natally both in mice and in clones (Renard *et al.* 1999; Chavatte-Palmer *et al.* 2002; Tamashiro *et al.* 2002) suggest that part of the long-term effects are due to fetal programming. Indeed, obesity and the metabolic syndrome, but also immune deficiencies (Moore *et al.* 1999) can be induced by abnormal, in general reduced, fetal conditions. Although bovine clones are in general larger than controls at birth, fetal and placental growth is often delayed in early pregnancy and the fetal/placental weight is reduced in the third trimester, suggesting that placental efficiency is decreased in these animals (Constant *et al.* 2006).

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SESSION VII:

Chairman:
Ron Hunter

EFFECTS OF MATERNAL NUTRITIONAL STATE DURING PREGNANCY ON INSULIN SECRETION AND SENSITIVITY IN NEONATAL FOALS

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INTRODUCTION

The horse is a precocious species, capable of standing and running within hours of birth. To support these activities the foal must maintain adequate blood glucose levels which requires a functional endocrine pancreas (Fowden *et al.* 1984). In other species, maternal nutrition plays an important role in determining fetal and placental growth and also influences insulin secretion and sensitivity in the offspring after birth and in later life (Hoet and Hanson 1999; Armitage *et al.* 2004). Previous studies in horses have shown that increasing fetal nutrient availability by transferring smaller Pony embryos into larger Thoroughbred (TB) recipient mares, increased placental size and fetal growth, and was associated with enhanced pancreatic β cell function in neonatal foals (Forhead *et al.* 2004). In contrast, restricting fetal and placental growth by transferring TB embryos into smaller Pony mares did not alter neonatal pancreatic β cell function despite a significant reduction in foal body weight at birth compared with control TB foals (Forhead *et al.* 2004).

Many TB broodmares are fed high energy rations throughout pregnancy in an attempt to maximise fetal growth for future athletic performance. In adolescent humans and sheep, obesity during pregnancy tends to restrict, rather than enhance, placental and fetal growth whilst over-feeding rats during pregnancy, particularly with high fat diets, causes insulin resistance in the offspring (Wallace *et al.* 2001; Armitage *et al.* 2004). Little is known about the effects of maternal nutrition per se on equine post natal insulin secretion and sensitivity. Therefore, the aim of this study was to identify the effects of maternal nutritional status throughout gestation on insulin secretion and sensitivity in neonatal Thoroughbred foals.

EXPERIMENTAL DESIGN

Ten TB mares were mated to one TB stallion, and fed either at (n=5), or above (n=5), maintenance throughout gestation. Feed intake was adjusted to produce a moderate or obese body condition (score 4–5 or 8–9, respectively). The diet contained 11.7 MJ DE and 10.2 % crude protein. The mares also received supplemental vitamins and minerals. At the end of the first trimester of pregnancy (mean ± sem: 125 ± 10 days), all the mares became inadvertently infected with *Streptococcus equi* and, as a result of the illness lost, on average, 10 % of body weight in both mare nutrition groups (Table 1). The period between onset of weight loss and recovery of pre-infection values was approximately 6 weeks.

Mares were weighed weekly throughout pregnancy and blood sampled for insulin and glucose measurements. Foals were weighed at birth, and the weights and surface area of the placentae were measured (Wilsher and Allen 2006). Pancreatic b cell responses to glucose (0.5 mg/kg, iv, 40% w:v) were investigated on Day 2 post partum. One day later, insulin sensitivity was assessed by measuring the decrement in plasma glucose concentrations in response to a bolus injection of insulin (0.05 IU/kg iv). Blood samples were collected at 5–15 min intervals, from 30 min before to 60 min after, administration and foals were muzzled during the test period. Plasma glucose concentrations were measured using a glucose analyser and plasma insulin concentrations were measured using a radioimmunoassay validated for equine plasma

TABLE 1: Mean ± sem body weight changes in pregnant mares during *S. equi* infection and in newborn foals, and various placental parameters

Nutrition group	Moderate (n=5)	High (n=5)
Mare body weight at conception (kg)	459 ± 5.6	474 ± 16.6
Mare body weight pre-partum (kg)	549 ± 7.0*	603 ± 15.6*
Body weight gain (kg)	90 ± 7.1**	129 ± 3.5**
Body weight lost during infection (kg)	54 ± 12.6	53 ± 15.2
Body weight lost during infection (%)	10.8 ± 2.3	9.8 ± 2.8
Period of weight loss (days)	40 ± 9.0	46 ± 7.5
Foal body weight at birth (kg)	42.1 ± 2.1	45.5 ± 1.4
Gestation length (days)	339 ± 5.2	338 ± 0.6
Placental weight (kg)	3.4 ± 0.22	3.3 ± 0.4
Placental surface area (m^2)	10.7 ± 0.61	9.44 ± 0.46
Foal body weight / placental area (kg/m^2)	4.5 ± 0.3	4.3 ± 0.3

*P<0.05; **P<0.01

(Fowden *et al.* 1984). All the foals examined in the current study were males.

Results

Mares

Maternal glucose concentrations were unchanged over the period of illness and all the mares remained normoglycaemic (Fig 1a). Serum insulin concentrations were significantly (P<0.01) higher in the High than the Moderate mare group one month before, during, and one month after, the period of weight loss (Fig 1b). With the onset of weight loss, serum insulin concentrations decreased significantly (P<0.05) in both mare groups and remained below pre-infection levels for up to one month after normal body weight was regained. All the mares recovered from the infection and maintained their pregnancies. The mares on the High nutrient intake gained significantly (P<0.01) more weight during pregnancy and were heavier at birth compared with the Moderate mares (Table 1). There were no significant differences in placental weight, surface area or placental efficiency (placental area [m^2]/ foal weight [kg]) between the 2 nutrition groups (Table 1).

Foals

All foals were mature and healthy at birth. Gestation lengths and foal body weights at birth were unaffected by maternal diet during pregnancy (Table 1). There were no significant differences in plasma glucose concentrations between the 2 groups of foals, during basal conditions or in response to glucose or insulin challenge tests. However, plasma insulin concentrations at 5, 10 and 15 min after glucose administration, and insulin area under curve, were significantly (P<0.05) higher in foals from Moderately fed mares than those from the High group (Fig 2). The insulin response exhibited by the foals from the obese mothers was similar to that seen previously in 2-day-old TB foals from well-fed mares that did not contract *S. equi*. The peak insulin response and insulin AUC were not significantly correlated to maternal body weight, either at birth or during illness, or to foal body weight or placental parameters.

Discussion

These data suggest that the plane of nutrition during pregnancy altered the insulin responses in the neonatal foals. In particular, the acute nutritional insult caused by maternal illness, superimposed on a moderate nutrient intake, caused a transient hyperinsulinaemia in the foals when challenged with exogenous glucose, an effect which was not apparent in the foals from obese mares. This response was apparently unrelated to maternal weight changes, foal birth weight or to placental weight or surface area. The insulin response to GTT in the foals from the obese mares was similar to that found previously for foals from well-fed mares that were healthy throughout pregnancy, suggesting that the acute weight loss incurred during the *S. equi* infection in the Moderate group may have influenced fetal pancreatic β cell secretion or cell numbers. In

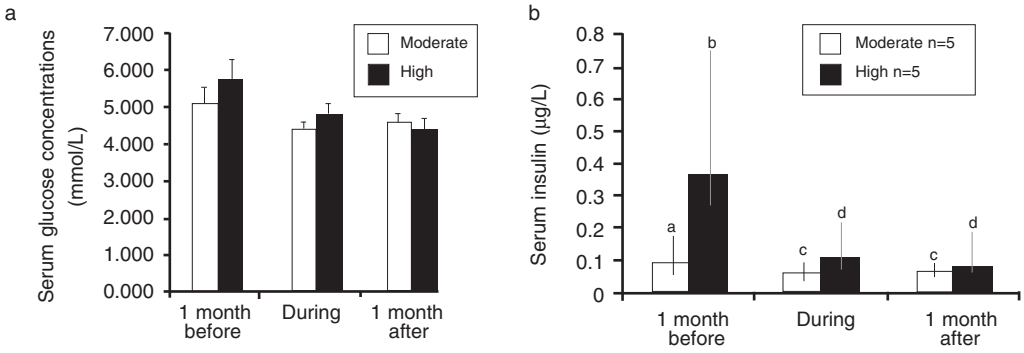

Fig 1: Mean ± sem serum glucose (a) and median ± IQR insulin (b) concentrations in 2 mare nutrition groups for one month before, during and one month after the weight loss period associated with S. equi infection. (Different letters denote significant difference between groups and time; P<0.01).

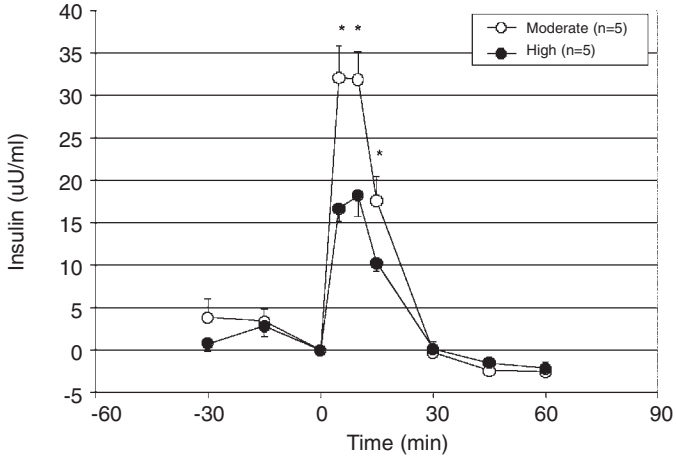

*Fig 2: Increment in plasma insulin concentrations following exogenous glucose (0.5 mg/kg bwt) at time 0 in foals from 2 mare nutrition groups (Values mean ± sem. * P<0.05).*

several species, discrete periods of maternal under-nutrition during pregnancy causes increased insulin secretion in the offspring during pre- and post natal life, possibly through altered β cell sensitivity to glucose, or hypertrophy of the β cells at a critical stage of development (Alvarez *et al.* 1997; Oliver *et al.* 2001; Gardner *et al.* 2005). Although insulin is found in the equine circulation from around 100 days of gestation, little is known about development of equine pancreatic β cells before birth (Forhead *et al.* 2004).

In animals and man, under-nutrition during late pregnancy alters insulin secretion in the offspring with little effect on birth weight (Alvarez *et al.* 1997; Hoet and Hanson 1999; Oliver *et al.* 2001; Gardner *et al.* 2005). Similarly, in the present study, Moderate nutrient levels throughout gestation did not appear to limit fetal growth. Indeed, since insulin is an important growth factor *in utero* (Fowden 1997), the enhanced β cell response to glucose in the foals of mares limited to a moderate nutrient intake may help to maintain fetal growth. Post natal diets can also alter insulin and IGF concentrations in horses and other species (Cymbaluk and Laarveld 1996). In the present study, mares' milk production and nutrient content was no different between the dietary groups during the first week post partum (Ousey and Cundy: unpublished observations), which suggests that post natal diet was unlikely to account for the observed differences in β cell function in the newborn foals.

Although maternal glucose concentrations were not affected by diet or illness, maternal insulin concentrations were significantly reduced in mares fed a moderate plane of nutrition, and in both mare groups following maternal illness. Similar observations have been reported for pregnant sheep subjected to acute nutrient restriction in mid-pregnancy (McMullen *et al.*

2005). These authors suggested a reduction in anabolic drive in order to maintain the supply of glucose to the fetus. It is likely that a similar adaptation occurred in the mares in the present study in response to the acute nutrient restriction. The equine fetus is more reliant than the sheep fetus on maternally derived glucose during gestation and even short (<24 h) periods of starvation may cause fetal abortion in the mare (Fowden 1997).

Conclusions

A moderate plane of maternal nutrition during pregnancy increases equine neonatal insulin secretion. The results suggest that acute weight loss in pregnant mares on maintenance feeding may lead to increased glucose sensitivity of the pancreatic β cells in their foals immediately after birth.

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PRE-NATAL PROGRAMMING OF POST NATAL PERFORMANCE IN SWINE

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In a polytocous species such as the pig, litter size is the end result of several interacting components including ovulation rate, embryonic survival, and uterine capacity. In situations of severe maternal catabolism, irrespective of its cause, litter size in the subsequent parity is limited by both a reduced ovulation rate and decreased embryonic survival. An example of this is the detrimental effect of lactational catabolism on the maturity of oocytes recovered from the presumptive pre-ovulatory follicles recovered after weaning. There is a substantial body of information that links such deficits in oocyte developmental competence with subsequent asynchronous embryonic development, as a key factor in determining embryonic survival. Further limitations to nutrient availability to the embryo, or other factors that compromise normal embryonic or fetal development, will impose additional negative effects on both the number of live-born piglets and their developmental potential. Uterine capacity is the ultimate constraint on litter size in the pig. Evidence suggests that high numbers of conceptuses *in utero* may have negative consequences on post natal growth and development that may not be apparent by an examination of fetal weight alone. Studies of post natal growth in the pig have shown a positive correlation between total muscle fibre number and growth potential, and also that littermates with higher muscle fiber number grow faster and more efficiently than littermates with a lower total fibre number. This within-litter variation in growth potential is established in the early stages of gestation (Day 27 to Day 35) and high numbers of conceptuses *in utero* have a significant negative impact on post natal growth. The authors have shown, through histological studies on porcine fetuses at Day 90 of gestation, that high numbers of conceptuses lead to lower secondary muscle fibre number. In the present study, the effect of uterine crowding on the expression of myogenic regulatory factors (MRFs) in the embryo at Day 30 of gestation was investigated to establish a mechanistic basis for understanding the reduced muscle fibre number in fetuses from 'crowded' uteri. Uterine crowding was hypothesised to lead to differences in transcript abundance or a shortening of the time frame of expression of MRFs and consequent reduction in the potential for muscle fibre development. Unilateral oviduct ligation was performed on sows to limit the numbers of embryos *in utero* and conceptuses were collected from 10 ligated and 10 non-ligated (control) sows. RNA was extracted from all embryos and the transcript abundance of 2 MRFs, MyoD and myogenin, were compared between the ligated and control groups using quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). Although no differences between treatments were seen in transcript abundance for MyoD, there was a significant increase in the level of myogenin transcript expression in conceptuses from less crowded uterine environments. These results suggest that uterine crowding, by compromising nutrient supply to the conceptus most likely through impaired placental development, can affect the expression of genes that regulate muscle development and that this difference can be detected as early as Day 30 of gestation.

THE EFFECT OF GROWTH IN EARLY LIFE ON METABOLIC HOMEOSTASIS IN LATER LIFE

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The incidence of metabolic diseases such as impaired glucose tolerance, insulin resistance and non-insulin-dependent diabetes is increasing and the influence of 'lifestyle' factors, such as diet and physical activity, cannot fully explain the variation in occurrence of these diseases. In addition, there is a so-called 'epidemic' of obesity in affluent countries that will have a strong influence on the aetiology of these diseases. Epidemiological studies in well-defined human populations have demonstrated a role for the early life environment in predicting later life risk of metabolic disease, as well as cardiovascular disease, obesity and dyslipidaemia (Osmond and Barker 2000).

Initial epidemiological studies focussed on the effects of reduced fetal growth and low birth weight and showed that the risk of type 2 diabetes mellitus increased with decreasing birth weight across the normal range (Hales *et al.* 1991). Studies of the long-term effects of famine have established that maternal nutrition plays a role in associations between the early life environment and later disease risk. Individuals exposed to the Dutch famine (1944–1945) in late gestation were lighter at birth and had impaired glucose tolerance in adult life, which worsened with obesity (Roseboom *et al.* 2001). Poor maternal nutrient supply in late gestation is often associated with asymmetrical growth retardation and it is now clear that thinness (low body mass index) at birth is a particular risk factor for glucose intolerance and insulin resistance in later life (Phillips *et al.* 1994). However, growth failure in late gestation may also originate from influences acting earlier in gestation, such as during placental development. Indeed, a poor maternal environment in early gestation, for example during famine, increases the incidence of coronary heart disease, in the absence of any effect on birth weight (Roseboom *et al.* 2001).

Epidemiological studies also show that growth trajectories and the environment in post natal life are implicated in the early life origins of adult disease and may amplify the effect of low birth weight. Poor early growth, as measured by persistent thinness in infancy, followed by catch-up growth into adolescence above and beyond average is associated with poor glucose tolerance in adulthood (Forsen *et al.* 2000; Barker *et al.* 2005). By contrast, other studies have suggested that accelerated growth in very early post natal life is detrimental to insulin sensitivity in later life and that this may explain the associations between low birth weight and impaired glucose tolerance in adulthood (Singhal *et al.* 2003).

Several animal models have been employed to assess whether birth weight or size is a good indicator of conditions during fetal life and to

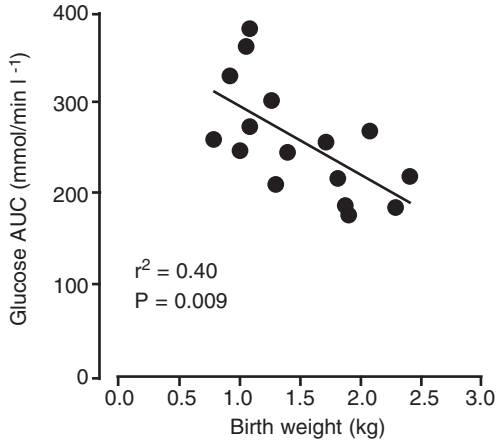

Fig 1: The association between birth weight and the area (AUC) under the glucose curve following bolus intravenous administration of glucose (0.5 g/kg body weight) at 12 months of age in pigs (Poore and Fowden 2004).

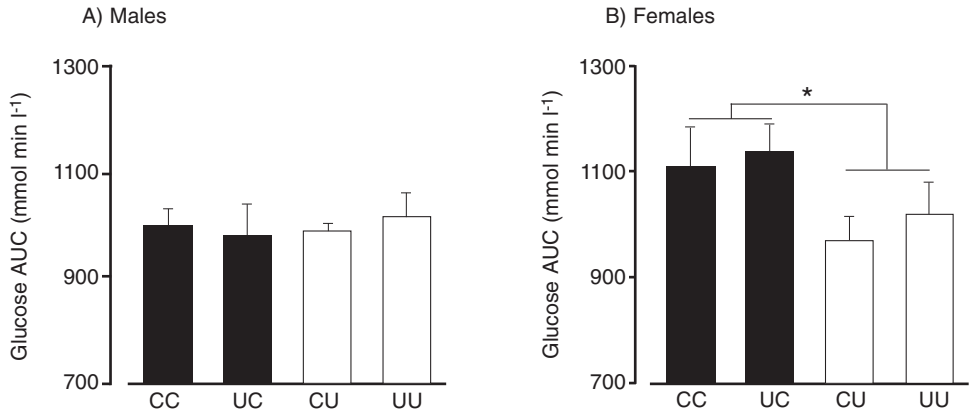

*Fig 2: Area under the glucose curve (AUC) during a glucose tolerance test in male (A) and female (B) sheep at 2.5 years of age. Sheep were born to ewes that received either 100% (group C: male, n=21, female, n=18) or 50% of nutritional requirements (group U: male, n=18, female, n=21) from day 1 to day 31 of gestation, and 100% thereafter. Offspring were fed ad libitum (■; CC: male, n=13, female, n=9; UC: male, n=9, female, n=10) or to reduce body weight to 85% of target from 12 to 25 weeks post natal age and ad libitum thereafter (❑; CU: male, n=8, female, n=9; UU: male, n=11, female, n=11). * p<0.05, ANOVA (Poore et al. 2007).*

determine the relative roles of the pre-natal vs. post natal environment on later disease risk. The naturally-occurring variation in birth weights within pig litters has enabled us to show that both birth weight and post natal growth patterns influence glucose-insulin metabolism in this species. Glucose tolerance in adults worsened with reducing birth weight (Fig 1) and catch-up growth during suckling was associated with reduced insulin sensitivity (Poore and Fowden 2002; Poore and Fowden 2004). In sheep, low birth weight lambs in twins discordant for birth weight have improved glucose tolerance in juvenile but not adult life (Clarke *et al.* 2000), while others have shown that glucose tolerance is negatively influenced by low birth weight, rather than the plane of maternal nutrition in late gestation (Oliver *et al.* 2002). However, adult offspring of ewes undernourished in late gestation have impaired glucose-insulin metabolism and increased body fat, in the absence of reduced birth weight, which may be due to defective glucose uptake into adipose tissue (Gardner *et al.* 2005). Low maternal body condition, a more long-term indicator of maternal nutritional status, is also associated with reduced glucose tolerance in adult sheep (Cripps *et al.* 2005).

Therefore, there is evidence that both birth weight and the early environment can have long-term effects on metabolic homeostasis in large animal models. However, it is also clear that gross adaptations to the early environment, such as reduced birth weight, are not necessary for altered function in later life. The developing organism is able to respond and adapt to suboptimal environmental conditions in several ways: to promote immediate survival and/or to make predictions about the environment in later life. The degree of mismatch between the environment predicted in fetal life, based on how the fetus interprets maternal conditions at the time, and the actual environment experienced by an individual in later life may increase the risk of adult-onset diseases (Gluckman and Hanson 2004).

Current studies from the authors' laboratory, using a new sheep model, aim to determine the relative roles for the pre-natal and post natal environments, and their possible interaction, on post natal growth patterns, glucose handling and insulin sensitivity in adult life. In this model, it is found that nutrient restriction in early postnatal life, immediately following weaning, causes a long-lasting improvement in glucose tolerance (at least until 2.5 years of age; Fig 2). This effect was independent of birth weight or the nutrient environment in early gestation and occurred only in females (Poore *et al.* 2004). The authors speculate that such an increase in glucose handling may predispose to an increase in body fat and a subsequent deterioration of glucose tolerance in later life, particularly in the presence of an abundant diet.

Thus, human and animal studies have demonstrated that there are a number of different critical windows during early life, both pre- and post natal, that can have long-term effects on metabolic homeostasis in later life. These effects may be sex-specific and do not necessarily involve changes in birth size. Ongoing studies in this field will require a life-course approach.

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SUMMARY

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SUMMARY

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These few short pages can offer no more than a taste of a privileged and fruitful Workshop, valued as much for the vigour and quality of the discussions as for the formal presentations themselves. Indeed, even allowing for various molecular contributions, there was a strong suspicion that most of what was said in the papers had already been published or otherwise entered the public domain. However, a major attraction of the relatively long periods of discussion was that enthusiastic participants could not always resist mentioning their latest unpublished findings. Speakers and chairmen prompted interactions which more than justified these immensely special Havemeyer assemblies in their idyllic geographical settings. Thus, the format of the Workshops remains highly effective.

Matters commenced in the female gonads and in tissues taken therefrom. We were repeatedly informed that ... "oocyte quality governs embryo potential", which is undoubtedly true, but it is surely not banal to recall the contribution of the male gamete. Spermatozoa were scarcely mentioned throughout the Workshop, yet genomic interactions at the time of zygote formation and soon thereafter are critical determinants of embryonic and fetal potential. That remark aside, diverse chemicals such as organochlorides present in the environment of most industrialised countries could target the mammalian ovary, and sound evidence was presented for a deleterious influence of such compounds on oocytes matured in *in vitro* systems of culture: meiotic progression was perturbed, degeneration of nuclear chromatin enhanced, and subsequent evolution into Day 8 blastocysts was depressed in bovine embryos generated *in vitro*. *In vivo* evidence for harmful influences on meiotic maturation of oocytes in pigs fed mycotoxins was also summarised, and endorsed with observations on subsequent embryonic development.

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Even so, in terms of a current understanding of ovarian physiology, what could these results mean or those suggesting that pre-ovulatory nutritional régimes can influence oocyte quality? One had always assumed, if perhaps naïvely, that the enormous complement of oocytes in the mammalian ovary, coupled with endless waves of follicular maturation and atresia, would afford a form of pre-ovulatory selection and enable essentially viable oocytes to be shed at ovulation. It would seem a poor biological strategy for a species to spend a protracted period in reaching puberty, then investing further weeks in an oestrous or menstrual cycle, only to release an incompetent oocyte at ovulation when, presumably, there remain so many viable ones in reserve.

If an oocyte can be rendered susceptible or labile during the pre-ovulatory interval, as we must now believe to be true, why should this be so? Does it reflect modifications to the vascular network of the follicle wall with an altered potential of the theca cells and/or is there a modified molecular exchange between cumulus-corona cells and the oocyte? As an alternative though certainly not exclusive consideration, could the relatively enormous size of both the vitellus and its germinal vesicle provide exceptional scope for the short-term accumulation of metabolic insults? In a broader perspective, should one now consider that the gonad acts as a sensitive monitor of health and nutritional status at around the time of oestrus, at least in terms of maturing Graafian follicles and the oocyte within? Whether yes or no, it must be accepted that apoptosis eventually becomes a feature of the above scenario, with diverse insults prompting changes in the underlying gene cascades. And apoptosis, it should be recalled, plays a subtle and not entirely predictable hand.

There was concern that the notion of oocyte quality was offered as a reasonably novel topic of investigation. In fact, it has a long and

distinguished pedigree stemming especially from the experimental studies of Professor Charles Thibault in Paris on both nuclear and cytoplasmic maturation (Thibault and Gérard 1970), and it was the subject of various meetings and symposia in that city during the 1970s. Even the present author finds that he used the term 'oocyte quality' in the title of a publication of that period. In similar vein, another of the opening papers in the Workshop dwelt at some length on the inhibitory influence of follicular granulosa cells on resumption of meiosis in cultured primary oocytes, with the feeling strongly conveyed that this was a recent finding. Not so. Using elegant techniques of whole follicle culture or culture of bisected Graafian follicles, the now classical publication of Foote and Thibault (1969) demonstrated unequivocally that follicular somatic cells in contact with the oocyte acted to suppress resumption of meiosis. In the slightly ungenerous turn of phrase, those who do not know the history of their field of research tend to repeat it.

Moving to the molecular level, a systematic series of studies demonstrated perturbations of gene expression in young bovine embryos brought about by *in vitro* culture of oocytes and/or embryos. Once again, the specific nature of the *in vitro* insult(s) requires clarification, although the physiological milieu of the oviduct lumen wherein the bovine embryo resides for 3 days can clearly not be taken for granted. As a way forward, components of the culture medium could be compared with the composition of oviduct fluid but this approach has seemingly not been helpful. Why? Doubtless because studies have examined oviduct fluid as a whole instead of as a series of microenvironments along the duct, not least as influenced by the suspension of ovarian follicular cells that remains in the vicinity of the embryos throughout their oviduct sojourn. At least as important as components of the culture medium could be considerations of temperature in the culture system. Modern approaches continue to use 38.5°C when there is a growing body of evidence that 1) this does not represent intra-follicular temperature during resumption of meiosis; and 2) small deviations from physiological temperature can have a profound influence on subsequent patterns of gene expression.

Remaining with the topic of gene expression in a context of oocyte maturation and early embryonic development, there were persuasive reports of up-regulation, down-regulation, and other forms of waywardness. Such perturbations are currently seen as one explanation for the Large Offspring Syndrome associated with a prolonged period of gestation. Discussion turned repeatedly to the key involvement of DNA methylation in controlling gene expression and yet one was left wondering (a) why methylation status had assumed such a prominent role and (b) to what extent there are other more subtle regulators of gene activity still to be revealed. There was also the critical question as to whether aberrations in early gene activity can be compensated for as the embryonic genome commences to be expressed more fully. The thrust of several discussions appeared to be that no such flexibility exists – an aspect on which this reviewer remains to be convinced. Nonetheless, evidence from somatic cloning would indicate that long-term deleterious influences of the cloning procedures are associated, at least in part, with epigenetic modifications.

Nutritional considerations during the various phases of gestation quite naturally occupied much of the Workshop and, although not specifically mentioned, these recalled and built upon the Hammond principle of metabolic priorities across the placenta and within the fetus, ie CNS, bone, muscle, fat. Having listened to Sir John Hammond lecture in the early 1960s (he died in 1964), there was once again the feeling that some of the younger participants in the Workshop might, to advantage, glance at Hammond's vintage writings on metabolic gradients and on the simultaneous metabolic demands of lactation and early gestation. Of course, modern methods of endocrine and molecular analysis have added much more detail, on occasions rather too much to enable specific conclusions to be drawn and potential therapies to be proposed. However, one is no longer surprised that energy status can influence the performance of reproductive tissues, be it within the gonads or in different compartments of the genital tract.

Incisive research at the nutrition-reproduction interface is seldom straightforward. Bearing in mind that the mother will have a fully-functional endocrine system and that an embryo will fast be developing its own endocrine potential, together with the consideration that one is attempting to describe dynamic physiological events, then it is not difficult to appreciate why nutritional experiments that clarify rather than complicate the

picture are an exception. There is also the paradox that the greater the number of molecules examined in the quest for precision, the more difficult the interpretation of such results becomes, let alone the development of some useful therapy.

This feeling of bewilderment, or perhaps better expressed as wonderment, leads us appropriately to that hallmark of higher mammals (Eutheria), the placenta. In words that flowed from this very same fountain-pen (who also uses such perfect technology apart from Twink?):

"Viviparity involves a high level of organisation in the genital duct system in conjunction with a corresponding development of the organ of exchange and sustenance, the placenta. In the play and interplay of evolutionary forces, who could have imagined that the ancestral paramesonephric duct system would one day embrace such a highly developed and complex organ as the eutherian placenta as one consequence of the switch from external to internal shedding of the gametes?" (Hunter 1995).

And of course, in such complexity lies the experimental dilemma: what really constitutes a meaningful measurement and what is feasible with the latest technology?

Timeless favourites were to the fore in diverse experimental models focusing on placental and fetal growth. These included growth hormone, insulin, IGF, IGF-binding proteins, glucose, leptin, prostaglandins, ACTH and cortisol in a range of tissues subjected to different conditions. And yet, despite skilful designs and a huge body of results, it was not obvious to this reviewer what would constitute an inspired next step within a conventional framework nor whether a quantum leap in experimental technology would bear fruit. As to the last point, and offering a perspective from the Royal Veterinary University in Copenhagen, monitoring passage of labelled molecules and precursors at the placental interface certainly appears possible using micro-imaging technology (a sphere in which Danish developments are pre-eminent). Dynamic measurements might thus be made with minimal interference.

But first back to the present. Progesterone, as the time-honoured hormone of pregnancy, has not lost its appeal. Its influence at the commencement of sheep conceptus attachment to the endometrium was revisited in detail, with Jacques Martal's trophoblastin – now termed interferon tau – recalled in the various steps to suppression of the luteolytic pulses of uterine $PGF_{2\alpha}$ (Martal *et al.* 1979). Inappropriate timing in the endocrine events, perhaps reflecting retarded embryonic development, is appreciated to result in a luteolytic cascade despite the presence of an embryo in the uterine lumen. Components of this potential scenario of embryonic loss require further dissection, although the involvement of specific maternal and embryonic genes is currently proceeding, not least using gene knock-out models. A key question appeared to be the extent to which nutritional regimes could act to influence placental gene expression. Within the over-nourished adolescent sheep model, there was a further novel contribution suggesting that wayward values for circulating progesterone and ovine placental lactogen might be associated with inappropriate microdifferentiation of the embryonic placenta at stages during which trophoblast binuclear cells should fuse with the maternal syncytium; reduced trophoblast proliferation was noted in support.

Placental morphology was also subjected to detailed examination as a backcloth primarily to the effectiveness of placental transfer of oxygen. The report focussed on both villous surface area and membrane thickness. Although various useful guidelines were reached, it was not completely clear to what extent the very substantial amount of laboratory work so involved was able to deliver new insights, over and above the fact that modified placental micromorphology could compromise the efficiency of transfer within this organ system.

Moving on to the Havemeyer species of choice, and in particular to the equine endometrium, a video-endoscope was used to guide us across the undulating surface of this remarkable tissue and into the neck of its glands before the extensive embryo-endometrial interactions were described. Thereafter, the endocrine backcloth and specific contributions of diverse growth factors to embryonic and early fetal development were reviewed at exceptional speed! Whilst such material was invaluable in its own right, knowledge of the viability of an embryo at given moments of assay might have added further insights. Rephrasing this thought, if embryo-endometrial interactions could have been probed using embryos destined to die at known stages of gestation, for example after mild X-irradiation at early cleavage stages, might it have been possible to tease out further valuable aspects of the interaction?

What appeared at first glance as an aside became a further useful contribution from the 2 organisers of the Workshop. This concerned an influence of *Streptococcus equi* infection-mediated nutritional insult on development of the placental microcotyledons in Thoroughbred fillies. Inappetance brought on by *Streptococcus equi* infection prompted measurable changes in placental development and thereby suggested other experimental models in equids for modifying placental efficiency and thus fetal development. Scaling steeply down the size of model, a youthful contribution from France employed the rabbit to explore the influence of diets with elevated lipid and cholesterol content on the offspring of adolescent mothers. Intra-uterine growth retardation and lesions in the aorta could be demonstrated, but it was uncertain to what extent this species and experimental design could be exploited fruitfully in the future.

Of specific value in broadening the perspective of reproductive physiologists, discussions moved to aspects of organogenesis and function in non-genital tissues. Concerning kidney function, a notable finding has been a reduction in the number of nephrons at birth as a response to a low-protein diet fed to pregnant rats. Such reduction in number is apparently irreversible after birth and is linked to perturbations in the renin-angiotensin system, with progressive renal dysfunction and hypertension demonstrable in the offspring. A relationship between nephron deficit and maternal glucocorticoid synthesis was indicated since blocking such glucocorticoid synthesis prevented a nephron deficit and hypertension in the pre-natal low protein rat model. Subtleties within this relationship are currently being unravelled at the molecular level.

Perhaps not too surprisingly, thyroid function in the fetus also influences development and post natal potential. Studies using catheterised sheep fetuses reveal that the classical cortisol-induced preparations for birth are mediated, at least in part, by glucocorticoid-regulated changes in the thyroxine metabolite, the so-called T_3. Once again, poor intra-uterine nutrition has an influence, acting to reduce circulating concentrations of thyroid hormones as term approaches.

Turning to our own species, modern micromorphometric techniques applied to the conditions of Sudden Infant Death Syndrome (SIDS) and Intra-Uterine Growth Restriction (IUGR) have revealed quantitative deficiencies in specific sub-organ components, the number of nephrons being once again a case in point. A key fact is that none of the organic lesions was open to post natal compensation, whereas accelerated or catch-up growth in early post natal life may mask potential problems. The novel proposition was offered that the interaction between fetal and post natal environments is critical, some form of recall in the neonate enabling mismatch to be perceived and potentiating the risk of disease being expressed in the adult. This could represent a form of mammalian metabolic memory.

Returning almost to the beginning, the oviduct wherein fertilisation takes place and early embryonic development commences, it was pleasing to be reassured that the fluid milieu of this portion of the duct system is vital for successful development of the zygote. By means of embryo culture and subsequent transfer experiments using a sheep model, an approach known to reduce fetal weight in twins, even a very short period of exposure (1 h) to human blood serum in a standard culture system could modify subsequent gene expression in both placenta and fetus. Extrapolating from such observations and those mentioned earlier, the period of embryonic residence in the oviduct (2–7 days, according to species) undoubtedly has a profound influence on later gene expression in the embryo and fetus. Transferring embryos directly into the uterus will not necessarily deprive an embryo of the putative programming influence of the oviduct fluid environment, assuming that the ducts remain patent and have not been compromised by inflammation.

The preceding paragraph is presented at this late stage of the Summary for quite specific reasons. As a concluding comment on matters academic, perhaps we now need to move our plane of thought to that of the gifted American maize geneticist, Barbara McClintock. Her proposition back in the 1930s was that all the genes of an organism are potentially in conversation with one another. Bearing in mind the number of genes in a mammalian species, say 30,000, and the potential for interactions therein, then unravelling the changing conversations at and within successive days of pregnancy should keep molecular biologists and computer scientists usefully occupied for some little time yet. However, this is not to overlook the absolute requirement for a

meaningful balance to be struck between molecular studies and physiological ones – those concerned with dynamic processes in the living animal.

Overall, and looking back, the conclusion must be that this was a particularly valuable Workshop with all of the formal speakers giving uniformly excellent presentations. It is therefore superfluous to state that the meeting was a success, but not to say that it would not have been without the energetic Newmarket input of Professor Twink Allen and his special research partner, Miss Sandra Wilsher. Nor could the Workshop have succeeded without the gifted organisational skills of Jan Wade and her colleagues at R&W Communications. And behind the scenes – alas, completely so on this occasion – the continued enthusiastic and dedicated support of Mr Gene Pranzo, President of the Havemeyer Foundation, must receive our warmest and very best thanks.

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