



*Havemeyer Foundation
Monograph Series No. 2*

Proceedings of a Workshop on

FETOMATERNAL CONTROL OF PREGNANCY

14th - 16th November 1999

Barbados, West Indies

Editors: T. A. E. Stout and J. F. Wade



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EDITORS' FOREWORD

In many ways, the evolution of viviparity and placentation by the eutherian mammals was a remarkable solution to the challenge of providing ones offspring with the best possible start in life, not least because it necessitated the development of a number of intricate physiological and immunological strategies to ensure maintenance of the pregnant state and to prevent rejection of the antigenically half-foreign fetus and placenta. Indeed, when one considers the numerous biological obstacles that the developing horse embryo, conceptus and fetus has to clear during 11 months of gestation, it seems incredible that any manage to develop successfully to full term; fortunately most do. There remains, however, a significant if relatively small proportion of cases in which either the pregnancy fails or the resulting foal is not capable of its intended athletic function. This is all the more frustrating when it is not possible to identify a reason for the failure or, perhaps more pertinently, there is insufficient information to develop a strategy for reducing the likelihood of its recurrence. Of course, to appreciate how and why things go wrong, it is first necessary to understand more fully what happens during normal pregnancy. In this respect, the horse with its numerous peculiarities is a fascinating animal to study.

In 1997, the Dorothy Russell Havemeyer Foundation generously sponsored a workshop on

'Maternal Recognition of Pregnancy in the Mare' in which a specific aspect of the interaction between the equine fetus and its dam, namely the prolongation of the lifespan of the primary corpus luteum, was examined. The meeting was a great success scientifically, and initiated a number of international collaborative initiatives. This was largely due to the input of scientists working on other large domestic animal species.

The aim of the current workshop on 'Fetomaternal Control of Pregnancy' was to expand this topic to cover the dialogue that must continue throughout pregnancy and the potential consequences of biochemical or physical perturbations to this interaction.

The willingness of the Havemeyer Foundation to support workshops of this nature is applauded by all concerned; and the recent initiative to introduce a series of monographs will ensure that the information gained is disseminated to the widest possible audience. We must express sincere thanks to the Foundation and, in particular, to Mr Gene Pranzo, President of the Foundation, whose continued enthusiasm and encouragement are much appreciated.

T. A. E. Stout
J. F. Wade

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
- Third International Symposium on Equine Embryo Transfer**
February - Buenos Aires, Argentina
Organisers : Drs D. F. Antczak, W. R. Allen, J. G. Oriol and R. Pashen

1995

Equine Perinatology

July - Cambridge, England

Organiser: Dr P. D. Rossdale

Second International Equine Leucocyte Antigen Workshop

July - Lake Tahoe, California, USA

Organisers : Drs D. F. Antczak, P. Lunn and M. Holmes

First International Workshop on Equine Gene Mapping

October - Lexington, Kentucky, USA

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

Erection and Ejaculation in the Human Male and Stallion: A Comparative Study

October - Mount Joy, Pennsylvania, USA

Organiser: Dr S. M. McDonnell

Bone Remodelling Workshop

October - Corcord, Massachusetts, USA

Organiser: Dr H. Seeherman

1997

Second International Workshop on Equine Gene Mapping

October - San Diego, California, USA

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

Maternal Recognition of Pregnancy in the Mare

January - Dominican Republic

Organisers: Drs W. R. Allen and T. A. E. Stout

Uterine Clearance

March - Gainesville, Florida, USA

Organiser: Dr M. M. LeBlanc

Trophoblast Differentiation

September - Edinburgh, Scotland

Organisers: Drs D. F. Antczak and F. Stewart

1998

Third International Genome Workshop

January - San Diego, California, USA

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

Third International Workshop on Perinatology: Genesis and Post Natal Consequences of Abnormal Intrauterine Developments: Comparative Aspects

February - Sydney, Australia

Organiser: Dr P. D. Rossdale

Horse Genomics and the Genetic Factors Affecting Race Horse Performance

March - Banbury Center, Cold Spring Harbor, New York, USA

Organisers: Drs D. F. Antczak, E. Bailey and J. Witkowski

Allergic Diseases of the Horse

April - Lipica, Slovenia

Organisers: Drs D. F. Antczak, S. Lazary and E. Marti

Equine Placentitis Workshop

October - Lexington, Kentucky, USA

Organisers: Drs D. F. Antczak, W. R. Allen and W. Zent

Septicemia II Workshop

November - Boston, Massachusetts, USA

Organiser: Dr M. R. Paradis

1999

Equine Genome Project

January - San Diego, California, USA

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

Third International Equine Genome Workshop

June - Uppsala, Sweden

Organisers: Drs D. F. Antczak, E. Bailey and K. Sandberg

Fourth International Meeting of OIE and WHO Experts on Control of Equine Influenza

August - Miami, Florida, USA

Organiser: Dr. J. Mumford

European Equine Gamete Workshop

September - Lopuszna, Poland

Organisers: Drs W. R. Allen and M. Tischner

Fetomaternal Control of Pregnancy

November - Barbados, West Indies

Organisers: Drs T. Stout and W. R. Allen

2000

Equine Genome Project

January - San Diego, California, USA

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

Uterine Infections in Mares and Women: A Comparative Study

March - Naples, Florida, USA

Organiser: Dr M. M. LeBlanc

*Expanded abstracts of presentations at the
Havemeyer Foundation Workshop on*

**FETOMATERNAL CONTROL OF
PREGNANCY**

TEMPERATURE CONSIDERATIONS FOR *IN VITRO* STUDIES WITH EQUINE GAMETES

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In statistical terms, *in vitro* maturation and fertilisation of oocytes, coupled with procedures of embryo transplantation, have seldom proved a fruitful approach to generating full-term pregnancies, impressive though the techniques themselves may be. Even in our own species, with the world-wide establishment of *in vitro* fertilisation clinics and a relatively simple means of embryo transplantation, the overall success in generating viable fetuses rarely exceeds 15–20%. Why should this be so? An all embracing answer might be that the conditions of culture diverge significantly from those in the physiological situation. A vitally important component here is the temperature of the culture system. Incubators for the maturation of mammalian gametes are generally set at 39°C, not because such a temperature is known to mimic that of the Graafian follicle or Fallopian tube ampulla but rather because it has been found to stimulate sperm motility and penetration of the egg investments (Cheng *et al.* 1986). Although the resultant incidence of fertilisation may frequently be high, the viability of zygotes is seemingly compromised. Inappropriate temperatures in the culture system may impose a degree of nuclear and cytoplasmic damage that is only revealed subsequently during specific phases of gene expression that underlie normal differentiation.

Previous studies using fine thermistor probes revealed a pre- and peri-ovulatory temperature gradient in the Fallopian tubes of pigs and rabbits, with the caudal isthmus being cooler than the cranial ampulla by a mean 0.7°C in mated pigs (Hunter and Nichol 1986). Such a temperature gradient was presumed to contribute to the pre-ovulatory phase of sperm storage in the caudal isthmus, during which time viable gametes show suppressed motility, stabilised cell membranes and

specific adhesion to endosalpingeal microvilli by the apex of the sperm head. Generation of a temperature gradient between the 2 ends of the Fallopian tube may depend on: 1) differences in the vascular bed between the relatively thin-walled ampulla and thick muscular isthmus; 2) enhanced contractile activity in the ampulla generating local heat production. In the light of recent studies, the possibility of endothermic reactions in the secretions accumulating as a form of mucus in the caudal isthmus before ovulation must also be considered. Whether a temperature gradient guides a partially or fully capacitated spermatozoon towards the site of fertilisation has still to be resolved. Nonetheless, it seems reasonable to regard a sperm cell in the process of being activated and released from storage in the caudal isthmus shortly before ovulation as especially temperature sensitive. Indeed, there could be heat-seeking programmes specifically revealed within its cell membranes. Such a physiological response might be viewed as an evolutionary shift from chemotaxis in external (aquatic) models of fertilisation to temperature sensitivity with internal fertilisation, particularly in the Fallopian tubes of eutherian mammals.

Thermistor probes have also been used to measure temperatures within the ovarian tissues of pigs, but results revealing a gradient between mature Graafian follicles and stroma of >1.0°C were only published more recently (Hunter *et al.* 1995). In the present context, a question arises as to temperatures within mature Graafian follicles or the lumen of the Fallopian tube in normally cyclic mares. Although the morphology of the equine ovary differs from that of other large domestic species with respect to arrangement of the cortex and medulla, a temperature gradient may still be revealed between ovarian stroma and Graafian

follicle, and indeed between ovarian and deep rectal temperatures. Recent measurements in cows and pigs (Hunter *et al.* 1997) and earlier studies in rabbits and women (Grinsted *et al.* 1980, 1985) revealed temperature gradients between mature follicle(s) and stroma of 1.3°–1.7°C or more, with the pre-ovulatory follicle(s) always being cooler. Arresting ovarian bloodflow for 5 min had no more than a negligible influence on follicle-stroma temperature differentials, nor did ovariectomy followed by thermo-imaging of the detached living tissues suspended within the abdomen. By contrast, killing tissues by plunging a detached ovary into liquid nitrogen and then thermo-imaging within the abdomen, after a period of equilibration, removed all temperature gradients. Cooling rate curves measured during 30 or 60 s for ovaries sited deep within a mid-ventral incision were essentially parallel for stroma and follicles. Temperature differentials observed via an infra-red transmitting endoscope revealed follicle-stroma differences of 0.6°C–1.1°C according to stage of the oestrous cycle, mature pre-ovulatory follicles being cooler than smaller follicles (Hunter *et al.* 1999).

Temperature gradients between follicles and stroma would be maintained by a counter-current heat exchange system in appropriate ovarian blood vessels. Persuasive anatomical evidence exists for such systems. Cooling would be generated by endothermic chemical reactions within mature Graafian follicles. *In vitro* evidence for such endothermic reactions has now been obtained (Luck and Griffiths 1998; Luck *et al.* 1999), although a possible cooling contribution from leucocyte activity in the wall of pre-ovulatory follicles has still to be assessed. Thus, measurement of ovarian temperatures in mares would seem an important research objective of considerable practical significance.

Approaches would involve infra-red sensing of the ovaries with a thermo-camera. This could be performed at laparotomy with a flank rather than mid-ventral approach or by a transvaginal approach. However, sensing would best be performed in a sealed abdomen using an infra-red transmitting endoscope linked to the thermo-camera. Thermo-probes introduced into the follicular antrum are not necessarily appropriate because they would puncture the basement membrane and thereby modify the vascular bed

and follicular fluid composition. Temperature measurements obtained by infra-red sensing of an exposed follicular surface should be relevant to culture procedures underlying *in vitro* maturation and fertilisation of equine oocytes, to techniques of intra-cytoplasmic sperm injection (ICSI) and, indeed, to *in vitro* manipulations associated with cloning.

Finally, it is worth re-emphasising that inappropriate culture temperatures may not so much influence the normality of fertilisation or initial development of embryos to the blastocyst stage as provoke a belated loss during differentiation when the underlying gene programme requires precise chronological and quantitative expression. Temperatures incorrect by as little as 1.0°C or less could have devastating influences on cell organelles and especially on the protein chemistry and folding that regulate development.

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MATERNAL FACTORS INFLUENCING FERTILISATION AND EARLY EMBRYONIC DEVELOPMENT IN THE OVIDUCT: AN OVERVIEW

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The maternal contribution to embryonic development is determined during the formation and maturation of the female gamete, the oocyte, which, early in oogenesis, acquires the ability to achieve sperm-egg fusion. Oogenesis is followed by a period of oocyte growth, during which the latter must reach a minimum diameter of 110–150 µm in sheep and cattle before it can resume meiosis. Furthermore, the oogenetic products synthesised during oocyte growth must also be sufficient to support embryonic development from fertilisation until activation of the embryonic genome. In response to the pre-ovulatory surge of gonadotrophins, resumption of meiosis in the oocyte begins with the breakdown of the germinal vesicle. This is followed by a series of nuclear and cytoplasmic events, which result in the formation of a nuclear metaphase II plate, extrusion of the first polar body, migration of the cortical granules to the periphery of the ooplasm and the production of cytoplasmic proteins. These will regulate later meiotic events, activation of the oocyte, incorporation of sperm components, initiation of cleavage and the completion of embryonic development prior to activation of the embryonic genome.

Successful fertilisation must be co-ordinated with both the nuclear and cytoplasmic maturation of the oocyte. Then, at the site of fertilisation at the ampullary-isthmic junction, the zona pellucida of the mature oocyte regulates the binding of capacitated spermatozoa, induces the acrosome reaction, the block to polyspermy, and acts as a final barrier to infection. After fertilisation, the embryo undergoes cleavage divisions and, in the ewe, the first 3 cell cycles occur within the oviduct. It is during this period of development that protein synthesis within the embryo depends

upon the storage of mRNA that was acquired during oogenesis. Thereafter, a rapid degradation of the maternal mRNA occurs, which coincides with the developmental block observed *in vitro* that is characteristic of 8-cell stage embryos undergoing activation of the embryonic genome.

During transition from its dependence upon the maternal genome to the embryonic genome, the embryo is very susceptible to its environment, particularly heat shock and oxidative damage. Once the embryonic genome has been activated, further development of the embryo depends upon the transcription of proteins derived from the combination of the paternal and maternal genomes.

There are many circumstances *in vivo* which influence the quality of the ovulated oocytes and their potential for normal embryonic development. The lifespan of an ovulated oocyte, during which the oocyte can undergo normal fertilisation and development, varies among species, ranging from 8–10 h in the mare to 10–15 h in the ewe (Hunter 1988). Pregnancy rates are reduced in species such as the horse due to post ovulatory ageing of the oocytes which occurs when insemination is performed more than 12 h after ovulation (Woods *et al.* 1990). Ball *et al.* (1989) revealed higher embryonic loss rates in subfertile mares than in normal fertile mares. Later, it was demonstrated that oocytes aspirated from pre-ovulatory follicles of old and subfertile mares had reduced embryogenic potential after gamete intrafallopian transfer (GIFT) into young recipients (Carnevale and Ginther 1995). Environmental conditions, such as dietary stress, high levels of ammonia and excessive heat, present when a population of oocytes is undergoing its 2–3 month period of growth prior to selection, may also influence the

transcription of maternally derived cytoplasmic proteins and subsequently result in increased rates of embryonic loss.

Recently, many studies comparing the developmental competence of oocytes from pre-pubertal animals with those of adult animals have revealed the reduced ability of pre-pubertal oocytes, despite hormonal stimulation, to undergo synchronised and appropriate cytoplasmic and nuclear maturation and satisfactory embryonic development (Levesque 1994). Defects in the migration of the cortical granules in the ooplasm during *in vitro* maturation of pre-pubertal oocytes result in an increase in the incidence of polyspermic fertilisation and subsequent failure of embryonic development (O'Brien *et al.* 1996). It also appears that these pre-pubertal oocytes have reduced stability of their maternally derived mRNA and there is also an increase in the occurrence of premature interruptions between the cumulus cells and the oocyte (Gandolfi 1998).

Oocytes can also be obtained for *in vitro* maturation studies from either *in vivo* aspiration of follicles or from the aspiration or slicing of follicles of ovaries collected from the slaughter house. Despite high levels of nuclear maturation being achieved after *in vitro* maturation of these oocytes, there is still a large reduction in the embryonic potential of these oocytes that becomes evident some time between the early cleavage divisions and blastocyst formation. Most of these oocytes have been obtained from a very heterogeneous population of follicles from within the ovary, many of which would otherwise undergo atresia during the normal *in vivo* processes of recruitment and selection. Furthermore, the relatively static environment provided during the *in vitro* maturation of the oocytes is quite different from the dynamic environment provided by the follicle and oviduct which is capable of altering the endocrine milieu constantly and removing metabolic wastes surrounding the oocyte.

Not only are the culture conditions suboptimal for oocyte nuclear and cytoplasmic maturation, but the ovaries must undergo a period of transport to the laboratory for processing. Earlier studies have revealed an effect of temperature on the progression of normal nuclear maturation and the organisation of the metaphase plates in sheep oocytes (Moor and Crosby 1985). Therefore, to investigate the effects of the temperature and duration of transit on the maturation potential of

equine oocytes, ovaries were transported to the laboratory in phosphate buffered saline at either 15°C or 30°C and maintained for 0–2 h, 4 h or 24 h prior to processing (unpublished data). The oocytes were then washed and transferred in groups of 5–10 to 500 µl wells of TCM199 supplemented with FSH, LH, oestradiol and 10% FCS and cultured in an atmosphere of 5% CO₂ for 36 h. The results of the nuclear maturation assessment are described in Figure 1.

The highest nuclear maturation rates were obtained when the oocytes were collected immediately after removal of the ovaries from the mare. It appears that there may be a beneficial effect of transporting the ovaries at 30°C if the duration of transit is 4 h. However, after 24 h of transport there was a significant decline in the nuclear maturation potential of the oocytes that could not be compensated for by maintaining the ovaries at either 30°C or 15°C. Therefore, it appears that there was an overall decline in the nuclear maturation rates of oocytes as the duration of storage before processing increased; and that this superseded any potential compensatory effect of the 2 different transport temperatures. Further studies are in progress to determine the developmental potential of these oocytes after intra-cytoplasmic sperm injection (ICSI) of the MII oocytes.

In conclusion, endogenous and exogenous factors which affect oocyte growth and maturation, both *in vivo* and *in vitro*, ultimately influence normal fertilisation, early cleavage divisions, and also embryonic development after activation of the embryonic genome. An understanding of these factors will enhance further studies into the causes of early embryonic loss in domestic species.

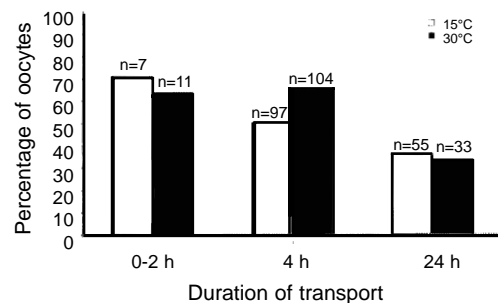


Fig 1: The effect of time and temperature of transport on oocyte nuclear maturation (MII) rates.

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THE ROLE OF ENDOMETRIAL OXYTOCIN SENSITIVITY IN MATERNAL RECOGNITION OF PREGNANCY IN THE MARE

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In the domestic ruminants, it is well established that oxytocin, secreted primarily by the corpus luteum (CL), stimulates the pulsatile releases of $\text{PGF}_{2\alpha}$ from the endometrium that cause cyclical luteolysis. In addition, the developing ruminant conceptus signals its presence to its dam by secreting interferon-tau ($\text{IFN-}\tau$) which inhibits the development of endometrial oxytocin responsiveness and thus blocks the development of the oxytocin- $\text{PGF}_{2\alpha}$ positive feedback loop.

As a result of this block, during early pregnancy in ruminants, $\text{PGF}_{2\alpha}$ is secreted not in the distinct, high amplitude pulses characteristic of late dioestrus but instead in a more constant manner. The resulting absence of luteolytic 'spikes' of $\text{PGF}_{2\alpha}$ release allows the CL to persist and thus ensures the continuation of the high circulating progesterone concentrations that are essential to the maintenance of pregnancy.

The failure of exogenous oxytocin to induce premature luteolysis reliably in cycling mares, and the absence of this peptide hormone from equine luteal tissue, led early authors to conclude that oxytocin was not involved in the luteolytic pathway in equids. However, more recent studies have demonstrated that oxytocin secreted by the endometrium and the hypothalamus almost certainly plays an important role in driving cyclical luteolysis in the mare and, furthermore, it is probable that the development of endometrial oxytocin responsiveness at around Days 10–11 after ovulation is the critical first step in the initiation of the luteolytic cascade in this species (see Stout and Allen 1999 for review).

Disappointingly, the conceptus signal(s) which averts luteolysis in pregnant equids has not been identified but, given the apparent similarities in the control of the luteolytic cascade between mares and ruminants, it is tempting to propose that the

equine conceptus exerts its influence primarily by inhibiting the late dioestrous increase in endometrial receptors for oxytocin. In this respect it is clear that, during Days 10–16 after ovulation, the endometrium of a pregnant mare does not release $\text{PGF}_{2\alpha}$ in response to oxytocin challenge in the way that the uterus of a cycling mare does so dramatically (Goff *et al.* 1987). Furthermore, the oxytocin binding capacity of equine endometrium is lower during early pregnancy than in late dioestrus (Starbuck *et al.* 1998), a finding that strengthens considerably the hypothesis that the equine conceptus exerts its anti-luteolytic action by inhibiting the cyclical development of endometrial oxytocin responsiveness. However, the conceptus-induced suppression of oxytocin binding capacity is not absolute and in any case it is not, on its own, likely to account for the almost total inhibition of $\text{PGF}_{2\alpha}$ secretion observed during early pregnancy in the mare. Therefore, it is likely that additional mechanisms contribute to the luteostatic action of the horse conceptus. At least in theory, these additional luteostatic actions may include an uncoupling of the interaction between the oxytocin-oxytocin receptor complex and endometrial $\text{PGF}_{2\alpha}$ release. Alternatively, or additionally, the conceptus might inhibit endometrial $\text{PGF}_{2\alpha}$ release directly or by inducing the production of an endometrium-derived prostaglandin synthesis inhibitor (EPSI), just as $\text{IFN-}\tau$ induces the secretion of the prostaglandin synthesis inhibiting linoleic acid in the endometrium of pregnant cattle (Thatcher *et al.* 1995). To date, however, there is no compelling evidence for the existence of an equine EPSI. Indeed, the failure of endometrium recovered from early pregnant mares to secrete less $\text{PGF}_{2\alpha}$ than endometrium from cycling animals when incubated *in vitro* (Stout 1998) makes the

existence of such a molecule unlikely. On the other hand, because oxytocin appears to stimulate luteolytic PGF_{2α} release, a reduction in oxytocin secretion would be expected to weaken the luteolytic drive and, in this respect, there is good evidence that both the production (Behrendt *et al.* 1997) and release (Stout 1998) of oxytocin by the mare's endometrium are reduced during early pregnancy, compared with equivalent stages of the oestrous cycle.

As one would expect, PGF_{2α} is not detectable in uterine luminal flushings recovered, by transcervical videoendoscopic lavage, from pregnant mares during Days 12–16 after ovulation. Somewhat surprisingly, however, the concentrations of luteolysin measured in flushings recovered in the same way during Days 18–30 of gestation are high and strikingly similar to those recorded during the luteolytic phase of the oestrous cycle (Stout and Allen 1998). Although it is possible that the PGF_{2α} measured in the uteri of Day 18–30 mares was a result of conceptus PGF_{2α} secretion (Watson and Sertich 1989; Stout 1998) it is more likely that this hormone was endometrial in origin and that its release was triggered by the cervical dilation and uterine trauma involved in the collection procedure; both of these stimuli are known to trigger oxytocin release (Sharp *et al.* 1997). This latter suggestion that, after Day 18 of gestation, endometrial PGF_{2α} release can be provoked by oxytocin secretion induced by cervical or uterine manipulation is supported strongly by the temporally coincident increases in endometrial oxytocin binding capacity and oxytocin responsiveness that occur on Day 18 after ovulation in pregnant mares (Starbuck *et al.* 1998). As a result, it is proposed that maternal recognition of pregnancy in the mare involves a delay in, rather than a total abolition of, the development of uterine oxytocin responsiveness. The reason for this alteration, which appears to occur between Days 16 and 18 of gestation, is almost certainly the cessation of conceptus mobility at around Day 17 after ovulation; mobility appears to be essential to enable the early equine conceptus to distribute its initial antiluteolytic signal (McDowell *et al.* 1985).

In conclusion, it appears that during Days 10–16 of gestation the mobile horse conceptus ensures luteostasis by preventing endometrial

PGF_{2α} release, a goal which it achieves primarily by delaying the development of oxytocin sensitivity. Beyond this time, during Days 18–30, a second strategy must be employed to prevent an apparently functional oxytocin-induced PGF_{2α} release pathway from being triggered. For this reason it seems likely that, during this Day 18–30 period, the mare is at a heightened risk of pregnancy loss which, in theory, could be triggered by hormonal fluctuations or uterine trauma occasioning the release of oxytocin.

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POSSIBLE ROLES OF OXYTOCIN IN REGULATING UTERINE PROSTAGLANDIN SECRETION DURING THE OESTROUS CYCLE AND EARLY PREGNANCY IN SOWS

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In sows, the critical event in maternal recognition of pregnancy is the prevention of luteolysis and maintenance of the corpus luteum. The corpus luteum is required to provide progesterational support throughout pregnancy (du Mesnil du Buisson and Dauzier 1957; Ellicott and Dzuik 1973). Luteolysis is caused by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Bazer *et al.* 1984). Prostaglandin $F_{2\alpha}$ is secreted from the porcine uterus as a series of pulses that coincide temporally with luteal regression (Schille *et al.* 1979). Uterine secretion of $PGF_{2\alpha}$ is regulated by at least 3 hormones: progesterone, oestradiol-17 and oxytocin. Serum concentrations of oxytocin increase at luteolysis, when $PGF_{2\alpha}$ is being secreted actively (Kotwica *et al.* 1990). Oxytocin is an acute stimulus for $PGF_{2\alpha}$ secretion from the uterus (Gross *et al.* 1988; Kieborz *et al.* 1991). An increase in uterine secretion of $PGF_{2\alpha}$ can be detected within 2 min after an iv injection of oxytocin (Kieborz *et al.* 1991). Repeated injections of oxytocin induce premature luteolysis and shorten the inter-oestrous interval (Prince *et al.* 1995). Prostaglandin $F_{2\alpha}$ can also induce secretion of oxytocin, primarily from the posterior pituitary gland (Ellendorf *et al.* 1979; Kotwica *et al.* 1990). Thus neurohypophyseal oxytocin and uterine $PGF_{2\alpha}$ exist in a positive feedback loop, each capable of stimulating secretion of the other. This relationship probably accounts for the high concentrations of each hormone observed during luteolysis.

In addition to the posterior pituitary gland, the uterine endometrium also synthesises and secretes oxytocin (Trout *et al.* 1995; Boulton *et al.* 1996; Vallet *et al.* 1998). Although the endometrial origin of this oxytocin strongly suggests that it contributes to the regulation of uterine $PGF_{2\alpha}$ secretion, its precise role, if any, remains to be established. In

contrast to ruminants, the corpus luteum of the pig synthesises relatively small quantities of oxytocin (Pitzel *et al.* 1984; Choy and Watkins 1988) that probably do not contribute to the regulation of endometrial $PGF_{2\alpha}$ secretion. An essential role for oxytocin in luteolysis has not been established. Recent attempts to ablate oxytocin from the circulation pharmacologically, using an oxytocin antagonist, have resulted in a diminution of $PGF_{2\alpha}$ pulses but no delay in the timing of luteolysis (Kotwica *et al.* 1998). Further studies of this kind are required to determine conclusively whether or not oxytocin is essential for luteolysis. The endometrium of sows also has receptors for luteinising hormone (Ziecik *et al.* 1986). In cattle, gonadotropins have been shown to stimulate uterine secretion of $PGF_{2\alpha}$ (M. Shemesh and M.J. Fields, personal communication). The role of gonadotropins in $PGF_{2\alpha}$ secretion has not been investigated in pigs. The ability of the uterus to secrete $PGF_{2\alpha}$ in response to oxytocin develops between Days 12 and 14 post oestrus, coinciding with the onset of endogenous $PGF_{2\alpha}$ secretion and luteolysis (Kieborz *et al.* 1991; Carnahan *et al.* 1996; Edgerton *et al.* 1996). We believe that the acquisition of secretory responsiveness to oxytocin is the final developmental step that permits uterine $PGF_{2\alpha}$ secretion to commence. Progesterone is required for the uterus to develop the enzymatic capabilities to synthesise and secrete luteolytic concentrations of $PGF_{2\alpha}$, particularly in response to oxytocin (Edgerton *et al.* 2000). This response to progesterone develops slowly, appearing on or about Day 15 post oestrus. It can be induced to occur prematurely by progesterone supplementation on Days 1–5 post oestrus (Printz *et al.* 1994). Oestradiol augments the uterine secretory response to oxytocin when administered at 'normal' luteal-

phase levels in the presence of progesterone. However, at this level, it has little effect in the absence of progesterone. The cellular mechanisms by which the progesterone and oestradiol act to enhance endocrine $\text{PGF}_{2\alpha}$ secretion in response to oxytocin are not completely understood. There is no significant increase in the endometrial concentration of oxytocin receptors at luteolysis to account for the increase in secretory responsiveness to oxytocin (Ludwig *et al.* 1998). However, the ability of oxytocin to stimulate activity of phospholipase (PL) C increases, suggesting that coupling of the receptor to this second messenger generating enzyme is deficient prior to Day 15. During early pregnancy, the conceptus prevents luteolysis by inhibiting the endocrine secretion of $\text{PGF}_{2\alpha}$. This is accomplished through 2 effects. First, the conceptus reduces the ability of the uterus to synthesise and secrete $\text{PGF}_{2\alpha}$ (Watson and Patek 1979; Guthrie and Rexroad 1981). Secondly, it alters the direction of the residual $\text{PGF}_{2\alpha}$ secretion so that it moves primarily into the lumen of the uterus (in an exocrine direction), rather than into the circulation (in an endocrine direction). The latter effect of the conceptus has been termed the endocrine:exocrine theory for maternal recognition of pregnancy in swine (Bazer and Thatcher 1977). These effects of conceptus can be induced simply by administration of high doses of oestradiol-17 during the mid-luteal phase of the oestrous cycle (Frank *et al.* 1977; Guthrie and Rexroad 1981; Geisert *et al.* 1982). Because the porcine conceptus is known to secrete oestradiol-17 at this time (Perry *et al.* 1973; Gadsby *et al.* 1980), it has been suggested that oestradiol-17 is the principal signal for maternal recognition of pregnancy in pigs. The cellular mechanism by which the conceptus and oestradiol suppress endocrine $\text{PGF}_{2\alpha}$ secretion in response to oxytocin is not completely understood. As during the oestrous cycle, there is no significant decrease in the endometrial concentration of oxytocin receptors to account for the lack of response to oxytocin during early pregnancy (Ludwig *et al.* 1998). Rather, the ability of oxytocin to stimulate activity of PLC is suppressed, again suggesting that regulation occurs at the interaction of the receptor with this key regulatory enzyme. One factor that may contribute to the change in uterine secretory responsiveness to oxytocin is endometrial secretion of oxytocin. Endometrial secretion of oxytocin increases dramatically in the presence of the conceptus (Boulton *et al.* 1996; Trout *et al.* 1995). This high level of secretion may

contribute to the suppression of $\text{PGF}_{2\alpha}$ secretion by inducing a desensitisation of post receptor, second messenger pathways that are required for $\text{PGF}_{2\alpha}$ synthesis. It remains to be determined if the stimulatory effect of the conceptus on endometrial oxytocin secretion is mediated through oestradiol-17.

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LUTEAL-UTERINE INTERACTIONS DURING THE PERI-IMPLANTATION PERIOD OF PREGNANCY: A MODEL FOR EMBRYO LOSS IN THE COW

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Recent studies indicate that a high incidence of pregnancy loss occurs between Days 20 and 50 of bovine pregnancy (16.8%, Vasconcelos *et al.* 1997; 14% to 29%, Shore *et al.* 1998). The detection of these losses was made possible by application of ultrasound technology in the monitoring of early pregnancy. An earlier study utilising milk progesterone analysis also showed a high pregnancy loss during peri-implantation (8% to 25%, Bulman and Lamming 1979). Similar losses (38%) occur in the peri-implantation mare as reported by Morris *et al.* (2000). Our working hypothesis is that luteal secretion of oxytocin during the window of implantation drives the uterus to secrete PGF_{2α}, may contribute to the demise of the corpus luteum and ultimately deprives pregnancy of the obligatory progesterone.

In the presence of a viable embryo the concentrations of endometrial oxytocin receptors (OT_R) are strongly suppressed in the third week of pregnancy. However, they reappear towards the end of the peri-implantation phase and increase progressively until term (Fuchs *et al.* 1992). Late peri-implantation OT_R are functionally linked to induction of prostaglandin H synthase-2 gene expression because OT was shown to increase secretion of endometrial PGF_{2α} (Fuchs *et al.* 1996). It has been observed that, following injection of a single bolus of OT, the more advanced the cow is in pregnancy the more heightened her response is. Interestingly, plasma concentrations of progesterone declined after OT injection and 2 of the 6 animals treated on Day 50 unexpectedly aborted indicating a raised sensitivity to PGF_{2α} at this stage of pregnancy.

It is during this peri-implantation phase that luteal OT-containing secretory granules are released (Fields *et al.* 1996). This is in contrast to their release on Days 14–19 in the non-gravid cycle (Fields *et al.* 1992). Thus at Days 14–19 of pregnancy, luteal stores of OT are retained, OT_R are down-regulated to non-detectable levels and the prostanoid system is quiescent. The shift in discharge of luteal OT, the apparent increase in uterine OT_R and the prostanoid responsiveness of the uterus to OT during the peri-implantation window of pregnancy indicates an OT-driven prostanoid system that may contribute to the high pregnancy loss at this time. This hypothesis is currently being tested with a single OT injection via the jugular vein on Days 20–50 of pregnancy. Non-pregnant cows on Day 17 of the oestrous cycle, chosen as a positive control, responded to OT with a dramatic increase in plasma concentrations of PGF_{2α} metabolite (PGFM) that subsequently declined gradually to baseline (see Fig 1).

All 4 non-gravid cows responded in a similar manner as indicated by the small standard error around the mean. The peri-implantation cows, Day 20–50, also responded with a sharp increase in PGFM that was more variable in the individual cow response and that appeared to remain elevated over a longer period of time (Fig 1). These preliminary results indicate that the peri-implantation cow has an OT-uterine responsive prostanoid system. This supports the hypothesis that the peri-implantation luteal discharge of OT and the upregulation of uterine OT_R drives the prostanoid system to secrete PGF_{2α} and disrupt

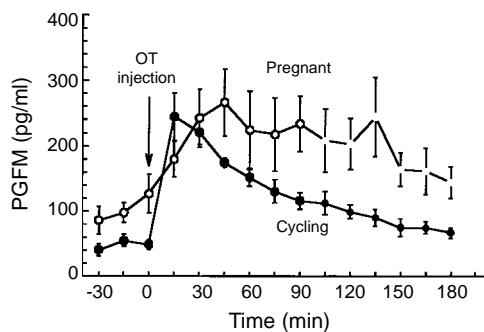


Fig 1: Plasma $PGF_{2\alpha}$ metabolite (PGFM) in response to a single injection of 100 units oxytocin administered via the jugular vein of cyclic Day 17 ($n = 4$) and peri-implantation cows Day 20 to 50 ($n = 5$) of pregnancy. Jugular blood samples were taken at 15 min intervals starting 30 min prior to OT injection and ending 3 h post injection. All cows responded, indicating a functional OT-prostanoid system in the peri-implantation cow.

luteal secretion of progesterone, resulting in loss of the pregnancy.

Because oestrogens increase bovine endometrial OT_R concentrations in the presence of progesterone, oestrogenic compounds in the feed may be involved in the observed peri-implantation embryonic losses by induction of uterine OT_R and the prostanoid system. Shore *et al.* (1998) reported that cows fed legumes had significantly higher peri-implantation loss than cows fed a non-legume grass. The authors suggested phytoestrogens may have played a role in the embryonic loss. In Florida there have been reports of premature hardening of the bone in young cattle processed through the abattoir. One possible explanation is that these animals were exposed to an oestrogenic environment which could have been in the form of phytoestrogens. There is also the possibility of mycotoxins in mouldy feed producing oestrogenic compounds, such as zearalenone, and the use of reclaimed sewage water having reportedly high oestrogenic activity.

We propose that environmental estrogens up-regulate endometrial and cervical OT_R , resulting in the release of $PGF_{2\alpha}$ and PGE_2 , respectively, in response to the discharge of luteal secretory granules. This, in turn, leads to luteolysis and termination of peri-implantation pregnancy. However, in the case of a robust conceptus, this

stimulus is overridden and the pregnancy is maintained. In other cases an OT-driven prostanoid system results in loss of the pregnancy. We propose that the OT-prostanoid system is primarily an evolutionary self-preservation mechanism for clearing a less-than-robust conceptus out of the uterus in preparation for a new ovulation and opportunity for a stronger pregnancy. In today's managed herds, the ill-timed introduction of factors that enhance the OT-prostanoid system may, in fact, unknowingly cause excessive synthesis of prostanoids by both the endometrium and cervix leading to high levels of pregnancy loss during the critical window of implantation.

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OESTROGENS DURING EARLY PREGNANCY IN THE MARE: KILL OR CURE?

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It is generally held that oestrogens secreted by the rapidly elongating trophoblast of the developing conceptus are responsible for maternal recognition of pregnancy in the sow. It is also accepted that the prevention of luteolysis is achieved primarily by an oestrogen-induced redirection of endometrial PGF_{2α} secretion, from a myometrium directed 'endocrine' route to a predominantly luminal or 'exocrine' pattern of release (Bazer and Thatcher 1977). Thus, rather than entering the uterine vein and systemic circulation as it does in cycling sows, the luteolysin is sequestered in the uterine lumen where it is unable to exert its effects on the corpus luteum. This hypothesis is supported strongly by numerous studies which have demonstrated that administration of oestrogens to cycling sows, during Days 11–15 after ovulation, results in prolonged luteal survival and elevated uterine luminal concentrations of PGF_{2α}.

Horse conceptuses, like their porcine equivalents but unlike the pre-implantation blastocysts of most other large domestic animal species, secrete copious quantities of oestrogens from a very early stage of development and, as a result, numerous authors have proposed that conceptus oestrogens may play a similarly pivotal role in luteostasis in the pregnant mare. However, the pregnancy-induced reversal in the direction of endometrial PGF_{2α} secretion, that underlies the anti-luteolytic action of oestrogens in the sow, does not occur in the mare. More alarmingly, studies of the effect of exogenous oestrogens on luteal survival in cycling mares have produced widely conflicting results, ranging from prolonged luteal survival (Berg and Ginther 1978) to unaltered corpus luteum lifespan or, more recently, a contrary stimulation of uterine PGF_{2α} release (Goff *et al.* 1993). Our own studies suggest that the marked differences

observed in the effects of exogenous oestrogens on luteal survival stem from the wide range of doses and formulations used and the timing and duration of their administration. In summary, it appears that continuous administration of high doses of oestrogens from before Day 10 after ovulation and for at least one week tends to result in prolonged luteal lifespan; whereas single doses of oestrogens given at later stages either do not affect luteolysis or hasten its onset by facilitating PGF_{2α} release (Stout 1998). A similar pattern of responses to oestrogen administration has been observed in ruminants, in which oestrogens are not produced by the early conceptus but are released by ovarian follicles and are generally thought to promote luteolysis. For example, in cycling ewes, a single mid-dioestrous injection of oestradiol stimulates luteolytic PGF_{2α} release (Hixon and Flint 1987) but the daily administration of 0.5 mg oestradiol during Days 3–20 after ovulation prolongs the luteal phase (Denamur *et al.* 1970). In any case, high systemic doses of oestrogens do not closely mimic the physiological pattern of oestrogen delivery to the uterus of the early pregnant mare; circulating oestrogen concentrations do not rise above dioestrous levels until the formation of the endometrial cups at approximately Day 35 of gestation. To mimic the effect of oestrogens produced by the developing conceptus more accurately, intra-uterine administration of physiological doses of oestrogens to cycling mares has been undertaken. However, this route of administration has also provided inconclusive results (Vanderwall *et al.* 1994; Stout 1998) which almost certainly reflect the potentially confounding influence of introducing the oestrogen-releasing device through the cervix and the additional difficulty of delivering

oestrogens to the whole uterine surface, in the way that a mobile Day 10–17 horse conceptus does.

There have, however, been few studies which have examined the effects of exogenous oestrogens on luteal maintenance in pregnant mares. In this respect, we found recently that the administration of a single 0.03 mg/kg dose of oestradiol-benzoate to pregnant mares on Day 14 or 22 after ovulation, followed 6h later by a 20 iu 500 kg oxytocin challenge, triggered an immediate decrease in the peripheral serum progesterone concentrations. Although progesterone concentrations then stabilised and pregnancy loss was avoided, in a previous experiment which aimed to examine the effect of oestrogens on placental development, the administration of oestradiol benzoate on 3 consecutive days, starting from Day 25 after ovulation, led to a greater fall in circulating progesterone levels followed, in 2 of 4 treated animals, by pregnancy loss (C. Gerstenberg, personal communication). On the other hand, in the present study we found that a single iv bolus of soluble oestradiol- β did not affect serum progesterone concentrations in mares on either Day 14 or 22 of gestation and, thus, it remains to be determined whether oestrogens really do stimulate PGF $_{2\alpha}$ secretion and, if so, whether they do this directly or indirectly, for example by stimulating an increase in endometrial receptors for oxytocin and/or by stimulating oxytocin release *per se*. This may be clarified by the measurements of peripheral plasma PGFM concentrations that we are currently performing on blood samples recovered before and after oestrogen and oxytocin treatment of pregnant mares.

In summary, these data suggest that transient elevations in systemic oestrogen concentrations may compromise the survival of the primary corpus luteum during early equine pregnancy and, in this light, it is surely significant that very few oestrogens, of conceptus or ovarian origin, appear to enter the systemic circulation until formation of the endometrial cups.

However, the postulated negative effect of oestrogens should not be taken to imply that conceptus-produced oestrogens do not play vital roles in the establishment and maintenance of pregnancy in the mare. On the contrary, oestrogens are known to act synergistically with progesterone to stimulate qualitative and quantitative changes in protein secretion by the endometrial glands

(McDowell *et al.* 1987) and this is undoubtedly important in providing adequate histotrophic nutrition to the unimplanted conceptus. Furthermore, it is likely that oestrogens provoke some of these changes in nutrient secretion by inducing changes in uterine blood flow and vascular permeability, as has been shown in other species but has yet to be proven in the horse. In addition, conceptus oestrogens have been proposed to contribute both to the mobility of the Day 10–17 conceptus and to the dramatic increase in uterine tone which occurs at the end of the mobile period; both of these phenomena are characteristic of early equine pregnancy and of considerable functional significance. In conclusion, we have still to explain how and why elevations in systemic oestrogen concentrations of relatively short duration may be detrimental to luteal survival while oestrogens released by the conceptus into the uterine lumen almost certainly help to maintain pregnancy by a variety of mechanisms which may include anti-luteolytic actions.

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CONCEPTUS CONTENTS AND COVERINGS IN RELATION TO THE MAINTENANCE OF EARLY PREGNANCY IN THE MARE

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The unusual buoyancy and mobility of the equine conceptus, and the fact that the horse is one of the 'fructogenic' species, have prompted us to continue our studies of yolk-sac fluid by: a) analysing it for its content of oxytocin (OT), arginine vasopressin (AVP) and fructose; and b) initiating studies of possible local variations in concentrations of ions by cryo-scanning electron microscopy (cryo-SEM). The evident functional importance of the capsule both before and after 'fixation' of the conceptus has led us to determine the patterns of expression of mRNA for MUC1 (one of the genes controlling mucin production) in both the trophoblast and endometrium. Some of the data reported here have also been presented elsewhere in abstract form (Gillies *et al.* 1999; Ruddock *et al.* 2000; Waelchli *et al.* 2000).

Hypothesising that OT contained in the yolk sac could be involved in pre-fixation conceptus mobility, and/or the down-regulation of endometrial OT receptors thought to be a factor in the prevention of luteolysis in the pregnant mare (Stout *et al.* 1999), we have measured the concentrations of this peptide in yolk sac fluids collected between Days 13 and 31 of pregnancy. Arginine vasopressin (AVP) has been measured in the same samples with a view to explaining unusual aspects of conceptus expansion in the horse (Waelchli *et al.* 1997) and because it, too, may be involved in the regulation of luteolysis. The samples of yolk-sac fluid were collected from 40 conceptuses from 17 mares over a period of 5 years and stored at -30°C as described by (Waelchli and Betteridge 1996). Thirty of the conceptuses were recovered by transcervical lavage, 8 by hysteroscopy and 2 at necropsy. They were assayed in 2 separate series. For the first,

frozen samples were transported in LN₂ to New Zealand where they were extracted with acetonitrile (mean recoveries 84 and 88% for OT and AVP, respectively) for radioimmunoassay (RIA). For the second series, the extractions were performed in Guelph. The RIA for OT (Alexander *et al.* 1995) is highly specific for OT, and shows little cross reactivity with AVP (0.005%). The RIA for AVP (Alexander *et al.* 1991) shows negligible cross reactivity with OT (<0.01%) and only minor cross reaction with deamino-Cys1, D-Arg 8-AVP. The detection limits of the assays are 1.0 pg/ml and 0.5 pmol/l for OT and AVP, respectively. Within-assay coefficients of variation were 6.0% for OT and 4.6% for AVP (for each hormone in each series, all samples were assayed together). The concentrations of both OT and AVP increased with the age of conceptus (Fig 1).

OT concentrations in series 1 and 2 were, respectively, 6.2 ± 0.8 (mean \pm se) and 12.9 ± 0.3 pg/ml from Days 13 to 16, and 11.1 ± 0.8 and 21.9 ± 0.9 pg/ml from Days 17 to 31. AVP concentrations in series 1 and 2 for the same 2 periods were 0.48 ± 0.02 and 0.44 ± 0.01 pmol/l and 0.81 ± 0.08 and 0.59 ± 0.03 pmol/l, respectively (absolute AVP concentrations for series 1 reported by Waelchli *et al.* 2000 are erroneously inflated \times 4). For both hormones and in both series, the differences between the 2 periods were statistically significant ($P < 0.001$; *t*-test). The pattern of increase in concentrations of both peptides within the conceptus is similar to those of osmolality (Waelchli *et al.* 1996) and of fructose concentrations (see below), suggesting that conceptus fixation at Day 16–17 is associated with important metabolic changes essential to the maintenance of pregnancy. The source(s) of OT

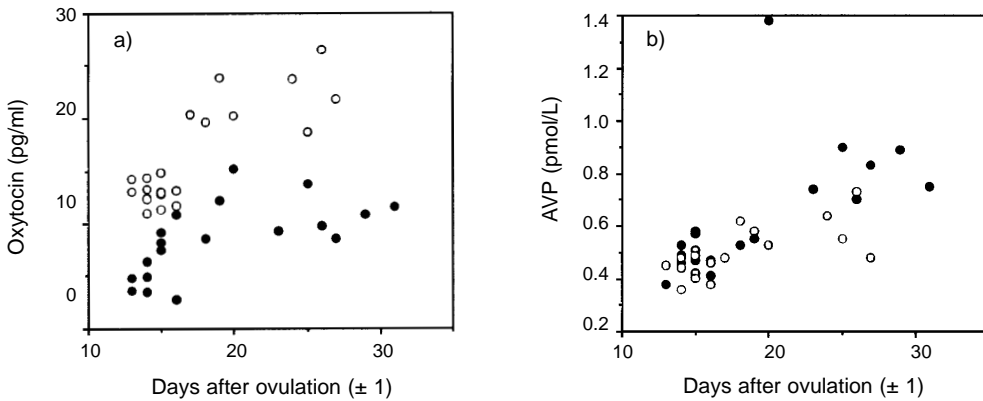


Fig 1: The concentrations of (a) oxytocin and (b) arginine vasopressin in yolk-sac fluid in series 1 (filled symbols) and series 2 (open symbols).

and AVP found in the conceptuses have not yet been established. The increase in OT concentrations after fixation argues against a principal role for this peptide in earlier mobility of the conceptus but in favour of its involvement, perhaps in conjunction with AVP, in the prevention of luteolysis. Further, an association between the presence of AVP and the tonicity of equine yolk sac fluid seems possible.

Horses are among the 'fructogenic' species in which the predominant blood sugar of the fetus is fructose (Goodwin 1956). During earlier stages of pregnancy, aldose reductase, a key enzyme in fructogenesis, is present in ovine pre-implantation embryos (Lee *et al.* 1998) and fructose itself has been detected in yolk sac fluid from equine conceptuses on Day 18 (ovulation = Day 0; Weithenauer *et al.* 1989) as well as in uterine flushings of pregnant mares on Days 14–20 (Zavy *et al.* 1982). There is no information, however, on how fructose concentrations vary with developmental stage in equine conceptuses, nor on the origin of that fructose. The objective of this study was to answer those 2 questions, using the same group of fluids described above. Fructose and glucose concentrations in aliquants of the fluids, and in series of standard solutions of both sugars, were measured by the D-Glucose/D-Fructose Enzymatic BioAnalysis, UV method (Boehringer Mannheim). Figure 2 shows that fructose concentrations between Days 11 and 15 ranged from 4–5 mM (median, mean, se = 10.3, 9.7, 1.0). From Day 16–27 the range of fructose concentrations increased to between 26 and 62 mM (46.4, 45.0, 3.5). The 2 medians were

significantly different ($P < 0.0001$). Glucose concentrations did not vary with stage of development, ranging between 0.1 and 2.7 mM. Preliminary incubation experiments suggest fructose production by conceptus tissues. Fructose could account for up to 25% of the yolk-sac fluid osmolality throughout weeks 2–4 of pregnancy.

MUC1 is expressed abundantly in the human and rabbit uterus, to the exclusion of other mucins except for small amounts of MUC6 in women (Gipson *et al.* 1997; Hoffman *et al.* 1998). The expression of MUC1 appears to be regulated by progesterone and oestrogen and to be related to the anti-adhesive effects of the uterus in preventing premature implantation. It was of interest, therefore, to determine whether the pattern of MUC1 expression in the equine endometrium and/or trophoblast is consistent with a potential role for this mucin in the development and function of the embryonic capsule. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of equine lung RNA using a H2MUC1 primer set yielded a 188 base pair sequence which was 94% homologous with the human MUC1 cDNA sequence (Gillies *et al.* 1999). RT-PCR amplification of the endometrial RNA revealed the presence of equine MUC1 in all endometrial biopsies taken between Days 11 and 25 of pregnancy. The PCR product showed sequence identity to the PCR product from the horse lung. MUC1 expression was observed in the trophoblast of 6 of 8 conceptuses collected over this time period. These findings indicate that MUC1 is expressed in the endometrium of pregnant and non-pregnant mares, and in the

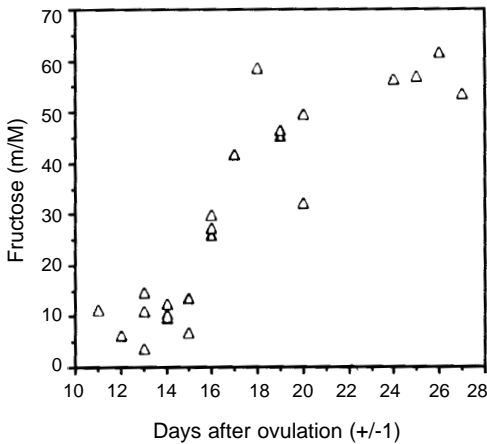


Fig 2: Fructose concentrations in equine yolk-sac fluid during early pregnancy.

trophoblast of most equine embryos between Days 11 and 25 of pregnancy. Although this is consistent with a role for this mucin in capsule formation, it remains to be determined whether or not MUC1 is the predominant mucin of the capsule and/or the endometrial glycocalyx.

Preliminary results have confirmed the usefulness of cryo-SEM and energy dispersive X-ray (EDX) microanalysis (Huang *et al.* 1994) for revealing differences in concentrations of ions in various compartments of developing conceptuses.

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PREGNANCY-ASSOCIATED GLYCOPROTEINS: STRUCTURAL AND FUNCTIONAL DIVERSITY AMONG SPECIES

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Pregnancy-associated glycoprotein-1 (PAG-1), also known as pregnancy-specific protein B, was initially identified as a trophoblast antigen present in the maternal circulation of ruminant ungulate species (Zoli *et al.* 1992; Sasser *et al.* 1986). Consequently, the detection of PAG-1 has been used as the basis for a test of pregnancy in these species. Upon cloning of this antigen, it became apparent that PAG-1 was related to the aspartic proteinases, sharing greatest structural resemblance to pepsin. Subsequent work revealed that it belonged to a family of recently duplicated genes. To date, 21 bovine, 9 ovine and 11 caprine PAG cDNA have been identified (Xie *et al.* 1997). Furthermore, numerous PAGs have been purified from placental extracts and placental conditioned media. Most of the amino-terminal sequences from these purified proteins do not correspond to any cDNA that have been characterised so far, suggesting that the PAG family within the ruminant ungulates is extensive.

Phylogenetic analysis indicated that the ruminant PAG could be segmented into 2 groups, one being very large while the other group is represented by fewer members of the family. PAG expression has been examined by both *in situ* hybridisation and RNase protection assays. The 2 groups happen to correlate with the expression patterns of PAG within trophoblast. The smaller group is comprised of PAGs expressed throughout trophoblast and at all stages of pregnancy. In contrast, PAGs comprising the other, larger, group were found to be expressed predominantly, if not exclusively, in binucleate trophoblast cells. Furthermore, the temporal expression within this group was variable. Some of the binucleate cell-specific PAGs were expressed very early in pregnancy, around the time at which binucleate cells first begin to appear (Day 17–19 in

domestic cattle). Other PAGs did not become expressed until much later in pregnancy.

The binucleate trophoblast cells are a hallmark of the ruminant placenta within the *Artiodactyla* order. Consequently, the PAG gene products expressed exclusively in these cells must have arisen relatively recently in evolution. Given that these genes comprise the bulk of the PAG family, the number of binucleate cell-specific PAG genes is being amplified rapidly within the ruminant ungulates. Interestingly, many of these PAGs have accumulated substitutions within and around the catalytic site that suggest that they are unable to function as aspartic proteinases. However, most can bind to the aspartic proteinase inhibitor, pepstatin, with a range of affinities. Consequently, it appears that the peptide-binding clefts are intact, but vary in binding specificity. Finally, diversity within this group is not restricted to simply an increase in gene number or to minor substitutions within the catalytic region. They also exhibit diversity in sequence, particularly in hypervariable regions that happen to correspond to solvent-exposed loops on the proteins. Non-synonymous (replacement) mutations in regions of the genes that encode these loop segments have accumulated at a higher rate than synonymous (silent) mutations. The most likely explanation is directional selection, favouring diversification at the amino acid level after gene duplication. Other gene families in which this type of diversification has been observed are IgG variable regions, T-cell receptors and defensins. The abundant presence of PAGs at the fetal-maternal interface, along with their array of peptide-binding specificities, suggests an involvement in fetomaternal interactions, possibly in immune modulation.

In contrast to the extensive PAG family in the *Artiodactyla*, only a single PAG-like gene has been

identified in the horse and zebra (*Perrisodactyla* order), cat (*Carnivora*), mouse (*Rodentia*) and rabbit (*Lagomorpha*). The protein product of this gene is an active aspartic proteinase. In the horse, it is detectable throughout pregnancy and is expressed in trophoblast (Green *et al.* 1999). Interestingly, in the mouse, the expression of its PAG-like gene (known as pepsinogen F [pepF] in mice and rabbits) was localised to the visceral yolk sac during the latter half of pregnancy. Its presence was detected by immuno-staining with an anti-equine PAG antiserum. The localisation of mouse pepF in the yolk sac suggests a role in proteolytic degradation of protein substrates taken up by the visceral yolk sac to provide for the amino acid requirements of the developing embryo. Mouse pepF was also found to be expressed in the glandular stomach of newborn mice, until the time of weaning. Presumably, it is functioning to degrade proteins in milk or to cleave casein itself to promote milk clotting. If so, it would be the functional homologue of chymosin, an aspartic proteinase expressed in the neonatal stomachs of ungulate species.

The functional relationship between the PAG-like protein in species outside the *Artiodactyla* and the PAG family within the *Artiodactyla* is not known. Equine PAG/pepF probably represents the primordial gene from which the PAG arose.

However, given the scope of the PAG family within the ruminant ungulates, along with the temporal and spatial expression patterns of the members within this family, it seems likely that they have diverged functionally from their pepF-like precursor and are probably filling a role unique to the placenta of the ruminant ungulates.

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REGULATION OF OVINE ENDOMETRIAL FUNCTION BY PLACENTAL LACTOGEN AND PLACENTAL GROWTH HORMONE

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In ungulates, establishment and maintenance of pregnancy requires integration of endocrine and paracrine signals from the ovary, conceptus and uterus. Establishment of pregnancy requires that the pre-implantation ovine conceptus (embryo/fetus and associated placental membranes) enter a receptive uterus and develop sufficiently to synthesise and release interferon tau (IFN τ), the pregnancy recognition signal. After pregnancy recognition, maintenance of pregnancy requires reciprocal communication between the conceptus and endometrium during implantation and synepithelio-chorial placentation. In sheep, superficial implantation and placentation is a lengthy process that begins on Days 15–16 and is not completed until Days 50–60 of pregnancy. During this period, the ovine uterus grows substantially in order to accommodate maximal rates of fetal development and growth in the latter half of pregnancy. In addition to placentomal development in the caruncular areas of the endometrium and changes in vascularity, the intercaruncular endometrial glands grow substantially in length (4-fold) and width (10-fold) during pregnancy in ewes. These uterine glands synthesise, secrete or transport a variety of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins and other substances which are collectively termed histotroph. Available evidence strongly supports the theory that secretions from the endometrial epithelia influence conceptus development, onset of pregnancy recognition signals and growth of the conceptus in species with an epitheliochorial type of placentation (Gray *et al.* 2000).

The hormonal, cellular and molecular mechanisms regulating ruminant endometrial

gland morphogenesis and function during pregnancy are not well investigated. In other epitheliomesenchymal organs, lactogenic and somatogenic hormones regulate epithelial proliferation, differentiation and function. In sheep, these hormones are produced by the pituitary (prolactin [PRL] and growth hormone [GH]) and placenta (placental lactogen [PL] and placental GH). During pregnancy, glandular epithelial (GE) morphogenesis and function is correlated with several placental events including production of PL (Day 16 to term) and placental GH (Day 35 to 60). Between Days 50 and 60, the uterine glands undergo rapid hyperplasia and begin to synthesise, transport and secrete large amounts of histotroph. The first 2 studies determined temporal and spatial alterations in expression of prolactin receptors (PRL-Rs) and uterine milk proteins (UTMPs), a marker of endometrial secretory activity, in the ovine endometrium during the oestrous cycle and pregnancy. Slot blot hybridisation analysis indicated that steady-state levels of ovine endometrial PRL-R mRNA increased almost 2-fold between Days 11 and 19 of early pregnancy and Days 20 and 120 of later pregnancy. *In situ* hybridisation and immunohistochemical analyses indicated that PRL-R mRNA and protein was expressed exclusively in the endometrial GE. No PRL-R mRNA expression was detected in luminal epithelium (LE), stroma, myometrium or conceptus trophoblast. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analyses determined that the endometrial GE expressed both long and short alternative splice forms of the ovine PRL-R gene.

Slot blot hybridisation analysis indicated that steady-state levels of intercaruncular endometrial UTMP mRNA increased about 3-fold between Days 20 and 60, another 3-fold between Days 60 and 80, and then declined slightly to Day 120. In pregnant ewes, UTMP mRNA expression was restricted to the endometrial GE in the stratum spongiosum (sGE), increased substantially between Days 15 and 17, and, between Days 17 to 50 of gestation, was markedly higher in upper than lower sGE. After Day 50, hyperplasia of the sGE was accompanied by increased UTMP mRNA expression by all endometrial glands. Collectively, results indicate that: 1) endometrial sGE is a primary target for actions of lactogenic hormones; and 2) UTMP mRNA expression is correlated with PL production by the trophoderm and state of sGE differentiation during pregnancy. Activation of PRL-R signal transduction pathways by PRL and PL is proposed to play a major role in endometrial GE remodelling and differentiated function during pregnancy, in support of fetoplacental growth and development.

Further studies tested the hypothesis that progesterone, IFN τ , PL and placental GH act in a sequential and paracrine manner to stimulate endometrial GE morphogenesis and secretory function during pregnancy (Spencer *et al.* 1999). In the third study, administration of IFN τ to cyclic ewes for one period (Days 11–15) resulted in an interoestrous interval of 30 days, whereas administration for 2 periods (Days 11–15 and Days 21–25) extended the interoestrous interval to greater than 50 days. Administration of IFN τ from Days 11–15 and PL or GH from Days 21–25 failed to extend the interoestrous interval more than for IFN τ alone. These studies do not support the hypothesis that PL plays a role in maintenance of corpus luteum function. In the fourth study, intrauterine effects of IFN τ , PL and GH on endometrial differentiation and function were determined in ovariectomised ewes receiving ovarian steroid replacement therapy. Using a uterine catheterised ewe model, the progestinised uterus was treated daily with recombinant ovine IFN τ from Days 11–20 and either control proteins,

recombinant ovine PL or recombinant ovine GH from Days 16–25. As expected, endometrial expression of mRNAs for oestrogen receptor (ER), progesterone receptor (PR) and oxytocin receptor (OT $_R$) were not affected by PL or GH treatment. Ewes receiving PL and GH had higher levels of endometrial UTMP mRNA compared to control ewes. Levels of osteopontin (OPN), a GE-specific secretory protein, mRNA were greater in ewes receiving PL than control or GH protein. Histomorphometrical and immunocytochemical analyses indicated that PL increased proliferation of deep glands in the stratum spongiosum, whereas GH affected primarily the upper glands of the stratum spongiosum. Collectively, results indicated that: 1) PL and GH do not regulate endometrial PR, ER and OT $_R$ expression or affect CL lifespan; 2) down-regulation of epithelial PR expression is requisite for progesterone induction of secretory gene expression in uterine GE; 3) effects of PL and GH on endometrial function require IFN τ ; and 4) PL and GH regulate endometrial gland proliferation and perhaps differentiated function. Overall, studies support the hypothesis that lactogenic and somatogenic hormones from the pituitary and placenta act directly on the endometrial GE to differentially regulate endometrial gland morphogenetic and secretory functions. Future research will be directed toward understanding: 1) the signal transduction pathways and genes regulated by PL and placental GH; and 2) effects of PL and placental GH on endometrial GE morphogenesis and secretory function as well as uterine development during pregnancy.

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FERTILITY PARAMETERS AND EARLY PREGNANCY LOSS RATES IN THOROUGHBRED MARES IN NEWMARKET

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Losses in reproductive efficiency in the Thoroughbred mare may occur at many stages throughout the breeding cycle and during gestation; namely, failure of ovarian cyclicity, failure of fertilisation, abortion or the resorption of pregnancy throughout gestation or failure of extrauterine survival (Sanderson and Allen 1987). In order to investigate the time of onset of pregnancy loss and to evaluate the overall reproductive efficiency of the Thoroughbred population, a retrospective analysis of the stud and veterinary records of 1,398 mares covered at 25 studs in the Newmarket region was performed.

By 15th July, 92% of the mares had been diagnosed pregnant at Days 15–30 of gestation. This pregnancy rate subsequently fell to 89% by 120–210 days of gestation when the mares were scanned again in October. From the currently available data, only 77% (609/788) of mares produced a live foal at term. This low foaling rate is similar to that found by Sanderson and Allen (1987). On a per cycle basis (1,555 oestrous cycles), by Day 15 of gestation a pregnancy rate of 62% was recorded but this had fallen to 56% by 30–45 days of gestation and 54% per cycle pregnancy rate by the time of the October scan. Indeed, throughout gestation, there was an overall pregnancy loss rate of 23% of oestrous cycles (Table 1) and around 10% of mares conceived more than once during the season after losing their pregnancy before Day 35 of gestation. As a result of either failure of conception or early pregnancy loss, the total number of times a mare was covered before she was diagnosed pregnant on Days 30–45 of gestation ranged from 1.6 to 2.5 depending upon her age and whether she was a maiden, barren or foaling mare.

These results reveal that over 50% of pregnancy losses occurred before formation of the

endometrial cups at around Day 36 of gestation. Most of these early losses (31.8%) occurred between Days 21 and 35, coinciding with the period of first appearance of the embryo and organogenesis. The incidence of pregnancy loss due to chromosomal abnormalities is yet to be elucidated in the horse, but would most likely coincide with this period of early fetal development. However, 21.2% of pregnancy losses occurred even earlier than this, between Days 10 and 20 after ovulation, most likely as a result of post breeding endometritis leading to prostaglandin release, luteolysis and the failure of the maternal recognition of pregnancy. Only 14.4% of pregnancy losses occurred between 50 and 120 Days of gestation, during the period of transition from luteal to placental sources of progestins to maintain pregnancy. The remaining 18% of pregnancy losses occurred after the placenta had formed its extensive and diffuse microcotyledonary attachment with the endometrium from 100 days of gestation.

The major factors that influenced the ability of a mare to become and remain pregnant included her age (Fig 1) and whether she was a maiden,

TABLE 1: Rate of pregnancy loss according to stage of gestation

Stage of gestation at time of pregnancy loss	% of overall pregnancy loss
<15 days	1.0
15–20 days	21.2
20–35 days	31.8
35–50 days	14.5
50–100 days	9.4
100d–120 days	5.0
120–250 days	5.6
250d–320 days	7.8
Dead foal at term	3.4

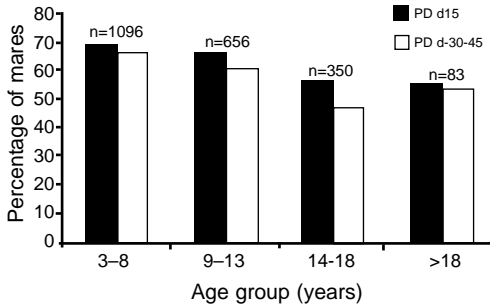


Fig 1: The influence of mare age on the per cycle pregnancy rate.

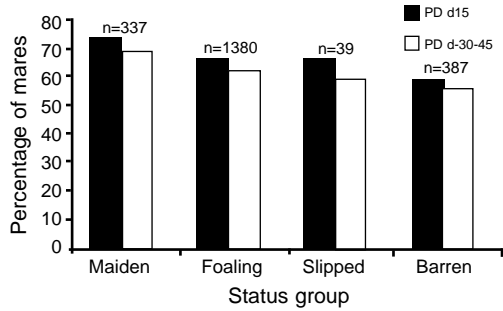


Fig 2: The influence of mare status on the per cycle conception rate.

barren or foaling mare (Fig 2). The proportion of mares that lost their pregnancies between Day 15 and Day 30 rose steadily with increasing age in a pattern somewhat similar to those of the Newmarket survey performed by Sanderson and Allen (1987). In aged mares with chronic degenerative endometritis there will be reduced placental function and consequently inadequate levels of progestins required for the maintenance of pregnancy until term. However, the most notable difference between the results of the 2 surveys is that there is a greatly reduced incidence of pregnancy loss among >18 year old mares compared with the results of the earlier survey. This is most likely a reflection of increased selection pressure based on fertility and the improved veterinary management of this older group of mares. There were also no significant differences in the pregnancy loss rates among the

mare status groups, whether maiden, barren or foaling, except that there was a higher incidence of loss in those mares that had slipped a foal in the previous breeding season (Fig 2).

In conclusion, Thoroughbred mares under good management and close veterinary attention can achieve an acceptable per cycle conception rate (62%) and a very high end of season pregnancy rate of 92%. However, this success is tempered by first trimester pregnancy losses that result in an overall reduction in the reproductive efficiency of this species.

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ORIGINS OF THE ALLANTOIS AND THE ROLE OF ALLANTOIC MESENCHYME IN DEVELOPMENT OF THE EQUINE CHORIONIC GIRDLE

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The allantois is a membranous sac that developed as a rapidly growing external bladder for the storage of embryonic waste products. However, via fusion with the chorion and extensive vascular development, it evolved into an efficient means of extracting nutrients and oxygen from the mother and it now forms the definitive (chorioallantoic) placenta in all eutherian mammals. Many placentae have become so efficient at passing nitrogenous wastes into the maternal bloodstream that the allantoic sac is no longer needed and it simply ends in a blind duct. Nevertheless, all eutherians form a chorioallantois in which allantoic mesenchyme develops via a common pathway to provide the fetal vasculature and umbilicus of the placenta.

Research in mice has revealed some unexpected findings as to the developmental origins of the mammalian allantois. Gene targeted (knockout) mouse embryos that lack Bone Morphogenetic Protein 4 (Bmp4) show several developmental defects, particularly in their extraembryonic mesoderm. They do not develop an allantois, they lack primordial germ cells (PGCs) and they die before mid-gestation (Winnier *et al.* 1995). In further experiments designed to investigate the role of Bmp4 in PGC development, Lawson *et al.* (1999) showed that Bmp4 expression in the trophoblast of mouse embryos induces the differentiation of precursor cells in the epiblast, some of which form the allantois whereas others give rise to the PGCs. It would appear, therefore, that a gene expressed in the trophoblast is responsible for 2 vital developmental processes in the embryo - that of the PGCs and the allantois. This is likely to be the case in all eutherian mammals and suggests that, in the horse, Bmp4 expression in polar trophoblast cells prior to their disappearance at about Day 12

after ovulation, might induce the differentiation of precursor cells in the embryo that subsequently give rise to the allantois and the PGCs.

In addition to providing the fetal vasculature of the placenta, allantoic mesenchyme appears to play a significant role in driving placental growth and development, largely by inducing proliferation and differentiation of trophoblast (Stewart 1996). Equine placentation illustrates an interesting example of this mesenchymal-epithelial interaction, whereby development of both invasive (chorionic girdle) and non-invasive (allantochorion) trophoblast can be studied. Previous investigations led to the hypothesis that chorionic girdle cells probably receive the same mitogenic signals as the non-invasive trophoblast of the allantochorion, but because the mesenchyme is not fused to the chorion in the girdle region, the mitogen-stimulated trophoblast cells pile up on one another instead of spreading out as a single layer (Stewart *et al.* 1995). However, further examination of the membranes in this region indicated that the mesenchyme might actually migrate beneath the region of the chorionic girdle and we have now used immunohistochemistry with a monoclonal antibody to vimentin (clone V9, Boehringer Mannheim) to show that this is indeed the case. The reason why the chorionic girdle cells pile up instead of spreading out might therefore be that the region in which they develop lacks an endoderm layer. This suggests that a chorioallantois formed from ectoderm, mesoderm and endoderm derivatives is always non-invasive, whereas a chorioallantois formed from ectoderm and mesoderm (ie no endoderm and no allantoic sac) is always invasive. This appears to apply to all species examined to date. The different developmental pathways also depend on interactions with the maternal endometrium and this interaction has clearly played a significant role in

evolution of the many different placental phenotypes in existence.

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MATERNAL ADAPTIVE RESPONSES TO THE EXPRESSION OF PATERNALLY INHERITED FETAL ANTIGENS IN THE PREGNANT MARE

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The success of the fetal allograft appears to depend upon several overlapping mechanisms of protection from maternal rejection, many involving modulation of the maternal immune system during gestation. This work sought to identify sources and profiles of cytokines that have the potential to influence the maternal immune response to the developing conceptus.

The expression of 5 cytokines was analysed in peripheral blood leucocytes from pregnant horse mares between Days 0 and 60 of gestation. Cytokine expression was extrapolated by amplification of specific mRNA via a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), using known quantities of cytokine

plasmid DNA to create a standard curve. Messenger RNA for interleukin 2 (IL-2), interferon γ (IFN- γ), interleukin 10 (IL-10), transforming growth factor β (TGF- β), and interleukin 4 (IL-4) was assayed in resting peripheral blood lymphocytes from non-pregnant animals. Upon maximal stimulation of the lymphocytes with mitogen *in vitro*, all cytokine levels increased between 8 and 10,000-fold, with the exception of IL-2 (Fig 1).

In mares pregnant with horse conceptuses, IL-4 and TGF- β message became upregulated in resting, non-stimulated lymphocytes between

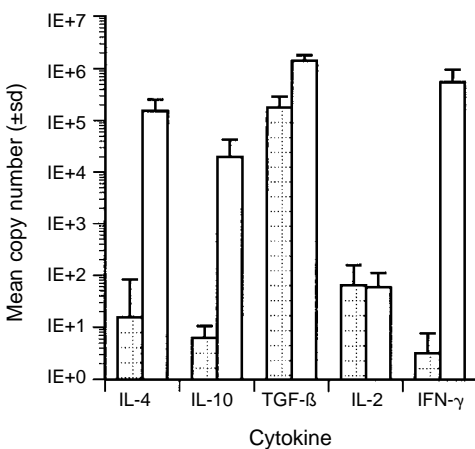


Fig 1: Comparison of cytokine expression by unstimulated vs. mitogen stimulated peripheral blood leucocytes from equids. Data are shown as the mean copy number of each cytokine \pm sd. Unstimulated samples (hatched bars) were placed in culture for 3 days with no additives, stimulated samples (open bars) were cultured for 3 days *in vitro* with pokeweed mitogen.

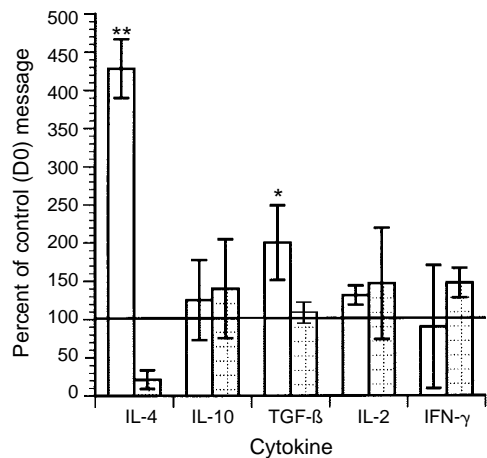


Fig 2: Cytokine message detected in unstimulated peripheral blood leucocytes (PBL) from pregnant mares. Data are shown as percent of control message \pm sd. Control represents cytokine cDNA amplification from the pre-insemination blood samples. Open bars: cytokine message detected in resting PBL from mares carrying intraspecies horse pregnancies, hatched bars: cytokine message in unstimulated PBL from mares carrying mule conceptuses. ** $P < 0.005$, * $P < 0.06$ compared to mule.

Days 14 and 35 of gestation. Mares carrying interspecies hybrid mule conceptuses had decreased IL-4 message with no significant change in other cytokine mRNA assayed, compared to mares carrying intraspecies conceptuses (Fig 2).

In vitro allogeneic stimulation of lymphocytes from pregnant mares resulted in specific upregulation of IFN- γ and TNF- α in lymphocytes from mares carrying mules and of IL-10 in mares carrying horse conceptuses (data not shown). Message for each cytokine in the resting peripheral blood leucocytes of pregnant mares did not approach the levels present in maximally mitogen stimulated lymphocytes.

Tissue samples from non-pregnant and pregnant mare uteri and developing conceptuses were tested for the presence of a panel of cytokines using qRT-PCR. When the relative quantities of fetal (conceptus) vs. maternal (endometrium and endometrial cup) cytokines were investigated, there were much larger amounts of mRNA for both Th1 and Th2-type cytokines in the maternal tissues, compared to fetal membranes, with the exception of TGF- β (Table 1).

The maternal cytokine mRNA production was temporally related to stages of pregnancy (data not shown). Comparison of relative cytokine expression between endometrial cup and endometrium in pregnant and non-pregnant mares

revealed that, with the exception of IL-5 and TGF- β , the production of mRNA for both Th1 and Th2-type cytokines was 5 to 100 times greater in the endometrial cups than either pregnant or non-pregnant endometrium. Much higher levels of IL-5 were detected in non-pregnant endometrium samples compared to pregnant endometrium and endometrial cup, and there was no change in TGF- β levels (Table 1). Next, comparison of cytokine mRNA produced locally in the uterus and distally in the peripheral blood of pregnant mares indicated that much greater levels of message for IFN- γ , IL-4 and IL-10 are found in the uterus (Table 1).

In the equid, there may be both peripheral and local production of cytokines which support tolerance vs immunity and antibody production over delayed type hypersensitivity responses during pregnancy. Locally, the upregulation of both Th1 and Th2-type cytokines may reflect an inflammatory (Th1) response occurring as a result of implantation and a modulatory reaction (Th2) to keep the inflammatory reaction from dominating and possibly harming the developing fetus. Because the message for most conceptus-derived cytokines was detected in such low frequency, it is doubtful that control over the maternal immune system is completely fetally derived. More likely, there is an event prior to the maternal recognition of pregnancy which primes the immune system

TABLE 1: Comparison of cytokine mRNA levels in conceptus tissues¹, uterine tissues and peripheral blood lymphocytes from pregnant mares

Tissue	Mean copy number/ μ l of cDNA						
	Th1-type cytokines			Th2/Th3-type cytokines			
	IL-2	IFN- γ	TNF- α	IL-4	IL-5	IL-10	TGF- β
Fetus	0.06	0.58	71.30	0.00	3321.05	2.96	1.17x10 ⁵
Chorion	0.50	0.01	6.08	0.00	720.37	3.36	1.30x10 ⁵
Allantochorion	0.45	6.08	37.30	0.00	499.09	0.20	3.76x10 ⁵
Chorionic girdle	0.19	0.02	33.00	0.00	236.10	0.25	2.35x10 ⁴
Yolk sac	0.03	24.40	68.00	0.00	678.54	1.45	1.00x10 ⁵
Bilaminar omphalopleur	0.45	0.00	143.00	0.00	538.73	0.08	4.98x10 ⁴
Endometrium ²	6.03	9.93	1951.44	23.33	1971.11	257.76	2.11x10 ⁵
Endometrial cup ³	30.62	997.93	1.14x10 ⁴	137.21	1652.22	830.11	2.27x10 ⁵
PBL ⁴	87.29	2.96	NT ⁶	26.69	3021.88	7.70	2.97x10 ⁵
PBL + mitogen ⁵	59.63	5.66x10 ⁵	2.67x10 ⁴	1.53x10 ⁵	6.28x10 ⁴	2.01x10 ⁴	1.45x10 ⁶

¹The mean copy number of mRNA detected in tissues isolated from 3 different intraspecies horse conceptuses at Day 33–35 of gestation; ²Pregnant endometrium, includes maternal endometrium and maternal leucocytes; ³Copy number in whole tissue sample; includes endometrial cup trophoblast cells, maternal endometrium and maternal leucocytes; ⁴Cytokine mRNA expression by non-stimulated peripheral blood lymphocytes from mares carrying intraspecies horse pregnancies. Data are pooled from various time points during gestation; ⁵Peripheral blood lymphocytes from non-pregnant males and females, cultured with pokeweed mitogen for 3 days; ⁶NT=not tested

toward a phenotype of tolerance, perhaps started with cytokines present in seminal plasma (Robertson *et al.* 1995), propagated by the hormones and enzymes associated with pregnancy (Stites *et al.* 1983; Bambra 1984; Munn *et al.* 1998), then maintained by peripheral blood and local uterine leucocyte production of Th2/Th3-type cytokines.

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FACTORS CONTROLLING GROWTH AND DEVELOPMENT OF THE EQUINE PLACENTA AND MATERNAL RESPONSES TO THESE CHANGES

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Between Days 25 and 35 after ovulation in pregnant equids the discrete, annulate chorionic girdle, consisting of a series of tightly packed ridges of hyperplastic specialised trophoblast cells, develops on the outer surface of the chorion of the spherical conceptus in the region of abutment of the enlarging allantois and regressing yolk sac. Between Days 36 and 38, the entire chorionic girdle separates from the fetal membranes and adheres to the underlying endometrium to enable the girdle cells to invade the maternal tissue. Once in the endometrial stroma, the girdle cells round up, enlarge greatly and clump together to form ulcer-like protuberances known as endometrial cups. These unique fetal structures persist in the endometrium until around Day 100–120 when they secrete large quantities of the gonadotrophic hormone, equine chorionic gonadotrophin (eCG), into the maternal bloodstream via large lymph sinuses. High concentrations of paternally inherited Class I major histocompatibility complex (MHC) antigens are expressed by the invading chorionic girdle cells, but not the non-invasive trophoblast cells of the allantochorion attached to the luminal epithelium of the endometrium. These stimulate the development of paternal-specific lymphocytotoxic antibodies in maternal serum that persist in high titres throughout gestation. A strong cell-mediated maternal response, composed of lymphocytes, plasma cells and eosinophils, is also mounted against the endometrial cups and this hastens their degeneration and desquamation from the endometrium before mid-gestation. The nature of the foreign antigens expressed by the endometrial cup cells which stimulate this maternal lymphocytic response remains uncertain.

Studies with hybrid (interspecies) and between-species embryo transferred (extraspecies) equine pregnancies have indicated some surprising

fetomaternal interactions in relation to both the development of the progenitor chorionic girdle and the maternal endocrinological, immunological and anatomical responses to the resulting endometrial cups. For example, serum eCG concentrations are 8–10 fold lower, and the lifespan of the endometrial cups is much shorter, in mares carrying interspecies mule (female horse x male donkey) conceptuses than in mares carrying

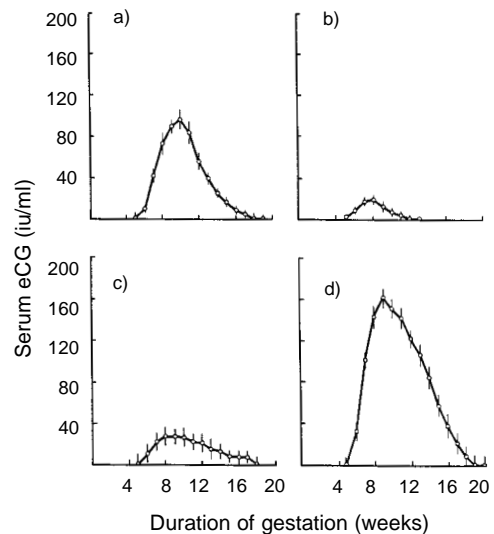


Fig 1: Mean (\pm se) concentrations of equine chorionic gonadotrophin (eCG) measured by haemagglutination inhibition assay in the serum of: a) 30 horse mares (*Equus caballus*, 2 n = 64) carrying intraspecies horse pregnancies; b) 11 mares carrying interspecies mule pregnancies; c) 14 Jenny donkeys (*E. asinus*, 2 n = 62) carrying intraspecies donkey pregnancies; and d) 6 Jenny donkeys carrying interspecies hinny pregnancies. Peak gonadotrophin concentrations are high (80–200 iu/ml) when the horse is the sire and low (5–40 iu/ml) when the donkey is the sire (Redrawn from Stewart and Allen 1981).

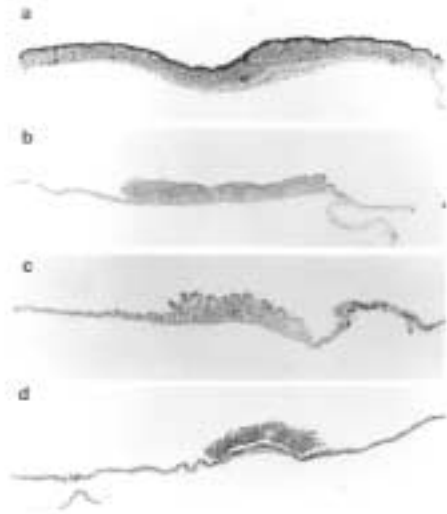


Fig 2: Low power photomicrographs of the chorionic girdles at Day 34 of gestation: a) a normal intraspecies horse conceptus (*E. caballus*, 2 n = 64); b) a normal intraspecies donkey conceptus (*E. asinus*, 2 n = 62); c) an interspecies mule conceptus *E. caballus* female x *E. asinus* male, 2 n = 63; and d) a donkey conceptus (*E. asinus*) transferred to the uterus of a horse mare (*E. caballus*).

normal intraspecies horse conceptuses, or in Jenny donkeys carrying reciprocal interspecies hinny (female donkey × male horse) conceptuses, both of which have a horse (*Equus caballus*, 2 n = 64) as the sire (Fig 1).

This stems from the development of a much smaller and narrower chorionic girdle on donkey and mule conceptuses (donkey sire; *E. asinus*, 2 n = 62), than on horse and hinny conceptuses (Fig 2), which leads to development of smaller and less productive endometrial cups in the donkeys carrying donkey and mares carrying mule pregnancies.

In addition, the maternal cell-mediated response to the endometrial cups is greatly enhanced and causes premature destruction and desquamation of the cups from the endometrium in the mares carrying mule conceptuses although, curiously, the humoral response to the endometrial cup cells is much reduced in this type of pregnancy.

On the face of it, these differences seem to suggest imprinted paternal genomic control of chorionic girdle development in equids. However, transfer of a mule demi-embryo into the uterus of a Jenny donkey results in the development of a broad and highly productive chorionic girdle on

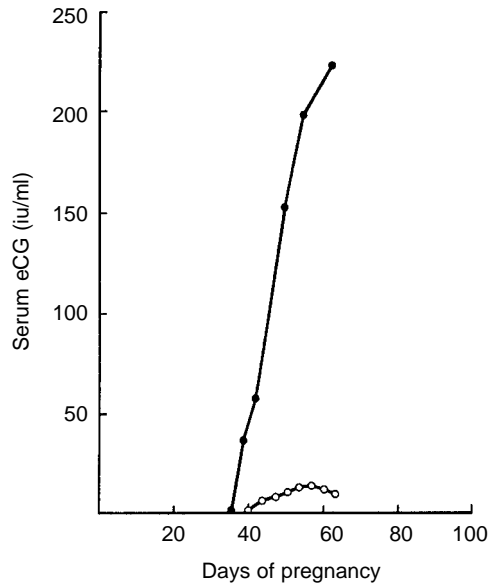


Fig 3: Concentration of eCG measured by amplified enzyme-linked immunoassay (AELISA) in the sera of a horse mare (○—○) and a Jenny donkey (●—●), each carrying one half of the same interspecies mule embryo bisected as a morula prior to transfer.

the mule conceptus gestating in the 'wrong' mother, thereby totally reversing the situation that pertains when the other mule demi-embryo is transferred back into a horse uterus. Thus, maternal uterine environment exerts an overriding control on the development of at least the invasive component of the equine placenta (Fig 3).

Extraspecific transfer of donkey embryos into the uteri of horse mares creates an interesting model of equine pregnancy failure which involves inadequacies of both placentation and maternal immunological responses. The donkey chorionic girdle develops even less well than that of the mule conceptus in the horse uterus such that it fails completely to invade the host endometrium. Endometrial cups do not form and no eCG is secreted. Subsequent non-invasive interdigitation of the donkey allantochorion with the horse endometrium between Days 60 and 90 of gestation ranges from normal, through patchy and inadequate, to completely absent, thereby resulting in fetal death and abortion during this period in around 70% of such extraspecies pregnancies.

This low rate of fetal survival is not improved by the exogenous administration of either eCG or progestagens to the surrogate horse mothers, but

it is increased by either infusing serum recovered from mares carrying normal horse conceptuses between Days 40 and 80 of gestation into the mares carrying donkey conceptuses, or by actively immunising these latter animals against donkey lymphocytes (Allen *et al.* 1987). Immune memory is clearly involved in the phenomenon, because abortions occur at increasingly earlier stages of gestation in individual mares carrying their second and third successive donkey-in-horse pregnancies. And at the placental interface, localised differences include an absence of the normal upregulation of expression of epidermal growth factor (EGF)-mRNA in the epithelium of the necks of the endometrial glands from Day 40 of gestation; an upregulation of expression of MHC Class I antigens by the non-invasive trophoblast cells of the allantochorion that are not interdigitated with the endometrial epithelium; and accumulations of lymphocytes in these areas of the endometrium in which intimate

interdigitated microvillous contact between trophoblast and endometrial epithelium is lacking.

The possible endocrinological and immunological roles of endometrial cup development in, and rejection from, the endometrium of pregnant equids in the establishment and adequate function of a stable microcotyledonary placenta that can sustain fetal growth and development throughout pregnancy have yet to be established clearly.

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THE ROLE OF ENDOMETRIAL GLANDS IN UTERINE FUNCTION: LESSONS LEARNED FROM THE UTERINE GLAND KNOCKOUT MODEL IN SHEEP

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Mammalian uterine morphogenesis begins in the fetus, but is only completed post natally with differentiation and development of endometrial glands. In ungulates, endometrial gland morphogenesis (adenogenesis) occurs after birth and is both steroid- and ovary-independent. Endometrial adenogenesis involves the emergence, proliferation and differentiation of the glandular epithelium (GE) from the luminal epithelium (LE) after birth. In neonatal ewes, endometrial adenogenesis is initiated between post natal day (PND) 0 (birth) and PND 7 and is essentially complete by PND 56.

In situ hybridisation, semi quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical analyses demonstrated that ovine endometrial adenogenesis involves the expression of short and long prolactin receptors (PRL-Rs) and high levels of oestrogen receptor alpha (ER- α) and progesterone receptor (PR) in the nascent and proliferating GE from PND 7 to PND 56. Our current working hypothesis is that circulating PRL stimulates short and long PRL-Rs in the GE which activates the mitogen activated protein kinase (MAPK) signalling pathway and, subsequently, ER- α in a ligand-independent manner. This hypothesis is supported by recent observations in sheep that exposure of the developing neonatal uterus to Norgestomet (Nor), a potent synthetic 19-norprogesterin, from birth to PND 28, suppresses uterine ER- α gene expression, particularly in the LE, and disrupts growth factor and receptors involved in epitheliomesenchymal interactions.

Indeed, ovine endometrial gland development is a post natal event that can be inhibited epigenetically by chronic exposure of ewe lambs to Nor from birth to puberty (Gray *et al.* 2000). As adults, these neonatally progesterin-treated ewes

lack endometrial glands and display a uterine gland knockout (UGKO) phenotype that is useful as a novel model to determine the role of endometrial glands in cyclic and pregnant uterine function. Studies were conducted to determine: 1) length of progesterin exposure necessary from birth to produce the UGKO phenotype in ewes; 2) whether UGKO ewes display normal oestrous cycles; and 3) if UGKO ewes could establish and/or maintain pregnancy. In the first study, ewe lambs (n=22) received a Nor implant at birth and every 2 weeks thereafter for 8 (Group I), 16 (Group II), or 32 (Groups III and IV) weeks. Control ewe lambs (n=13) received no Nor treatment (Groups V and VI). Group I, II, III and VI ewes were semihysterectomised (Hhx) at 16 weeks of age. After puberty, the remaining uterine horn in Hhx ewes was removed on either Day 9 or 15 of the oestrous cycle (Day 0 = oestrus). Histological analyses of uteri indicated that progesterin exposure for 8, 16 or 32 weeks prevented endometrial adenogenesis and produced the UGKO phenotype in adult ewes. Three endometrial phenotypes were consistently observed in Nor-treated ewes: 1) no glands; 2) slight glandular invaginations into the stroma; and 3) limited numbers of cyst- or gland-like structures in the stroma. Overall patterns of uterine PR and ER- α and oxytocin receptor (OT_R) expression were not different *in uteri* from adult cyclic control and UGKO ewes. In the endometrial epithelium, abundant levels of PR expression, but not ER- α or OT_R, were detected on Day 9. On Day 15, PRs were not detected in the LE or superficial GE (in control [CX] ewes), and abundant levels of ER- α and OT_R expression were detected in uterine epithelium from both CX and UGKO ewes. However, intact UGKO ewes displayed altered oestrous cycles with inter-

oestrous intervals of 17 to 43 days. Nevertheless, UGKO ewes responded to exogenous prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) with luteolysis and behavioural oestrus. During the oestrous cycle, plasma concentrations of progesterone in intact control and UGKO ewes were not different during metoestrus and dioestrus, but levels did not decline in many UGKO ewes during late dioestrus. Peak peripheral plasma concentrations of PGF metabolite, in response to an oxytocin challenge on Day 15, were 3-fold lower in UGKO compared to control ewes. Intact UGKO ewes bred repeatedly to intact rams did not display evidence of pregnancy based on results of ultrasound. These studies indicate that endometrial glands are required for normal uterine function during both the oestrous cycle and pregnancy.

Recent studies determined that embryo transfer of blastocysts from normal Day 7 pregnant ewes into synchronised UGKO recipients also fails to produce a successful pregnancy. Uterine flush of bred UGKO ewes on Day 14 post mating revealed the absence of embryos (4/8) or the presence of growth retarded embryos (4/8). Collectively, results indicated that: 1) transient, progestin-induced disruption of ovine uterine development from birth alters both structural and functional integrity of the adult endometrium; 2) normal adult endometrial integrity, including uterine glands, is required to ensure a luteolytic pattern of PGF production; and 3) the UGKO phenotype, characterised by the absence of endometrial glands and a compact, disorganised endometrial stroma, limits or inhibits the capacity of uterine tissues to support the establishment of pregnancy. Specifically, the endometrial glands appear to produce or transport substances required for conceptus elongation during the peri-implantation period.

The UGKO sheep endometrium has also been utilised to identify genes differentially expressed in the endometrial epithelium using the

molecular techniques of mRNA differential display (DD), PCR and suppression subtractive hybridisation (SSH) (Spencer *et al.* 1999). Sequence analyses of DD and SSH identified and cloned cDNAs indicated similarity of some to known mRNAs, including β -lactoglobulin, alkaline phosphatase, type B and D endogenous sheep retroviruses, gp330/megalin, matrix gla protein and others. Other cDNAs were not similar to any known sequences and are considered novel, although some of these match human expressed sequence tags (ESTs). *In situ* hybridisation analyses of uteri from cyclic and pregnant ewes indicated that all DD-PCR and SSH identified mRNAs were expressed in either the endometrial LE and/or GE, although some were also expressed in other uterine cell types. Northern and *in situ* hybridisation analyses revealed that patterns of mRNA expression for most clones were affected by day of the oestrous cycle and pregnancy in a manner consistent with regulation by progesterone. These studies demonstrate the utility of the UGKO sheep model to identify known and novel genes transcribed in the uterine endometrial epithelium. The cloned cDNAs with specific expression in the endometrial epithelium should be useful as potential markers of hormone action and uterine receptivity, EST reagents for comparative and physical genetic mapping, and to discover new factors and pathways regulating endometrial epithelial function.

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THE ROLE OF THE ADRENALS AND EFFECTS OF ACTH TREATMENT ON DEVELOPMENT AND MATURATION OF THE EQUINE FETUS IN LATE GESTATION

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The hypothalamo-pituitary adrenal (HPA) axis is essential for maturation of the fetus in late gestation in all species studied, including the horse. It is also responsible for initiating parturition in several species. The equine adrenal gland becomes more responsive to exogenous adrenocorticotrophic hormone (ACTH) over the last 2 or 3 weeks of gestation when it undergoes significant morphological and functional changes; it doubles in weight between 300 days gestation and term and shows increased steroidogenic enzyme activity close to term, in particular P450_{SCC}, 3 β -HSD and P450_{C17}, enzymes important for progesterone and cortisol production (Silver and Fowden 1994; Fowden and Silver 1995; Han *et al.* 1995). These changes occur in parallel with increases in circulating levels of ACTH and progesterone metabolites (progestagens). Cortisol itself increases substantially in the equine fetus only during the last 24–48 h before delivery (Fowden and Silver 1995). Cortisol promotes development of many fetal tissues and organs. In the horse, the pre-partum cortisol surge is associated with increased release of neutrophils, deposition of liver glycogen, thyroid development, improved thermoregulation and musculoskeletal development in the foal.

The role of the adrenal in fetal maturation and parturition has been demonstrated clearly in catheterised sheep preparations by surgical ablation of the fetal HPA axis (adrenalectomy, hypophysectomy or pituitary stalk section), which prolongs gestation, and by fetal iv infusion of ACTH or cortisol which promotes fetal maturation and shortens gestation. In the equine fetus, more clinical approaches have been used to investigate the role of the HPA axis in fetal maturation. In a preliminary study (Ousey *et al.* 1998), fetuses were injected *in utero* with bolus doses of ACTH Depot im (1 mg daily from 300 to 302 days

gestation) or water (controls), guided by transabdominal ultrasound. Following intra-fetal ACTH treatment, maternal plasma progestagen concentrations increased significantly above values for control mares. ACTH treated fetuses were delivered significantly ($P < 0.01$) earlier than control foals (gestational age = 314 and 327 days, respectively). They also had a lower body weight and higher mean red blood cell volume, suggesting delivery before full term, but were mature with respect to adrenocortical function and other endocrine and behavioural parameters (Rossdale *et al.* 1984). These results suggest that precocious delivery of mature foals may be stimulated by ACTH administration to the fetus. It is hypothesised that exogenous ACTH stimulates pregnenolone production, probably from the adrenal cortex, leading to enhanced production of progestagens and, ultimately, cortisol. However, 5 fetuses (31%) were aborted within 48 h of ACTH injection indicating that the technique was not safe for use in clinical practice.

To minimise the risk of fetal abortion, a second study examined the effects of maternally administered ACTH on gestational length and fetal maturity. Pony mares were given 1 mg (low dose, LD, $n = 6$) or 4–5 mg (high dose, HD, $n = 16$) Depot ACTH daily from 300 to 302 days gestation. Because conception dates are known to influence gestational age at delivery, the HD group was divided retrospectively into mares conceiving before (early) or after (late) 1st July. All LD mares conceived before 1st July. Maternal ACTH administration increased peripheral progestagen and cortisol concentrations in all mare groups but values were significantly higher in the HD compared with LD mares. Eight HD mares, conceiving after 1st July, delivered mature foals significantly ($P < 0.01$) earlier (318 ± 2 days) than

LD mares (335 ± 4 days) and 6 HD mares, conceiving before 1st July (340 ± 4 days). They also delivered significantly ($P < 0.01$) earlier than control (untreated) mares mated after 1st July (327 ± 1 days). Two mares aborted within 48 h of maternal ACTH administration but post mortem examination failed to reveal any specific abnormalities. These results suggest that maternal ACTH treatment may stimulate precocious fetal maturation but only in mares mated later in the year, ie those in which fetal development may already be more advanced. This 'seasonal' effect may have considerable importance for future studies on equine fetal maturation and development. The endocrine pathway involved cannot be elucidated without access to the fetal measurements.

Studies in other species (Wood 1988) suggest that ACTH is unlikely to cross the placenta and, therefore, any maturational effects on the fetus are likely to have been mediated via maternal cortisol acting either on the placenta, in a feed forward manner, or crossing the placenta and acting directly on the fetus. However, this latter hypothesis seems less plausible because the equine placenta contains 11β -hydroxysteroid dehydrogenase activity which converts cortisol to less active cortisone (Chavatte *et al.* 1995).

In conclusion, administration of ACTH Depot to the pre-term mare or fetus may help advance fetal maturation and delivery but both routes have

their limitations in terms of safety and efficacy. The procedures require further refinement before they can be applied in clinical practice.

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DEVELOPMENTAL DELAY OR ARREST IN UTERO AND POSSIBLE POST NATAL CONSEQUENCES

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Developmental delay or arrest occurring *in utero* or during infancy may have wide ranging consequences, not only for the neonatal infant but also during later life (Anon 1993). Such developmental delays or arrest may result from any number of insult(s) mediated through the mother. The onset, duration and severity of the insult(s) all play a crucial role in determining the extent to which an organ may show structural, physiological or metabolic changes post nately. An organ undergoing organogenesis may be highly susceptible to any insult(s) during critical periods of development (ie periods of greatest proliferative change, growth or maturation). An increased physiological demand placed on an organ with a reduced reserve capacity may lead to rapid homeostatic derailment resulting in death from no apparent cause or diminished mental or physical performance depending upon the organ affected. However, fetal growth has a certain degree of plasticity and, depending on the severity of the insult(s) *in utero*, the fetus may be able to utilise its reserve capacity to initiate compensatory responses often characterised by a post natal 'catch up' period.

In the past, the use of conventional 2D microscopy to analyse structural components contained within an organ has often produced spurious results due, primarily, to inherent inaccuracies in the sampling and final estimation. However, the use of 3D unbiased design based stereological techniques (Ansari 1998) has made it possible for the first time to obtain total values of number, volume, length or surface area that can be related to the structure as a whole, making comparison of organs between groups more meaningful.

Investigations into the development of a number of organs (brain, phrenic nerve, diaphragm,

lung and kidney), in various species (human, lamb, pig and horse), have revealed that the development of these organs at a micro-anatomical level was arrested or delayed, especially in animals considered to be growth retarded as defined by current clinical parameters (eg reduced body weight). Within each species there was a range in the severity of the developmental delay or arrest experienced by each organ. This may be the consequence of each organ being subjected to varying degrees of either prolonged or low level chronic insult(s) or periods of acute insult(s) *in utero*. Changes observed in a particular organ in one species and not repeated in another species may result from the variation in the developmental rate of each species

Human sudden infant death syndrome (SIDS) cases born with a birth weight above the 10th centile for gestational age, show a reduction in total neocortical neuron number and an increase in mean neocortical nuclear volume (Ansari 1998) when compared with control infants. Significant reduction in total neocortical neuron and glial cell number is also observed in runt or low birth weight piglets, compared with litter matched controls, but this is not true of low birth weight lambs. Because the lamb is highly precocious at birth in comparison to the human neonate and the newborn piglet, the process of evolutionary survival of the lamb may have resulted in specific organs, critical for survival *ex utero*, being less susceptible to developmental insult(s) occurring *in utero*. In the human fetus, neocortical neuron proliferation and migration occurs between 4 and 16 weeks of gestation. Any insult occurring when cells are undergoing proliferation may result in reduced neuron number with little possibility of post natal catch up. Should a similar insult(s) occur when neurons are undergoing maturation

(from 20 weeks gestation) it may result in a change in neuron size. Human infants affected by intra uterine growth retardation (IUGR) are known to be at risk of having minor motor neurological problems (Ens-Dokkum *et al.* 1983)

Reduction in myelinated axon number in the left phrenic nerve observed in human SIDS infants, runted piglets and low birth weight lambs (Ansari 1998), compared with appropriate controls, is thought to result partially from insults occurring during the period of phrenic nerve myelination (15–23 weeks of gestation). Possible consequences of reduced myelinated axon number may include altered or reduced electrical transmission along the nerve to the muscle fibres within the diaphragm. Anomalies have also been noted in the cross sectional area in both type I and type II muscle fibres in the diaphragm in SIDS infants in comparison to a control population (Ansari 1998). The hypertrophy of both muscle fibre types was accompanied by an increase in the ratio of type I to type II muscle fibres, suggesting that the changes were due to an insult(s) occurring when the muscle fibres were undergoing differentiation *in utero* (in the human fetus this occurs between 20 and 26 weeks of gestation).

There is a significant reduction in the total number of respiratory terminal ducts in SIDS normal birth weight (NBW) infants without any significant change in the amount of gas exchange surface area (Beech 1997). The low birth weight lamb has a significant reduction in terminal duct number and in the total gas exchange surface area. However, the runted piglet has a reduced gas exchange surface area but no change in the terminal duct number. In human SIDS NBW infants, a normal amount of gas exchange surface area is supplied by a reduced number of terminal ducts, a possible consequence of which may be increased air pressure within each terminal duct. Should the infant experience an increased respiratory demand this may result in the air within each terminal duct changing from a smooth laminar flow to more turbulent flow resulting in inadequate gas exchange. The physiological response to this would be to hyperventilate but hyperventilation may further compromise the air flow. Continued inadequate gas exchange may result in hypoxia in the first instance followed by anoxia and perhaps death. Children who survive the vulnerable period for SIDS, and have a reduced number of terminal ducts, may be at

increased risk subsequently of developing an asthmatic condition.

Results of a reduced glomerular number in SIDS low birth weight infants (Beech 1997), low birth weight lambs and runted piglets suggest that there is a strong correlation between low birth weight and reduced glomerular number. Analysis of low birth weight human stillbirths has revealed no shift in renin gene expression, suggesting that these fetuses, had they survived, may have been at an increased risk of developing neonatal oliguria (Kingdom *et al.* 1999). Maternal smoking, a known risk factor resulting in low birth weight, has been linked with increased blood pressure in children as early as 7.5–8 years of age (Anon 1993).

Because organogenesis of the organs studied so far is completed *in utero* (except for the gas exchange surface area of the lung which continues post nately) any deficiency in either number or size of the functional sub-components of an organ cannot be compensated for post nately. These developmental delays or arrest cannot be detected using existing clinical prognostic techniques, they can only be detected using post mortem tissue and sophisticated microscopical techniques. The placenta, as the major organ responsible for oxygen and nutrient delivery to the developing fetus, plays a crucial role in maintaining an adequate homeostatic environment *in utero*. It has been hypothesised that placental insufficiency is the most likely condition to result in delayed or arrested organogenesis.

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COMPARATIVE STEREOLOGICAL ANALYSIS OF ORGANOGENESIS IN THOROUGHBREDS AND PONIES

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Previous studies in human infants (Hinchcliffe *et al.* 1992, 1993), piglets and lambs (Beech *et al.* 1996; Sibbons *et al.* 1996) have demonstrated that the use of unbiased stereological techniques to estimate the volume, number and surface area of the functional units of several organ systems indicate delayed organogenesis in animals and children afflicted by low birthweight. Delayed organogenesis is also demonstrated by this technology in some infants of apparently 'normal' birthweight (Hinchcliffe *et al.* 1993). The presence of certain clinical conditions such as exercise-induced pulmonary haemorrhage (EIPH), which are manifested specifically in the Thoroughbred racehorse, have led to questions regarding the condition of organ development (organogenesis) in these animals. There is specific interest in the relationship between organogenesis and the competitive ability and therefore earning potential of these highly valued animals. Ponies provide a contrasting breed which is non-selectively bred, compared to the highly-selectively bred Thoroughbred racehorses.

This pilot study utilised stereological techniques to gain valuable information with regard to 'normal' baseline organogenesis in ponies and Thoroughbred racehorses.

Organs were retrieved from 45 equine post mortem examinations, from previously normal healthy horses, where cause of death was considered acute enough for the animals to be classed as controls. Fetuses were removed from pregnant mares by hysterectomy following euthanasia for acute clinical conditions such as bone fracture. Foals, yearlings and adults were subjected to euthanasia due to acute conditions such as bone fracture, grass sickness etc. A variety of organs were removed at post mortem examination and immersion fixed in 10% neutral buffered formalin. Stereological techniques were used to assess the development of the following organs - lung, kidney, phrenic nerve and adrenal gland. Cavalieri's Principle (Gundersen and Jensen 1987) was used to estimate the volume of: whole left lung, whole, cortex and medulla of left kidney; whole, cortex and medulla of left adrenal gland. The physical disector (Sterio 1984; Pakkenberg

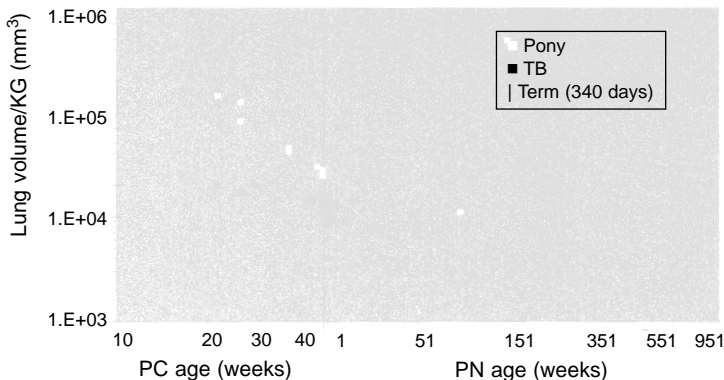


Fig 1: Lung volume/kg.

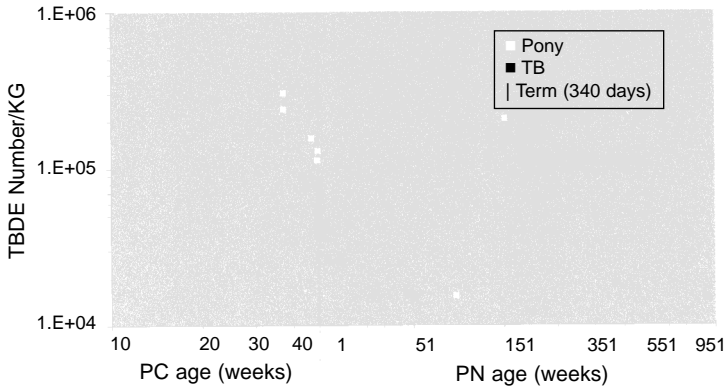


Fig 2: TBDE number/kg.

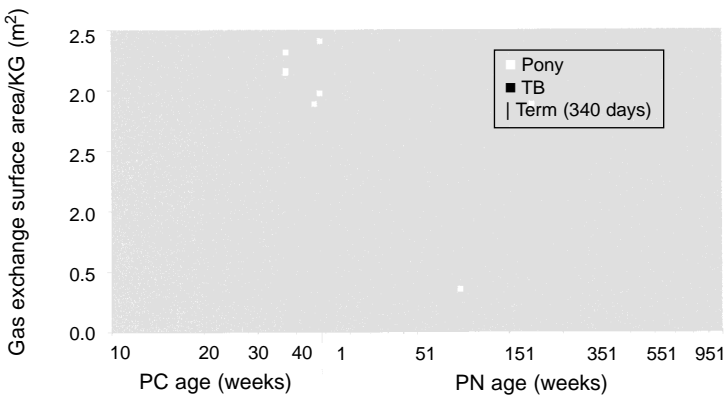


Fig 3: Gas exchange surface area/kg.

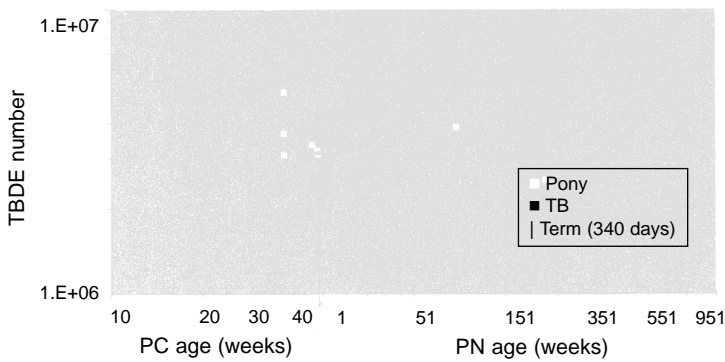


Fig 4: Terminal bronchiolar duct ending number.

and Gundersen 1988) was used to estimate total terminal bronchiolar duct ending number of the left lung and total glomerular number of the left kidney. The surface estimator (Baddeley *et al.* 1986) was used to estimate total gas exchange surface area of the left lung. The point-sampled intercept method (Gundersen and Jensen 1985) was used to estimate the mean glomerular volume

in the left kidney. In the phrenic nerve, the area of the nerve and the total number of myelinated axons were estimated.

The results demonstrated that lung development in the pony was more advanced at birth than in the Thoroughbred racehorse - lung volume, terminal bronchiolar duct ending number, gas exchange surface area and mean gas exchange

surface area per terminal bronchiolar duct ending were shown to be greater throughout gestation and at birth in ponies compared to Thoroughbred racehorses. This was particularly evident when correcting the estimates for the bodyweight of the animals (Figs 1–3).

By 2 years of post natal age, there was little difference between the breeds for these parameters. The development of the terminal bronchiolar duct endings was shown to continue until approximately one year of age in the Thoroughbred racehorse (Fig 4).

This has not been demonstrated in any other species (Bucher and Reid 1961; Beech *et al.* 1999) and is not present in the pony where terminal bronchiolar duct ending number development appears complete before birth. There was little difference in the development of the kidneys, phrenic nerves and adrenal glands of the 2 breeds. The phrenic nerves of the Thoroughbred racehorses had a very large range in their area and myelinated axon number. The adrenal glands of ponies and Thoroughbred racehorses showed an increased proportion of cortex within the last few weeks of gestation. This is most probably related to the increase in cortisol produced in the few days before parturition (Silver and Fowden 1988, 1994).

This study has produced baseline data regarding the 'normal' development of several organs of Thoroughbred racehorses and ponies and has identified several fundamental differences in specific organogenesis between these 2 breeds. The most striking of these is the advanced stage of lung development of the pony at term compared to the Thoroughbred racehorse and the post natal development of terminal bronchiolar duct ending number in Thoroughbred racehorses. Animals that suffer chronic and severe EIPH may be those which have an extraordinarily low number of terminal bronchiolar duct endings. Post natal development of terminal bronchiolar duct ending number may indicate a potential for the post natal manipulation of respiratory development and maturation in Thoroughbred racehorses. This manipulation would only be possible if it were possible to estimate terminal bronchiolar duct ending number in the live animal by some type of functional test.

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MICRO-ANATOMICAL DEVELOPMENT OF THE EQUINE KIDNEY IN NORMAL AND GROWTH RETARDED ANIMALS AND ASSOCIATED FUNCTIONAL TESTS

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INTRODUCTION

The effects of adverse intra-uterine conditions on fetal development have been well documented in man with regard to intra-uterine growth retardation (IUGR), sudden infant (cot) death syndrome (SIDS), and diseases occurring in later life (Buck *et al.* 1989). In man, IUGR has been defined as when an infant is born at a birth weight significantly below (10th percentile) the mean for gestation, irrespective of any clinical features shown (Battaglia and Lubchenco 1967). In spite of the absence of macroscopic organ defects, stereological techniques have revealed important micro-anatomical deficiencies and, in particular, glomerular number in the kidneys of infants suffering from IUGR and SIDS (Hinchcliffe *et al.* 1992, 1993).

Little is known about normal renal organogenesis in the horse and stereological techniques are novel in their application to equine tissue. Directly correlating structure to function is unique for any species and will provide further understanding of renal development during gestation and in the *post partum* period, and allow development of prognostic tests for micro-anatomical deficits in renal development with implications for subsequent survival, growth and athletic performance.

AIMS

i) To determine normal micro-anatomical development of the pony and Thoroughbred (TB) kidney

ii) To correlate the above anatomical findings to previous functional tests of the equine kidney
iii) To establish any micro-anatomical deficits in the kidneys of IUGR TB foals.

MATERIALS AND METHODS

For consistency, the left kidney was collected from 63 horses (control ponies n=24; control TBs n=32; IUGR TBs n=7), ranging from 60 days gestation to 8 months post partum. The IUGR animals were defined using parameters previously described in infants (Battaglia and Lubchenco 1967). Using design-based, unbiased stereological techniques (Hinchcliffe *et al.* 1993), whole kidney and cortex volume, total glomerular number and glomerular volume were determined. Functional tests, including amniotic and allantoic fluid analysis, insulin/para-aminohippuric acid and fractional excretion of electrolytes, have already been performed (Holdstock 1995), and were correlated to the stereological findings of the kidneys.

RESULTS

When stereological data were presented as values per kilogram bodyweight, no differences were seen from those presented in absolute value and, therefore, results in Figs 1–4 are shown in absolute values.

Kidney volume and cortex volume (Figs 1 and 2)

These parameters increased with post conceptual age in both breeds. In early to mid gestation kidney

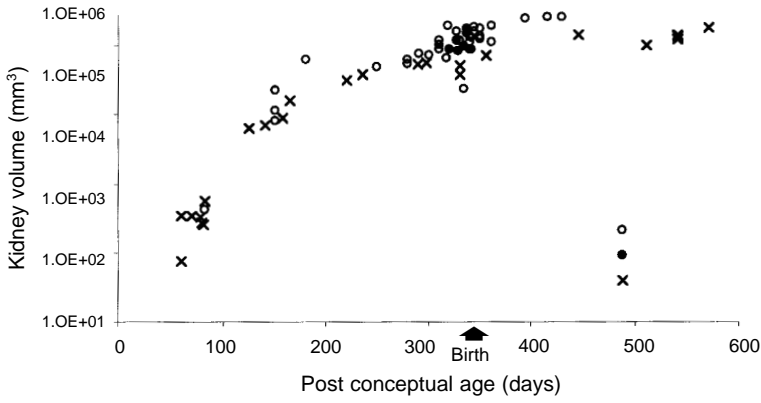


Fig 1: Kidney volume (mm³) against post conceptual age (days) in the horse.

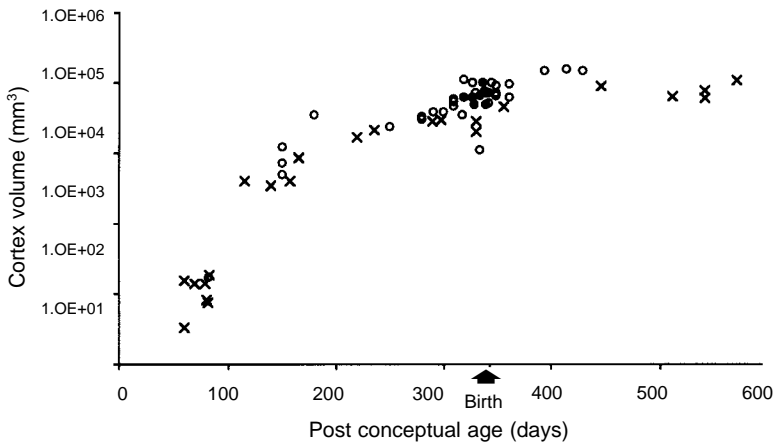


Fig 2: Cortex volume (mm³) against post conceptual age (days) in the horse.

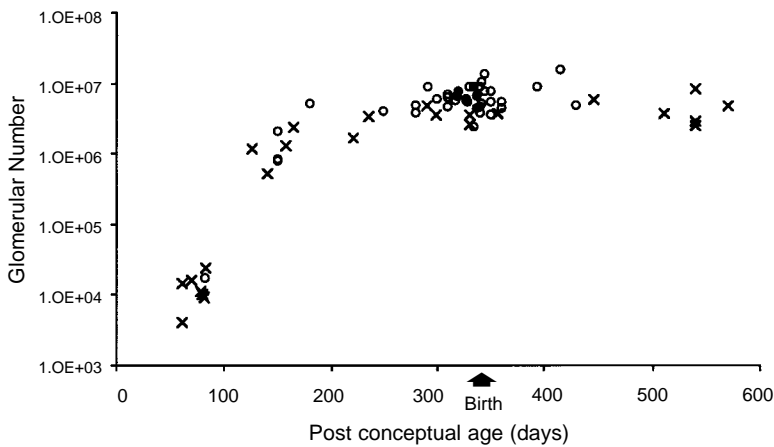


Fig 3: Glomerular number against post conceptual age (days) in the horse.

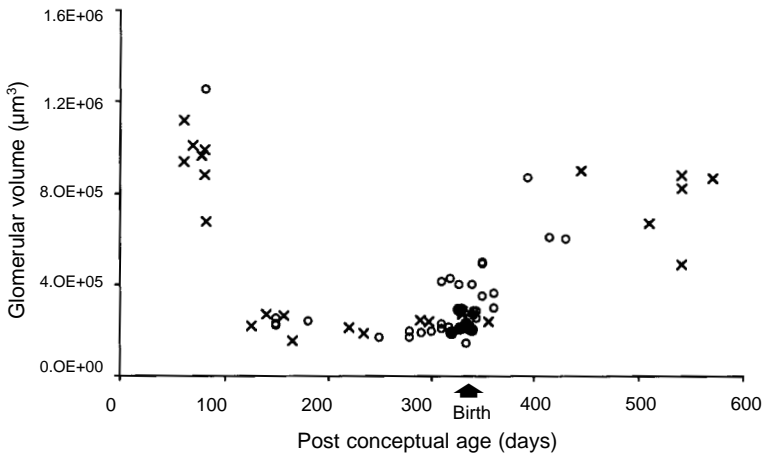


Fig 4: Glomerular volume (μm^3) against post conceptual age (days) in the horse.

development in ponies and TBs appeared to be similar whereas, by late gestation, the rate of increase in Ponies slowed down sooner, and at a lower value, than that of TB animals. At birth, one normal TB had kidney and cortex volumes lower ($P < 0.05$) than the normal TB range. The IUGR group appeared to fall within the normal TB range for both parameters.

Glomerular number (Fig 3)

There was an increase in glomerular number with post conceptual age in both breeds, although the rate of increase appeared to level off sooner, and at a lower value, in ponies where glomerular number increased from 9.18×10^3 at 60 days gestation to a plateau of 3.62×10^6 at 250 days gestation. In TBs the glomerular number increased from 1.66×10^4 at 82 days gestation to a plateau of 8.75×10^6 around 2 months post partum. The values for the IUGR TB group were within the normal TB range.

Glomerular volume (Fig 4)

The profile of glomerular volume development appeared to be similar throughout the time of study for both breeds. Glomerular volume decreased in ponies and TBs from $8.78 \times 10^5 \mu\text{m}^3$ and $1.25 \times 10^6 \mu\text{m}^3$, respectively, at 80 days gestation, to $2.19 \times 10^5 \mu\text{m}^3$ and $2.28 \times 10^5 \mu\text{m}^3$, respectively, at 150 days gestation, and then increased around birth to reach a plateau of $8.65 \times 10^5 \mu\text{m}^3$ and $8.68 \times 10^5 \mu\text{m}^3$, respectively, at 2 months post partum. At birth, one normal TB had a lower ($P < 0.05$) glomerular volume than the normal TB range. The IUGR TB foals had reduced ($P < 0.05$) mean

glomerular volume compared to the control TB group ($2.16 \times 10^5 \mu\text{m}^3$, $\pm 0.32 \times 10^5$, and $3.67 \times 10^5 \mu\text{m}^3$, $\pm 0.43 \times 10^5$, respectively) when compared for the same post conceptual and post natal age ranges.

DISCUSSION

Although the number of kidneys included in this study was limited, because the range of antenatal and post natal development was examined, the data gained has provided important information regarding normal micro-anatomical development in the horse. In early gestation there are no comparable studies available in other species. However, renal organogenesis has been shown to stop *in utero* or around birth in most species studied (Hinchcliffe *et al.* 1991; Bains *et al.* 1996), whereas kidney development in the horse appeared to continue for a relatively longer time in the post natal period.

Correlating the structural findings seen in the present study to previous functional tests of the equine kidney showed that the greatest developmental changes in glomerular number and volume occurred when fetal urine was first detected in the allantoic fluid, at approximately 150 days in ponies and TBs (Holdstock 1995). At birth, renal micro-anatomical maturation appeared to be more advanced in ponies compared to TBs, which also supports previous physiological studies (Holdstock 1995). The present study has highlighted differences in normal kidney development between native ponies and 'artificially' selected TBs.

Although micro-anatomical deficits in the kidneys of the IUGR TB foals were established in

the present study, the data suggested that these foals had deficiencies in glomerular volume, rather than reduced glomerular number as seen in IUGR animals of other species (Hinchcliffe *et al.* 1992; Bains *et al.* 1996). This study also showed that one normal TB had lower values for most stereological parameters studied, indicating the necessity for screening tests to identify these 'unexpected' normal animals which are at risk of organ limitation.

Until completion of organogenesis the horse may be at risk of exceeding its functional capacity and, as in IUGR infants (Barker 1994), altered/deficient organogenesis may help to explain some of the physiological incompetence seen in IUGR foals. However, it is possible that the continued post natal development in the horse may allow neonatal compensations for intra-uterine problems or, on the other hand, it may allow extra-uterine growth retardation. Further work is proposed to follow up these hypotheses.

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MATERNAL ADRENALECTOMY AS A MEANS OF STUDYING THE CONTROL OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS DURING PREGNANCY IN THE EWE

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During pregnancy there is a well-documented increase in the activity of the maternal hypothalamus-pituitary-adrenal (HPA) axis. In sheep, this results in the doubling of circulating concentrations of cortisol in maternal plasma. Our goal was to develop a model for the study of maternal adrenal gland regulation and the effects of maternal cortisol secretion on fetal homeostasis.

At about 108 days of gestation (term = 147 days), before the time of rapid fetal growth or fetal

adrenocortical maturation, ewes under halothane anaesthesia with controlled ventilation and positioned in sternal recumbancy, were bilaterally adrenalectomised. Ewes were treated with aldosterone by iv infusion (3 µg/kg bodyweight per day) to induce normal late-gestation aldosterone concentrations. Ewes were also treated with cortisol. For 2 days post operatively, this infusion (1–2 µg/kg/min) induced plasma concentrations similar to those associated with

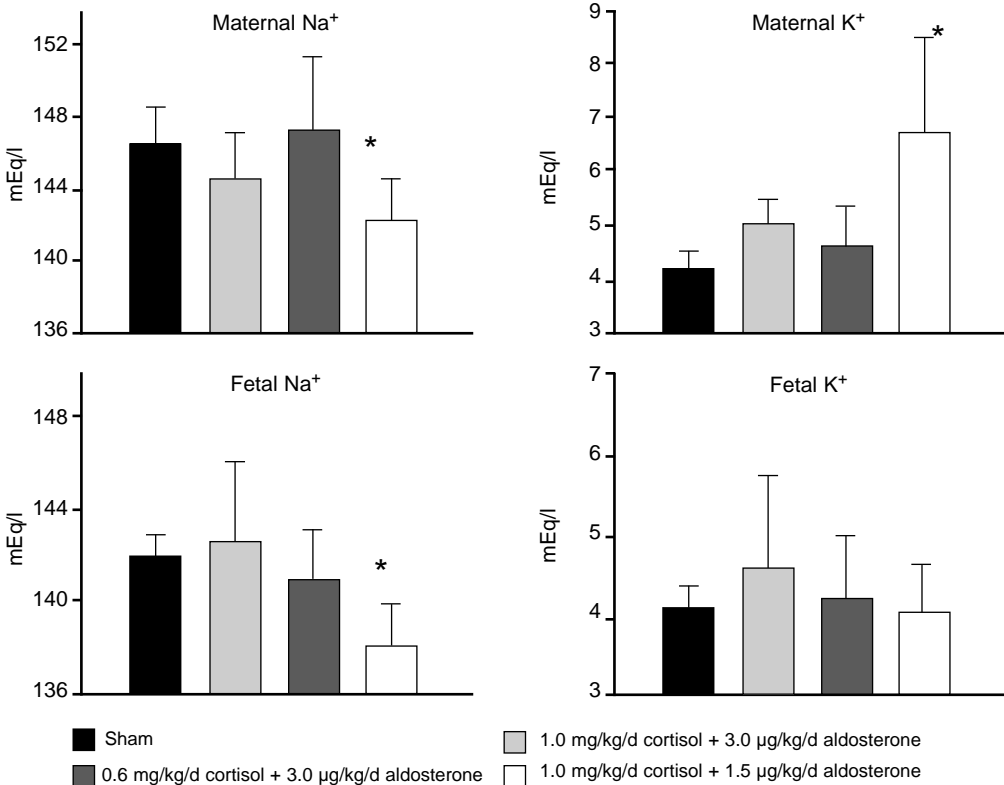


Fig 1: Electrolyte concentrations in adrenalectomised pregnant ewes receiving cortisol and aldosterone replacement therapy and in sham operated controls.

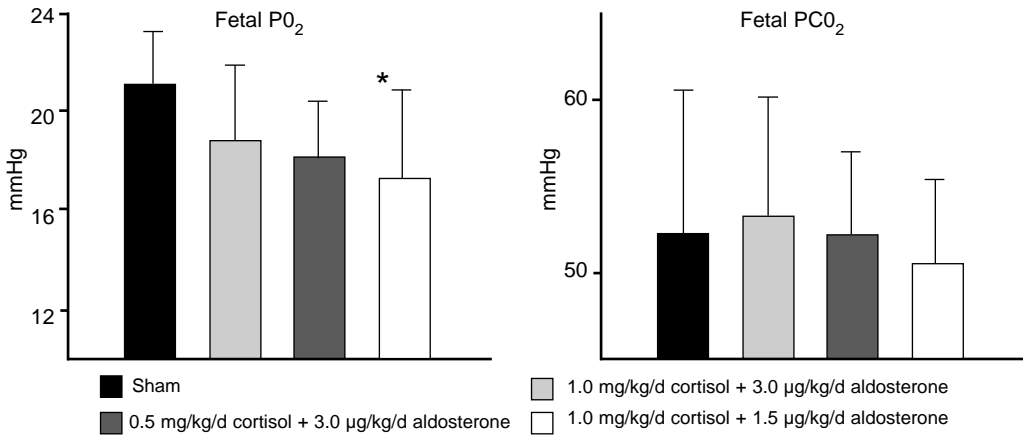


Fig 2: Fetal blood gases from adrenalectomised ewes receiving cortisol and aldosterone replacement therapy and in sham operated controls.

stress. Thereafter, the dose of cortisol was reduced to induce plasma concentrations similar to normal late gestation cortisol concentration in the maternal plasma (~10 ng/ml; 1 mg/kg/day), or to values in non-pregnant ewes (~5 ng/ml; 0.6 mg/kg/day).

Administration of cortisol and aldosterone was required to prevent electrolyte imbalance and signs of hypoadrenocorticism. With steroid replacement at the higher rate, plasma protein, electrolyte and glucose concentrations in adrenalectomised ewes were not different from those in sham-operated pregnant ewes. Of 11 adrenalectomised ewes, 2 aborted their fetuses; 3 delivered one live and one dead fetus; 2 delivered live singleton fetuses; 2 delivered live twins; and 2 ewes died. This model of relative hypoadrenocorticism in pregnancy is feasible and practical for studying the influence of maternal cortisol concentration on maternal and fetal homeostasis. In further experiments, we studied ewes with singleton fetuses. The ewes were bilaterally adrenalectomised and replaced to normal pregnancy plasma concentrations of both cortisol (infusion rate 1 mg/kg/day) and aldosterone (3 µg/kg/day), or to concentrations of cortisol (0.5 mg/kg/day) or aldosterone (1.5 µg/kg/day) which are normal for non-pregnant animals. We found that the reduction of maternal cortisol (but with normal pregnancy concentrations of aldosterone) did not significantly alter maternal electrolytes, but resulted in reduced maternal blood volume, increased maternal plasma concentrations of arginine vasopressin (AVP) and

adrenocorticotropin (ACTH), and an increased incidence of fetal hypoxia and abortion in these ewes. The reduction of maternal aldosterone (but with normal pregnancy concentrations of cortisol) resulted in fetal death in 2 of 6 pregnancies and fetal hypoxia in the other 4 fetuses. In this experimental group, maternal plasma potassium concentration was significantly elevated, as expected in hypoadrenocorticism. The ewes were relatively hypovolaemic compared to sham ewes and adrenalectomised ewes with the same cortisol replacement dose but higher plasma aldosterone concentrations. Ewes with normal pregnancy concentrations of both cortisol and aldosterone significantly increase plasma volume between 120 and 130 days gestation. This increase in plasma volume was prevented by reducing the plasma aldosterone concentration to non-pregnant plasma concentrations. We conclude that elevated (pregnant) concentrations of both steroids were needed for normal fetal homeostasis. However, the fetal blood pressure, blood gases and electrolytes were more critically dependent upon the elevation in aldosterone.

The results suggest that relative hypoadrenocorticism of the pregnant ewe impairs maternal blood volume expansion and, in turn, impairs fetal gas exchange and growth.

ACKNOWLEDGEMENTS

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EFFECTS OF MATERNAL SIZE ON PRE- AND POST NATAL DEVELOPMENT IN THE HORSE

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Walton and Hammond (1938) first used artificial insemination to create crosses between Shire horses and Shetland ponies. Tischner and Klimczak (1989) transferred pony embryos into the uteri of larger draft mares. These studies demonstrated a profound influence of maternal size on the birthweight of the foal and the maintenance of these differences into adult life.

The present study also utilised embryo transfer, between small Welsh Pony and Welsh Cob mares with a mean (\pm sd) body weight of 329.5 ± 69.5 kg and larger Thoroughbred mares with a mean \pm sd body weight of 592 ± 47.6 kg. The purpose of study was to examine the influences of: 1) intrauterine cramping and nutritional deprivation resulting from a smaller than normal placenta versus; 2) spatial luxury and nutritional excess *in utero* afforded by a larger than normal placenta, on the size and functional competence of the foals at birth and on their subsequent growth profiles.

Eight Pony-in-Thoroughbred (P-in-Tb) and 7 Thoroughbred-in-Pony (Tb-in-P) experimental foals, together with 7 P-in-P and 7 Tb-in-Tb control foals, were born spontaneously between Days 327 and 343 of gestation. During pregnancy, maternal serum eCG and total conjugated oestrogen profiles showed no striking differences between the 4 types of pregnancy but serum progesterone profiles during the 2 months prior to birth were at least 10-fold higher in the Tb-in-P than the P-in-Tb pregnancies, perhaps reflecting both the larger blood volume of the Thoroughbred recipient mares and involvement of the fetal adrenal cortex in response to increasing stress in the smaller pony recipients. At birth, the mean (\pm sd) weights of the foals were highest in the Tb-in-Tb foals (53.07 ± 6.80 kg) followed the P-in-Tb foals (37.9 ± 5.5 kg), the Tb-in-P foals (33.0 ± 6.8 kg), and the P-in-P foals were lowest (24.0 ± 3.3 kg). These differences were well correlated with maternal weight ($r=0.74$) and with the weight

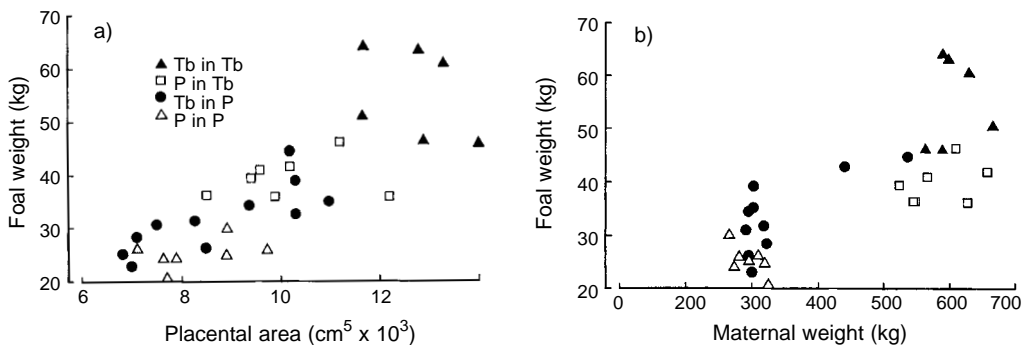


Fig 1: Foal weight vs placental area (a) and maternal weight (b).

($r=0.76$), gross area ($r=0.86$) and volume ($r=0.91$) of the placenta (Fig 1).

Height, weight and 11 other body and longbone measurements were made on all the foals at weekly intervals from birth to weaning at 6 months of age; and thereafter at monthly intervals. Prior to weaning, the birthweight differences were maintained and bodyweight profiles remained remarkably parallel. During the subsequent 12 months, however, the size difference between Tb-in-P ('deprived') offspring, and their Tb-inTb controls has persisted whereas the disparity between the reciprocal P-in-Tb group and their P-in-P controls has declined. It is predicted that the

degree of 'runting' occasioned *in utero* in Tb-in-P foals will persist throughout their lives. What effects, if any, these relative levels of deprivation versus excess *in utero* have had upon organ development and function and how this, in turn, may affect athletic ability, will be examined over the coming year.

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THE INFLUENCE OF MATERNAL SIZE, PARITY AND AGE ON MORPHOMETRIC PLACENTAL DEVELOPMENT AND FUNCTION IN THE MARE

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Using between-breed matings (Walton and Hammond 1938) and embryo transfer (Tischner and Klimczak 1989) uterine size has been shown to influence foal birthweight. Maternal age and parity are also thought to influence birthweight, with foals from primigravid, or very aged, mares being small (Platt 1984). These effects are thought to be mediated via differences in the size and/or exchange competence of the diffuse, non-invasive, epitheliochorial equine placenta. Rossdale (1966) observed that the birthweight of Thoroughbred foals is directly proportional to gross surface area of the placenta and Bracher *et al.* (1996) demonstrated a close correlation between age of the mare, health of the endometrium, density and regularity of the microcotyledons per area of placenta and fetal growth in the first two thirds of gestation. Reduced absorptive potential of the placenta has been shown to lead to small-for-date babies in women (Pivalizza *et al.* 1990) and fetal growth retardation in sheep (Alexander 1974).

In the present study, maternal weight, placental area and weight, birthweight, surface area of microcotyledons per unit volume and total area of fetomaternal contact across the placenta were measured in 2 cohorts of foals. Computer-assisted stereological morphometric analysis of 10 random biopsies from the placenta of each foal immediately after spontaneous third stage labour was used to measure the microscopic surface area of fetomaternal contact per unit volume of microcotyledons at the interface and, hence, the total area of fetomaternal contact across the placenta. These measurements were compared to maternal weight, placental weight and foal birthweight.

EXPERIMENT 1

Embryo transfer was used to create 8 Thoroughbred-in-Pony (Tb-in-P) and 7 P-in-Tb

pregnancies. This imposed placental/nutritional limitations on the genetically larger Thoroughbred foals developing in the pony mares and a larger than normal placenta and hence nutritional excess for the pony foals in the large Thoroughbred uterus. Seven Tb-in-Tb and 7 P-in-P pregnancies served as controls. Strong positive correlations were shown between maternal weight and foal birthweight and both the weight and gross area of the placenta. Microscopic surface area per unit volume (surface density) of the microcotyledons was also positively correlated with foal birthweight, with the highest density in the Tb-in-Tb control placentae and the lowest in the P-in-P control placentae. Surprisingly, there was no compensatory increase in microcotyledonary surface density in the 'deprived' Tb-in-P pregnancies, although the P-in-Tb pregnancies did show increased surface density compared to the P-in-P controls. The correlation between foal birthweight and total area of fetomaternal contact at the placental interface was particularly strong, thereby illustrating that surface density of microcotyledons and also the gross volume and area of the placenta are important in determining total haemotrophic nutrition across the placenta.

EXPERIMENT 2

The second cohort of foals came from 32 Thoroughbred mares that had conceived by natural mating in the previous year and were foaling at 4 studfarms in Newmarket, England. They ranged in age from 5 to 23 years and in parity from primigravid to multiparous. When grouped on the basis of parity and age, primigravid mares aged 5–7 years (Group A) showed a lower mean foal birthweight, mean placental weight, mean gross area of the placenta and mean total area of fetomaternal contact than the other 3 groups, none

of which were primigravid (Group B, 5–9 year olds; Group C, 10–15 year olds and Group D, \geq 16 year olds). The primigravid mares also showed a reduced surface density of microcotyledons compared to the Group B mares, despite the valid assumption of minimal degenerative changes in the endometrium of the primigravid animals. However, there were much larger individual differences within Group A. Nevertheless, the findings do support the widely held contention that the mare's uterus needs to be 'primed' in some way by the first pregnancy before it can achieve its full potential in terms of promoting placental and fetal growth. It was surprising to find that mean foal birthweight, mean placental area, mean placental weight and mean total area of fetomaternal contact at the placental interface were all slightly higher in the Group D mares (\geq 16 year olds) than the Group B mares (5–9 year olds), even though, as expected, the surface density of microcotyledons in the Group D animals was lower than in the other 2 groups of younger mares. Thus, the lower surface density of microcotyledons in the aged animals, occasioned no doubt by increased levels of degeneration (endometrosis) in their endometrium, was offset by both a higher volume and a higher gross area of placenta which, in turn, maintained the higher mean birthweight in this aged group even though, as with the primigravid mares, there were large individual differences in birthweight (Fig 1).

Thus, surprisingly in this experiment, primigravity exerted a greater negative influence on placental size and, hence, foal growth *in utero*, than did maternal age. However, it is prudent to remember that the total microscopic surface area of fetomaternal contact at the placental interface is not the only parameter governing placental competence and efficiency. For example, the adverse effects of maternal undernutrition during pregnancy on placental size in sheep are accompanied by hypoplasia of the cotyledons, but compensatory increases in haemoglobin and enhanced blood flow (Clarke *et al.* 1998). Thus, whereas maternal features such as size, age and parity all bear varying degrees of influence on gross and microscopic area of the placenta, other factors such as uterine blood flow and general vascularity of the placenta will also exert profound effects on nutritional and gaseous exchange at the

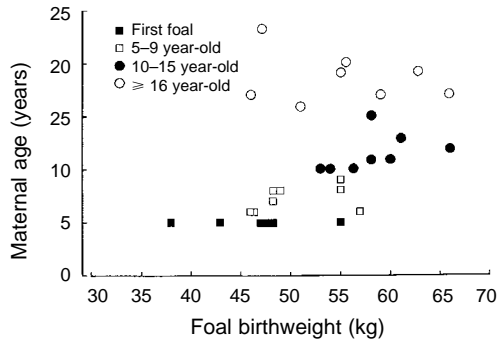


Fig 1: Correlation between the age of the mare and the birthweight of the foal across the 4 groups of Thoroughbred mares. Note the bigger spread between individual animals in the primigravid (■) and \geq 16 year old (○) groups.

fetomaternal interface and, consequently, fetal growth *in utero*.

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BIOLOGICAL FUNCTIONS OF SULPHOCONJUGATED OESTROGENS AND THEIR INFLUENCE ON FETAL DEVELOPMENT AND RESPONSIVENESS

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Ovine parturition is initiated by increases in fetal hypothalamus-pituitary-adrenal (HPA) axis activity which, in turn, increase placental oestrogen biosynthesis and ultimately increase uterine contractility. In addition to the action in the uterus, oestrogens augment fetal ACTH secretion. Treatment of fetal sheep for 5 days with 17 β -oestradiol (0.25 mg/day) for 5 days significantly and dramatically increases fetal ACTH secretion and the ACTH responsiveness to stress. In other experiments, we have found that androgen (androstenedione) treatment opens the negative feedback loop between cortisol and ACTH in the late-gestation fetal sheep. Combined treatment of fetuses with both oestradiol and androstenedione results in premature parturition. We hypothesised, therefore, that the endogenous increase in fetal plasma oestrogen and androgen concentrations prior to term participates in the signalling process which culminates in parturition in sheep. We investigated the sites within the central nervous system which are responsive to oestrogen. Immuno-histochemical studies demonstrated that oestradiol treatment increased the abundance of Fos in brain nuclei important for HPA axis function. These neurons responded to increases in fetal plasma oestrogen well within the physiological range of endogenous plasma concentrations. We recognised that the potential for oestrogen interaction with HPA function is even greater because, in late gestation, oestrone sulphate is more abundant in fetal plasma than is unconjugated oestrone. We studied hypothalamus, hippocampus and brain stem tissue from fetal, neonatal and adult sheep to test the hypothesis that the ovine brain contains the enzymatic machinery needed to interconvert sulphoconjugated and unconjugated oestrogens.

The hypothalamus is of interest because of the paraventricular nucleus, which contains the parvocellular neurons containing the releasing factors (arginine vasopressin and corticotropin releasing hormone) important for releasing ACTH from the fetal pituitary. The hippocampus is an important site of negative feedback control of HPA function by glucocorticoids. The brainstem is the site of afferent signalling from visceral afferents (stretch receptors, baroreceptors and chemoreceptors) which influence HPA responses to 'stress' and other homeostatic challenges. We investigated the presence of both oestrogen sulphatase (converts oestrone-3-sulphate to oestrone) and oestrogen sulphotransferase (converts oestrone to oestrone-3-sulphate) in these regions of ovine fetal brain. We found that sulphatase activity in the hippocampus was significantly increased in late gestation fetuses compared with both younger and older animals. No significant change in either hypothalamus or brain stem was revealed. However, the activity in all brain areas was high. We designed and raised a custom rabbit polyclonal antibody (Alpha Diagnostic, Texas, USA). The peptide sequence used from the human sulphatase gene, amino acids 294-309, was NH₂-FSSKDFAGKSQHG VYGC-COOH. We designed and raised an anti-sulphotransferase antibody to recognise a unique sequence within the enzyme, a sequence which was within a hydrophilic and antigenic part of the molecule, and a sequence with which there is no known homology. The peptide sequence used from the bovine sulphotransferase gene, amino acids 273-287, was NH₂-RERFEEHYQQMKDC-COOH. Primary antibodies were diluted to concentrations of 1:1000 in antibody diluent (1% BSA in phosphate buffered saline with 0.05% Tween 20). Visualisation of the

protein-antibody complex was accomplished utilising a chemiluminescence detection system (Renaissance, DuPont NEN, Boston, Massachusetts) and analysed by densitometry (Bio-Rad, California, USA). Antibody specificity was confirmed by pre-absorption of the primary antibody with peptides (1 µg/ml) also supplied by Alpha Diagnostic. The results of immunoblot analyses of sulphatase were consistent with the results of the kinetic assays. Fetal brains were prepared for immuno-histochemistry by perfusion fixation with 4% buffered paraformaldehyde, blocked in paraffin, and cut to approximately 5 µm sections on a rotary microtome. Immuno-histochemistry revealed the presence of oestrogen sulphatase in the paraventricular nucleus of the hypothalamus, the nucleus of the solitary tract and the rostral ventrolateral medulla. We also used the immunoblot and immuno-histochemistry techniques to study hypothalamus and brainstem tissue from fetal, neonatal and adult sheep to test the hypothesis that the ovine brain contains oestrogen sulphotransferase. Although no significant ontogenic pattern was revealed, the presence of oestrogen sulphotransferase within the hypothalamus and brainstem was detectable. Immuno-histochemistry revealed the presence of both oestrogen sulphatase and sulphotransferase in the paraventricular nucleus of the hypothalamus, the nucleus of the tractus solitarius and the rostral ventral lateral medulla. Preliminary results of double immunostaining experiments for sulphatase and oestrogen receptor (alpha form)

revealed that, in some neurons, there was immunostaining for sulphatase in the cellular cytoplasm and oestrogen receptor in the nucleus.

We conclude that ovine fetal hypothalamus, hippocampus and brain stem contain both oestrogen sulphatase and sulphotransferase. We further conclude that the activity of sulphatase in the hippocampus is developmentally regulated. We speculate that the interconversion of unconjugated and sulphoconjugated oestrogens alters the bioavailability of oestrogen within neurons which are important for the control of the fetal HPA axis and therefore important for both the responsiveness to stress and the initiation of parturition. We further speculate that the sulphoconjugated oestrogens which circulate in fetal plasma are biologically active within the fetal brain, and that increases in the concentrations of these sulphoconjugated steroids in the last 2 weeks of ovine gestation provide the afferent limb of a positive feedback loop which initiates parturition. Spontaneous mutation of the gene for steroid sulphatase results in prolonged gestation in women, suggesting that sulphatase and sulphotransferase-mediated alterations in the bioavailability of oestrogens could be a strategy common to several mammalian species.

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WINDOWS OF DEVELOPMENTAL OPPORTUNITY AND DISADVANTAGE THROUGHOUT PREGNANCY

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INTRODUCTION

Data relating to environmental effects on pre-natal growth and development illustrate that there are critical time periods of intrauterine life which impact a legacy of changes that can affect the size, viability and health of the neonate and the body composition and reproductive performance of the adult. Historically, the first illustrations of these effects came from studies of late pregnancy carried out in the 1950s. A notable example is the adverse effect of undernutrition in the last trimester of pregnancy on development of secondary wool follicles in Merino sheep fetuses and the accompanying reduction (-10%) in wool production as adults (Schinckel and Short 1961). Now examples are coming from earlier pregnancy, bringing a new dimension to our understanding of pre-natal growth and development and providing new opportunities for improving reproductive efficiency.

THE EARLY FETAL PERIOD

Recent studies in ewes illustrate that maternal undernutrition (0.5 vs. 1.0 x maintenance) in early pregnancy can influence the development of the fetal ovary and thus provide a mechanistic explanation for the reduced adult reproductive performance of ewe lambs conceived in harsh environments (Borwick *et al.* 1997). The alteration in the development of the ovary occurs in the absence of a reduction in fetal size and is characterised by a delay in oogonia degradation and a postponement in the arrest of meiotic activity.

There are also convincing data illustrating important effects of maternal food restriction in early pregnancy on fetal muscle development. For

example in the guinea pig, a species well recognised for its high metabolic demands during pregnancy, a level of food restriction in the first trimester of pregnancy that leads to a 10% reduction in maternal bodyweight causes a major reduction (20–30%) in fibre numbers in muscles such as the *biceps branchii* that have a high ratio of secondary to primary fibres (Dwyer *et al.* 1995). When the period of restriction is shortened to the first 15 days of the 68-day gestation, the adverse effect on fetal muscle development does not occur. These observations indicate that there is a critical window (~15–25 days) for the expression of nutritional influences on fetal muscle hyperplasia. The nutrients that are important for the full expression of muscle development at this time are carbohydrate and protein, but not fat (Dwyer and Stickland 1994).

In the pig, a species in which muscle fibre hyperplasia ceases by Day 90 of the 115-day gestation, doubling the recommended level of feeding for 25 days preceding the period of hyperplasia, which begins about Day 50 of gestation, increases production of secondary myofibres leading to significant improvements in post natal growth rate and food conversion efficiency (Dwyer *et al.* 1994). Imposing the same high-intake nutritional regimen during fibre hyperplasia (Days 50–80) stimulates only a small increase in the number of secondary muscle fibres. This observation implies that feed level prior to hyperplasia is critical in stimulating the proliferation of presumptive secondary myoblasts and thereby providing the potential for increasing secondary fibre formation.

Not all recently documented instances of the early fetal programming of a later response have a mechanistic explanation. An example is the adverse effects of subclinical cobalt deficiency

during early pregnancy on the vigour of lambs at birth (Fisher and MacPherson 1991), an effect which is not reversed by correcting the deficiency later in pregnancy. In this example the effects are very pronounced with mean (\pm se) time in minutes taken by lambs from deficient vs. control ewes to stand, find the udder and suck being 22 vs. 15 (\pm 4.9); 41 vs. 24 (\pm 5.8) and 76 vs. 31 (\pm 6.9), respectively. The effect of the reduced vigour is a depression in the acquisition of passive immunity by the lamb as illustrated by a mean serum IgG concentration (g/100 ml) at 2 weeks of age, of 2.5 vs. 3.6 for controls. This early-pregnancy effect of inadequate cobalt on the neonate is not mediated by a shift in birthweight. Therefore it is unlike that arising from sibling embryo mortality which occurs in the face of selenium/vitamin E deficiency and is characterised by significant reductions in birthweight for a fixed litter size as the difference between ovulation rate and litter size increases.

THE EMBRYO

Alterations in the environment of the early embryo are now providing evidence that effects on fetal growth and development can be programmed during the early cell cleavage stages that follow fertilisation. For example, temporary exposure of Day 3 sheep embryos to an advanced uterine environment (Day 6) for 3 days increases both the total number of fibres and the ratio of secondary to primary fibres in the skeletal muscles of the resulting fetuses (Maxfield *et al.* 1998b). This transitory alteration of the environment of the early embryo also influences the temporal expression in the fetus of the myogenic gene, *myf5*, implying that it may be involved in prolonging myoblast hyperplasia.

The temporary exposure of the embryo to an advanced uterine environment not only changes its nutrient supply directly but also indirectly through shifts in the maternal endocrine and growth factor systems. It is interesting, therefore, that the suppression in fetal growth that occurs in adolescent ewes overfed in early pregnancy can be alleviated partially by reversing the feeding level induced suppression of maternal progesterone with exogenous progesterone from Day 5 but not from Day 11 of pregnancy (Wallace *et al.* 1998, 1999).

In some instances *in vitro* culture of cattle and sheep embryos from the zygote to blastocyst

stage (Day 1 to Day 6 post fertilisation) leads to fetal oversize and to shifts in body development, with the magnitude of these effects being influenced by the composition of the embryo culture media (Sinclair *et al.* 1998, 1999). A characteristic feature of such oversize is that it does not occur in all fetuses; indeed when full-sibling zygotes are cultured together to the blastocyst stage under the same *in vitro* conditions, some produce normal-size and others over-size fetuses (Carolan *et al.* 1998). This implies that other factors, perhaps even operating prior to fertilisation, can predispose the embryo to environmental factors which alter subsequent fetal growth and development. The oversize is accompanied by significant increases in the allometric coefficients for key organs, most notably the heart with the effect persisting into adulthood (McEvoy *et al.* 1998). It is also accompanied by hypertrophy of both the primary and secondary fibres of fetal skeletal muscle and an approximate 20% increase in the ratio of secondary to primary muscle fibre numbers (Maxfield *et al.* 1998a). Although the factor in *in vitro* culture that programmes the down-stream effects on the fetus has not been identified, there is strong evidence that it is linked to the addition of serum to the culture medium (Sinclair *et al.* 1998, 1999).

In our studies, elevation of the ammonia concentrations (\sim 150 mmol/l) in the environment to which the embryos are exposed, either *in vivo* by manipulating the maternal diet (McEvoy *et al.* 1997) or *in vitro* through the addition of serum to the culture medium (Negrin-Pereira *et al.* 1997), is accompanied by up-regulation of embryo metabolism, and enhanced fetal growth. These observations suggest that there may be a common cause. Irrespective of the factors involved, there is now a requirement for establishing and testing new hypotheses to explain their modes of action. A possible mechanism is an alteration in the expression of some of the imprinted genes with likely candidates being *Igf-2*, *Igf-2r*, *Insulin-2* and *H19* (Young *et al.* 1997).

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WORKSHOP SUMMARY

WORKSHOP SUMMARY

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Conferences are of many kinds: assemblies of three hundred to five hundred people which, although perhaps exciting in a certain way, are really enormously frustrating with their distant speakers, simultaneous sessions and constant interruption of any useful conversation in the corridors. At the other end of the scale are small Workshops such as this, also exciting but very much so in a quieter and more fruitful way. In terms of exchanging information and having meaningful personal interactions, I am sure you will all agree that the format could not be bettered.

Hospitality also takes diverse forms, ranging from the somewhat spartan fare offered in northern Europe to the special delights of the warmer countries, not least those situated around the shores of the Mediterranean and Caribbean. Here in the Colony Club, we have been generously looked after, so once again a particularly warm word of thanks to Mr Gene Pranzo and the Havemeyer Foundation. We are immensely grateful.

Warm words also to Jan Wade and Louise Holder for perfect organisation and for many thoughtful arrangements put in hand with the minimum of intrusion. Similarly, of course, our best thanks to Dr Tom Stout and Professor Twink Allen and their close colleagues for planning a stimulating and well-integrated programme.

If we take a chronological glance back at the events – the excellent lectures – of the last two and a half days, then my interpretation of the pleasant little duty of summing up is to make general comments on some of the events described. I shall do this with poetic licence, with no intention at all of working my way through a list of topics or of being comprehensive, and certainly with no intention of mentioning names or of repeating the lectures. To adapt a phrase offered endlessly on

our radio station back in the United Kingdom, the BBC, we shall focus on policies not personalities.

Commencing with the topic of gametes, and extrapolating from the wide-ranging review we heard almost at the outset, there are still remarkable gaps in our knowledge concerning female germ cells. I don't know for certain but feel reasonably confident that there is little information for the equine fetus on the kinetics of the primordial germ cell population during its migration to the region of the hind gut and so to the embryonic genital ridges. By kinetics, we of course mean multiplication of the initial cohort of primordial germ cells *en route* to the genital ridges and whether there is cell-cell communication between equine germ cells by means of fine filaments at this stage as has been noted, for example, in the mouse.

There is reasonable information for large domestic species concerning the numbers of oogonia in the fetal gonads and the numbers of primary oocytes established by the time of birth, but we are still perplexed by the classical question of why so many oocytes are generated if so few will ever leave the ovaries and fewer still have the opportunity to be fertilised. The conundrum is whether there are specific processes of selection at work and, if so, the nature of the selection. Studies in recent years, especially relating to the ovaries of ruminants, have suggested that elements of chance are uppermost here in terms of waves of follicular recruitment, waves of follicular ripening, and of whether an oocyte finds itself in the happy situation of being encompassed within a follicle selected for ovulation. In a sense, the element of chance concerns both chronological and endocrine opportunity in the selected follicle.

However, the above focus takes us away from the concept of oocyte quality and the possibility

that a proportion of ovarian oocytes may carry genetic errors leading eventually to their elimination by means of an apoptotic cascade. So I believe we still have to reflect on the size of the fetal population of oocytes and search for explanations, reminding ourselves that we are talking of peak populations of hundreds of thousands of primary oocytes formed during oogenesis. Is this an evolutionary vestige of systems of external (aquatic) fertilisation or are there subtle elements of selection at work in terms of promoting competence in the ovulated oocyte?

Turning to male gametes and more particularly to their interactions with the oocyte, we do not yet know where the process of capacitation is completed in the fertilising spermatozoon nor whether the acrosome reaction is induced by the substance of the zona pellucida in the physiological situation. It can be induced by contact with the zona pellucida *in vitro*, but many other factors such as follicular fluid, a progesterone solution or calcium ionophore will also induce the acrosome reaction *in vitro*. Unfortunately, colleagues have extrapolated all too freely from events in the mouse without a sufficiently critical mind and certainly on the basis of inadequate experiments. The dogma concerning zona receptors for sperm in the mouse, that is the ZP3 – ZP2 system of Wassarman and the reported downgrading of ZP3 receptors by the fertilising spermatozoon, is not a sufficient explanation in the sense that polyspermic fertilisation can and does occur spontaneously *in vivo* in mice. In other words, if the fertilising spermatozoon downregulates the receptors for zona binding of any subsequent spermatozoa, then how can multiple sperm penetration of an egg occur? Furthermore, the nature of the zona reaction is clearly different in domestic species that can gain many accessory spermatozoa within the substance of the zona pellucida (eg sheep, cow, pig).

Several of us remain curious as to the extent of spontaneous polyspermic fertilisation in horse eggs and, experimentally, the incidence of polyspermy that can be generated by increasing the population of spermatozoa in the Fallopian tube ampulla. And I myself am not sure, perhaps others in the audience will know, to what extent abnormalities of fertilisation underlie embryonic loss in mares. Here, one is thinking of problems such as digyny, polyspermy, incomplete evacuation of cortical granules and defective microtubule arrangement on the meiotic spindle.

There is abundant evidence, for example in pigs, that abnormalities of fertilisation are expressed as early embryonic loss and may provoke prolonged oestrous cycles, frequently of 26–28 days' duration rather than of 21 days.

Jumping from the stage of fertilisation to that of a blastocyst and its subsequent development, remodelling and expansion into a conceptus, we were certainly impressed and seemingly troubled by its skills in regulating the movement of fluid across its membranes into the blastocoel. Perhaps even more impressive was a vision of the conceptus being moved, shunted around between the 2 horns of the uterus, to inform the maternal system of its presence and thereby to prevent termination of the luteal phase. As will be recalled from the discussion, there remains the question of precisely how coordinated myometrial contractions of sufficient effectiveness are generated to ensure displacement of the conceptus over appropriate areas of endometrial surface. In addition is the question of just how frequent visitations of the conceptus must be to different regions of the uterus to inform the maternal tissues of its presence and prevent initiation of luteolysis. There would seem to be major considerations of dimensions here – the size of the conceptus in relation to the total surface area of endometrium. There are also considerations of fluid volume in the uterine lumen, fluid movement over the endometrial surface, and the rate of dispersal of molecular messages from the conceptus. In sum, there would seem to be scope for mathematical modelling of the tactics employed by the conceptus at this most vulnerable of stages.

This brings us to question the specific nature of the conversation between conceptus and endometrium that suppresses the luteolytic process. One would like to believe that an appropriate protein or peptide molecule is secreted by the embryo so that there would be parallels, for example, between equids and ruminants in this regard. However, demonstration of a suitable trophic molecule seemingly remains a source of frustration. Even so, one imagines that we are all disciples of the luteotrophin-luteolysin concept in which an embryonic luteotrophin acts to suppress release of a uterine luteolysin, and that it is the balance between luteotrophic and luteolytic factors at the end of a luteal phase that regulates the establishment of pregnancy.

Both quantitative and chronological factors can compromise recognition of pregnancy:

quantitative in the sense of insufficient luteotrophin production; chronological in the sense of a retarded secretion, overall perhaps due to a poorly developed conceptus. I was a little surprised that we did not hear more on this theme, bearing in mind quite prominent studies in sheep and cattle. These indicate that a retarded conceptus is unable to express sufficient trophic activity ('noise') to prevent termination of the corpus luteum and its secretion of progesterone, thereby prompts its own demise.

By contrast, we heard a great deal concerning luteolytic mechanisms in terms of the $\text{PGF}_{2\alpha}$ -oxytocin feedback loop, regulation of the respective receptors for these hormones, of their messenger systems and of the prevailing steroid backcloth. Both the amount of work and the detail achieved were impressive, yet this extensive new body of information did not obscure the overriding embryonic strategy of prolonging the lifespan of the corpus luteum and thereby initiating a viable pregnancy. It is only the endocrine tactics that seem to vary between species.

Nor was it fully clear to this reviewer just how we should apply the molecular information in a clinical or animal breeding context to render the reproductive process more efficient. I would need guidance here, but even if – for example – the equine genome were to be sequenced as soon as the human genome will be, perhaps within the next 18 months, I cannot anticipate an immediate route to a therapy for overcoming early embryonic loss, if in fact that were considered desirable. There may be good biological reasons for not intervening in a proportion of instances. Identifying such instances is quite another matter.

Please do not conclude that I am unsympathetic to molecular studies – that would be very far from the truth. I should say immediately that I was particularly impressed by the presentation dealing with pregnancy-associated glycoproteins, knowing the critical importance of glycoprotein molecules in diverse reproductive events. Even so, having a long-term interest in animal breeding and the associated technologies, and having colleagues with clinical responsibility for a wide range of transgenic animals and their problems, I believe that enthusiasm for research techniques involving gene targeting, gene insertion, all sorts of knock-out preparations – these wonderful modern techniques – must be tempered by serious consideration of what it is that one is trying to achieve. Short-term

exploitation of the financial markets is one thing. A long-term contribution to animal breeding is almost certainly dramatically different and carries heavy responsibilities. All of you will appreciate this point, so perhaps it is unnecessary for me even to have mentioned it.

From various angles, we also heard much on the placenta and on uterine glands, especially concerning their development or underdevelopment, and in a sense these subjects were pleasingly complementary. One presumes and hopes that all the detail on offer, not least that concerning the influence of trophic hormones such as growth hormone and prolactin, was not only to understand the formation, control and synthetic abilities of such marvellous eutherian tissues but also had, as an eventual focus, the generation of a viable foetus. Fascinating lines of detail on the placenta were offered, including some provocative evolutionary thoughts vis à vis the equine placenta, yet there was very much the feeling that one of J. Hammond's, L.R. Wallace's or T.J. Robinson's vintage slides illustrating the relative growth of placenta and fetus at different stages of gestation would have been helpful as background. Sometimes the fine detail of a modern research endeavour obscures the broader and equally important picture.

The series of papers dealing with pre- and post natal organ development, fetal and neonatal maturity and various physiological and nutritional constraints upon these were treated in a novel and clear-sighted way. The application of stereological analysis in this context is a significant step forward. One feels that this body of work on anatomical and functional differences between breeds and indeed between species will grow rapidly because its clinical implications are substantial, not least when sensitive assay procedures become available for scanning the spectrum of organ maturity in an individual fetus. It is not too difficult to imagine, say in five years' time, a computer-assisted scan of the state of fetal organ maturity based upon analysis of a single blood sample. Alleviating any deficiencies so revealed may turn out to be a far more challenging problem.

You will have noticed that immunological considerations have not yet been alluded to, nor has the word 'cytokine' been mentioned a single time. This is not an oversight. I suspect that we are all fascinated by the immunological surveillance systems of the body, not least those of the pregnant animal, and of how the fetus plays its subtle hand to

enable us all to be sitting here today. Those of you who know so much more about reproductive immunology than I do will appreciate that we are only just beginning to understand the manner whereby, for example, genetic dissimilarity between mother and fetus, steroid hormone backcloth imposed by the gonads, placenta and adrenal glands, degree of stress and diverse other factors all act to modulate the nature of the immunological response and the degree of tolerance. From the inception of pregnancy – that is the presentation of sperm surface antigens – to the stage of a highly invasive placenta as found in certain carnivores and primates, it is surely not banal to say that we cannot yet give a straightforward explanation of the cell trafficking programmes or of the overall maternal tolerance of the fetus. So clearly reproductive immunology is a discipline with an assured future, and the specialised nature of the equine placenta, with its endometrial cups and local reactions, offers

a privileged model system to be exploited by modern techniques.

I must draw to a close. Anyone who feels that their work hasn't been mentioned in any sense at all must excuse me, although I suspect that it will be more due to my style of writing than to any specific omission. In any event, you will all be in a better position to reflect on the meeting when we have the forthcoming Monograph in front of us – another splendid initiative for which we again express our best thanks to the Havemeyer Foundation. On that note, I really must stop and simply wish you safe travels to your home countries, happy memories of an invaluable Workshop, and successful continuation of excellent and exciting research programmes. Thank you all.

R.H.F. Hunter
November 1999

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