The influence of maternal size on placental, fetal and postnatal growth in the horse. II. Endocrinology of pregnancy

W R Allen¹, S Wilsher¹, F Stewart^{1,2*}, J Ousey^{3,4} and A Fowden⁴

¹Thoroughbred Breeders' Association Equine Fertility Unit, Mertoun Paddocks, Woodditton Road, Newmarket, Suffolk CB8 9BH, UK

²The Babraham Institute, Babraham, Cambridge CB2 4HT, UK

³Beaufort Cottage Stables, High Street, Newmarket, Suffolk CB8 8JS, UK

⁴University of Cambridge, Department of Physiology, Downing Site, Cambridge CB2 3EG, UK

*Deceased

(Requests for offprints should be addressed to W R Allen)

(Part I, Development *in utero*, is published in *Reproduction*, March 2002, 123.)

Abstract

Within-breed artificial insemination and between-breed embryo transfer were carried out in small pony (P) and large Thoroughbred (Tb) mares to create 4 types of horse pregnancy in which the fetus experienced spatial and nutritional deprivation (Tb-in-P; $n=8$), luxury (P-in-Tb; $n=7$) or normality (Tb-in-Tb; $n=7$ and P-in-P; $n=7$) *in utero*.

Measurement of equine chorionic gonadotrophin (eCG), total conjugated oestrogens and progesterone concentrations in serial peripheral serum samples recovered from all the mares throughout gestation showed that the amount of eCG produced during the first half of gestation was dependent upon the breed of the mare rather than the breed of the fetus being carried. In contrast, the mean total amounts of oestrogens produced, as measured by area under the curve, were significantly greater ($P=0.003$) in

the two types of pregnancy in which a Thoroughbred fetus was being carried (Tb-in-Tb and Tb-in-P) than those in which a pony fetus was gestated (P-in-P and P-in-Tb); the evidence suggests that the Tb fetus may have larger gonads than the P fetus and thereby secrete more C-19 precursor steroids for aromatisation to oestrogens by the placenta. In the final weeks of pregnancy mean plasma progesterone concentrations rose much earlier, and to significantly higher levels ($P<0.001$), in the Tb-in-P than in the P-in-Tb pregnancies, thereby reflecting the increased fetal stress in the former causing premature maturation of the fetal adrenal gland. This, in turn, resulted in increased secretion of pregnenolone by the adrenal cortex for conversion to progesterones by the placenta.

Journal of Endocrinology (2002) **172**, 237–246

Introduction

Many aspects of the secretion of gonadotrophic and steroid hormones by the fetoplacental unit in pregnant equids are unique to this genus and are of special interest (Fig. 1). Between days 25 and 35 after ovulation a discrete area of the allantochorion, in the region of abutment of the enlarging allantoic and regressing yolk sac membranes, becomes a thickened, pale, annulate band known as the chorionic girdle due to hyperplasia of the trophoblast cells in that area (Ewart 1897; van Niekerk 1965). Between days 36 and 38 the girdle becomes adhered to the surface of the overlying endometrium by an exocrine secretory material which then allows the majority of the girdle cells to invade deeply into the endometrial stroma (Allen & Moor 1972, Allen *et al.* 1973). Here, they suddenly become sessile, enlarge greatly, begin to secrete the unique equine chorionic gonadotrophin (eCG) and become

clumped together in the endometrial stroma to form a structure known as an endometrial cup (Schauder 1912, Cole & Goss 1943). Collectively, these endometrial cups comprise a circle of pale, raised endometrial protuberances at the base of the gravid horn which, in time, become ulcer-like in appearance due to overgrowth at the periphery and commencing degeneration in the central region of the structure (Clegg *et al.* 1954, Amoroso 1955). They reach their maximum size and hormone secreting capacity between days 55 and 70 of gestation and thereafter become increasingly pale and degenerate in appearance until they are eventually sloughed off the surface of the endometrium between days 90 and 120 (Allen 1975).

eCG reaches the maternal circulation via a complex network of lymph vessels that develop beneath each endometrial cup, and gonadotrophin concentrations in maternal serum mirror the cycle of development and degeneration of the cups. They rise steeply from days

Figure 1 A diagrammatic summary of the principal endocrinological events of pregnancy in the mare.

38–40 to a definite, but individually very variable, peak of 20–200 i.u. eCG/ml serum between days 55 and 70 and thereafter they fall again steadily until gonadotrophic activity disappears entirely from the serum between days 100 and 140 (Cole & Hart 1930, Allen 1969a). eCG is a high molecular weight glycoprotein molecule (Gospodorowicz 1972) that expresses both follicle-stimulating hormone (FSH)-like and luteinising hormone (LH)-like biological activities in a ratio of approximately 1.4:1 (Stewart *et al.* 1976). It binds avidly to gonadal gonadotrophin receptors in most mammalian species but, in the horse itself, it shows a very low rate of binding to gonadal receptors (1–3%) compared with the rates exhibited by the equine pituitary gonadotrophins and the pituitary and chorionic gonadotrophins of other species (Stewart *et al.* 1977). In pregnant mares, the LH-like component of eCG induces ovulation and/or luteinisation of the dominant member of successive waves of follicles which are stimulated to develop in the mare's ovaries throughout the first half of gestation by the continuation of light-driven 10–12 day cyclical releases of pituitary FSH (Evans & Irvine 1975, Urwin & Allen 1982). These accessory luteal structures accumulate in the mare's ovaries between days 40 and 150 of gestation and the progesterone they secrete causes pronounced rises in peripheral serum progesterone concentrations throughout the period (Allen 2001). The process ceases, however, in mid-gestation due to the autumnal decline in FSH release from the pituitary and all the accumulated corpora lutea regress (Amoroso *et al.* 1948, Squires & Ginther 1975).

From around days 60–70, the allantochorion begins to secrete increasing quantities of 5 α -reduced pregnane derivatives of progesterone, the most significant of which are 5 α -pregnane-3,20-dione (5 α -dihydroprogesterone; 5 α DHP), 3 β -hydroxy-5 α -pregnane-20-one (3 β -5P) and 5 α -pregnane-3 β ,20 β -diol ($\beta\beta$ -diol; Holtan *et al.* 1991). By day 90, the allantochorion has expanded to fill the whole uterus and it is interdigitated with the entire luminal surface of the endometrium. The progesteragens it secretes are sufficient in quantity and biological activity to maintain the pregnancy without any further contribution from the maternal ovaries (Holtan *et al.* 1979) and, since the principal organ of supply, the allantochorion, is attached intimately to the target tissue, the endometrium, maternal serum progesteragen concentrations remain fairly low and constant between days 150 and 300 but bear little relationship to the higher concentrations of biologically active hormone at the fetomaternal interface. From around day 300, a further pronounced rise in maternal serum progesteragen concentrations commences (Hamon *et al.* 1991) and the levels remain elevated until 12–24 h before parturition when they fall dramatically to remove the long-standing progesterone block from the myometrium (Rossdale & Silver 1982, Thorburn 1993).

The third endocrinological feature of equine pregnancy is the relatively enormous concentrations of oestrogens that are present in the blood and urine of mares between days 100 and 320 of gestation. They include both the common phenolic oestrogens, oestrone and oestradiol-17 β , and the unusual equine-specific ring β unsaturated oestrogens,

Figure 2 A schematic diagram to show the methods used to create the four types of pregnancy.

equilin and equilenin, all of which stem from placental aromatisation of C-19 precursors secreted by the fetal gonads (Bhavnani *et al.* 1969, 1971, Bhavnani & Short 1973, Pashen & Allen 1979, Tait *et al.* 1983). These enlarge greatly from around day 80 of gestation due to hypertrophy and hyperplasia of the interstitial cells (Hay & Allen 1975) and they reach a combined weight of 300–400 g around days 200–230 (Cole *et al.* 1931, Allen 1970). Thereafter, they regress again steadily to term (Walt *et al.* 1979). The functions of these large quantities of fetoplacental oestrogens produced during the second half of equine pregnancy are not altogether clear although the fetal gonadectomy studies of Pashen and Allen (1979) indicated that, as in sheep (Resnick *et al.* 1974, Pupkin *et al.* 1975), oestrogen production in equine pregnancy may be important to stimulate the development of both endometrial and placental blood vessels to facilitate food and waste product exchange between mother and fetus. They are also likely to play an essential role in stimulating the synthesis and storage of prostaglandin $F_{2\alpha}$ in the myometrium and other uterine tissues (Pashen *et al.* 1982).

Thus, the concept of interdependent cooperation between the fetus, placenta and endometrium, to create the true fetoplacental unit, is clearly very significant in equine pregnancy in order to produce all the gonadotrophic and steroid hormones needed to maintain the

pregnancy state and promote fetal growth. In the present study, the opportunity arose to examine the interacting influences of fetal genotype and maternal uterine environment on these hormone synthetic capabilities of the equine fetoplacental unit by the use of within-breed artificial insemination and between-breed embryo transfer to create four distinct types of equine pregnancy that differed markedly in genetic and size relationships between the mother and the fetus she was carrying.

Materials and Methods

Establishment of the pregnancies

As described by Allen *et al.* (2001), the techniques of between-breed embryo transfer (ET) and within-breed artificial insemination (AI) were used to create totals of 11 Thoroughbred-in-pony (Tb-in-P) pregnancies, in which the genetically large Thoroughbred fetus experienced spatial and nutritional deprivation in the smaller pony uterus ('deprived' group), 8 pony-in-Thoroughbred (P-in-Tb) pregnancies in which, by contrast, the genetically smaller pony fetus was exposed to spatial and nutritional excess *in utero* (the 'luxurious' group), and 8 Tb-in-Tb and 7 P-in-P control groups (Fig. 2). The Thoroughbred mares had a mean $\pm$ S.E.M body weight of 589.6 ± 12.7 kg

($n=14$; range 524–683 kg) at the commencement of pregnancy while the pony mares, consisting of a mixture of Welsh Pony and Welsh Cob-type animals, weighed 327.6 ± 15.8 kg ($n=17$; range 264–537 kg). Donor pony mares were inseminated once with freshly extended semen collected from one of identical twin pony stallions while the Thoroughbred donor mares were all inseminated with extended semen from the same Thoroughbred stallion. Embryos were recovered on day 7 or 8 after ovulation and transferred non-surgically to recipient mares that had ovulated 1–3 days after the donor (Allen 1982) to create the between-breed pregnancies.

Blood sampling and hormone assays

Commencing at the time of initial pregnancy diagnosis by transrectal ultrasonographic examination of the uterus around day 14 after ovulation (Simpson *et al.* 1982), thrice weekly jugular vein blood samples (9 ml) were recovered from every pregnant mare until day 120, and thereafter at weekly intervals until mammary development indicated that parturition was impending, when the collection frequency rose again to once daily until foaling occurred. The samples were collected into disposable 10 ml plastic tubes containing plastic beads (2/10/T; L.I.P. Industries, Shipley, Yorks, UK) and centrifuged within 2 h. The serum was decanted and stored at -20°C until assayed for its concentrations of eCG, progesterone and related progestagens, and total conjugated and unconjugated oestrogens.

eCG assay An amplified enzyme-linked immunoassay (AELIA) was used to measure eCG concentrations in all the serum samples recovered between days 30 and 150 of gestation. The assay employed a polyclonal anti-eCG serum generated in a rabbit (MSII-C: W R Allen, Cambridge) against a highly purified extract of eCG (MSII; 16 000 i.u./mg; M S Stewart, Cambridge) to coat the wells of a 96-well polystyrene microtitre assay plate (F-96 Maxisorb; Life Technologies, Paisley, Glasgow, UK). An eCG-alkaline phosphatase complex was used as the conjugate, 0.2 mM NADPH in 0.9 M diethanolamine acted as the substrate and a mixture of alcohol dehydrogenase, lipoamide dehydrogenase, ethanol and iodonitrotetrazolium violet in a phosphate buffer functioned as the amplifier (Stanley *et al.* 1985). A partially purified extract of eCG (Folligon: Intervet Laboratories, Cambridge, Cambs, UK), that had been calibrated previously against the Second International Reference Preparation for Serum Gonadotrophin (Bangham & Woodward 1966) was used as standard. It was dissolved in phosphate-buffered saline to give concentrations that ranged from 0.5 to 200 i.u. eCG/ml. The assay had a total incubation time of 145 min. It showed a limit of sensitivity of 0.5 i.u. eCG/ml serum and it exhibited intra- and interassay coefficients of variation of, respectively, 8% and 11%.

Oestrogen assay Concentrations of total conjugated and unconjugated oestrogens were measured in the serum samples recovered between day 80 of gestation and foaling using a modification of the single antibody radioimmunoassay developed by Heap and Hamon (1979). The assay employed a polyclonal antiserum (BF461/6) generated in a goat against oestradiol-17 β -17-hemisuccinyl bovine serum albumen. This showed cross reactivities of 100%, 110% and 8% against, respectively, oestrone, oestradiol-17 β and oestradiol-17 α . Although it was not tested against the two ring β unsaturated equine-specific oestrogens, equilin and equilenin (Gaudrey & Glen 1958), the fact that it cross-reacted so completely with both oestrone and oestradiol-17 β and measured concentrations of total oestrogens in the pregnant mare serum samples that were very similar in magnitude to values published previously when using assays that were known to cross-react fully with the ring β unsaturated oestrogens (Cox 1975, Raeside & Liptrap 1975, Pashen & Allen 1979) gave confidence that the measurements made in this study were indeed of total oestrogens that included equilin and equilenin. [2,4,6,7 ^3H]Oestrone, with a specific activity of 3.74 TBq/mmol (TRK 321, Amersham Life Science, Amersham, Bucks, UK) was used as label, and antibody-bound and free oestrogens were separated using sieved dextran-coated charcoal. Oestrone (Sigma-Aldrich, Poole, Dorset, UK), dissolved in ethanol at concentrations of 0.15 to 100 ng/ml, was used as standard and a 1 μl aliquot of each serum sample diluted 1:100 in assay buffer was first hydrolysed by the addition of 100 μl of a 1:10 dilution of *Helix pomatia* sulphatase enzyme (Sigma-Aldrich) before it was extracted with 3 ml peroxide-free diethyl ether (BDH-R&L Slaughter, Stowmarket, Suffolk, UK). The assay showed a limit of sensitivity of 0.2 ng oestrone/ml serum and intra- and interassay coefficients of variation of, respectively, 6% and 9%.

Progestagen assay Concentrations of progesterone and related progestagens were measured in the serum samples collected throughout gestation, using the AELIA assay described by Allen and Sanderson (1987). This employed a monoclonal antibody generated in a mouse against progesterone (No. 11/32; Stanley *et al.* 1985) and bound to the surface of all the wells of a 96-well polystyrene microtitre assay plate (F-96 Maxisorb). Progesterone-alkaline phosphatase conjugate and the same 0.2 mM NADPH substrate and amplifier mixtures used in the eCG assay were also employed. The assay had a total incubation time of 35 min, showed a limit of sensitivity of 0.15 ng progesterone/ml serum and exhibited intra- and interassay coefficients of variation of, respectively, 6% and 8%. The anti-progesterone monoclonal antibody (11/32) showed cross reactivities of 100% with 5 β -pregnane-3,20-dione (5 β dihydropregesterone; 5 β DHP), 79% with 5 α DHP, 56% with 11 α -hydroxyprogesterone, 134% with 5 β -pregnandione and 18% with 5 α -pregnandione (Stanley

et al. 1986, Taussig *et al.* 1987). Since these 5 α - and 5 β -reduced pregnane derivatives are present in high concentrations in the peripheral blood of mares during the second half of pregnancy (Hamon *et al.* 1991, Holtan *et al.* 1991), the values obtained were considered to be of total progestagens, rather than progesterone alone.

Statistical analyses

The progestagen and total conjugated oestrogen measurements were analysed statistically (Sigma-Stat, v. 2.0; SPSS Inc, Chicago, IL, USA) using two-way repeated measures ANOVA followed by the Tukey test.

Results

Birth of the foals

One of the Tb-in-P and one of the P-in-Tb conceptuses were aborted spontaneously, one for no discernible reason and the other because of a twisted umbilical cord, at 299 and 275 days of gestation respectively. One Thoroughbred mare carrying a control Thoroughbred fetus was shot humanely on day 149 when it accidentally broke a hind leg. The results of the eCG measurements on these 3 animals were included in the analyses, but not in the overall mean progestagen or total conjugated oestrogen results. Two of the Tb-in-P foals were stillborn at, respectively, days 328 and 335 of gestation. The first of these was asphyxiated by the unruptured amnion when the mare foaled unattended in the paddock, and the other died during dystochia that required vigorous manual correction. The results of all three hormone measurements for these two animals are included in the statistical analyses.

The remaining 8 Tb-in-P, 7 P-in-Tb, 7 Tb-in-Tb and 7 P-in-P foals were born alive spontaneously between days 314 and 348 of gestation. As described by Allen *et al.* (2001), the Tb-in-P foals exhibited varying degrees of muscle wastage on the upper limbs and body and other signs of prematurity (Rossdale & Silver 1982). They required assistance to stand and suck during the first 24 h *post partum* only, but thereafter progressed normally. In contrast, the P-in-Tb foals were all physically and behaviourally mature and required no assistance whatsoever. The placenta was recovered intact from each mare at the end of spontaneous third stage labour. The weight, volume and gross surface area of the allantochorion were positively correlated with foal bodyweight at birth. Details of these placental parameters and the weights of the mares and newborn foals are described by Allen *et al.* (2001).

Hormone secretion patterns

eCG The individual eCG profiles exhibited during the first half of gestation by 35 mares from the four pregnancy

groups are shown in Fig. 3. eCG first became detectable in maternal blood between days 35 and 40 after ovulation, as has been described by Cole and Hart (1930), Allen (1969a) and others. Concentrations of the hormone then rose steeply to reach individually variable peaks of between 9 and 220 i.u./ml between days 51 and 69, after which levels declined again steadily until gonadotrophic activity disappeared from the serum between days 88 and 138. The profiles tended to rise and fall more steeply, reach higher peak concentrations and show greater individual variation in the smaller pony mares than in the larger Thoroughbreds, regardless of the type of fetus being carried (Fig. 3). Clearly, the amount of eCG produced was dependent upon the breed of the mare rather than the breed of the fetus.

Oestrogens The mean $\pm$ S.E.M. serum total oestrogen profiles of the 4 types of pregnancy, meaned and presented in relation to the stage of pregnancy to highlight the position of the mid-gestational peak, are shown in Fig. 4. In all the mares, concentrations began to increase rapidly from around day 100 of gestation to reach a variable peak between days 138 and 205; the mean peak concentrations in the Tb-in-P group (1411 ng/ml) occurred significantly later in gestation than the mean peak value of 1174 ng/ml in the P-in-P group (Fig. 3; 205 versus 138 days, $P=0.02$). Thereafter, levels declined again steadily to a nadir just before foaling. There was no statistical difference between the two Tb mare groups for the gestational age at which mean peak serum oestrogen concentrations were reached. However, as with the eCG profiles, marked individual variations existed in peak values attained, both within and between the groups. For example, maximum concentrations ranged from 844 to 1613 ng/ml in the P-in-Tb mares and from 980 to 2511 ng/ml in the Tb-in-P mares. Statistical analysis showed significant differences. Namely, the Tb-in-Tb profile differed significantly from the profiles of both the P-in-P ($P=0.014$) and the P-in-Tb ($P=0.046$) groups. In contrast to eCG production, the breed of the fetus did appear to influence maternal serum oestrogen concentrations in that the mean profiles exhibited when a Thoroughbred fetus was being carried, regardless of the breed of the mother in which it was gestating, were higher than the mean profiles produced when a pony fetus was *in utero*. Furthermore, measurement of the area under each curve similarly revealed that the mean total amounts of oestrogen produced by the two types of pregnancy in which a Thoroughbred fetus was being carried (i.e. Tb-in-Tb and Tb-in-P) was significantly greater ($P=0.003$) than that produced by the two types of pregnancy in which a pony fetus was being carried (P-in-P and P-in-Tb).

Progestagens The mean $\pm$ S.E.M. peripheral serum progestagen concentrations exhibited by the 4 groups of mares, meaned and presented in relation to foaling to

Figure 3 Individual eCG profiles measured in the serum of the Thoroughbred and pony mares carrying the four types of pregnancy between 30 and 150 days of gestation.

highlight the size and duration of the pre-parturient rise, are shown in Fig. 5. The differences between the groups were even greater than the oestrogen profiles, especially between the P-in-Tb and Tb-in-P experimental groups. Here also, the breed of the fetus, rather than that of the mother, appeared to be the controlling factor, i.e. TB fetuses tended to be associated with higher values than P fetuses. In the P-in-Tb (luxurious *in utero*) group mean progestagen concentrations were significantly lower than in all the other mare groups ($P < 0.001$); concentrations remained low and steady around 6–12 ng/ml for the majority of pregnancy and only began to rise rapidly after day 310 to reach a pre-foaling peak of only 16–18 ng/ml. In contrast, the mean progestagen concentration in the Tb-in-P pregnancies (deprived *in utero* group) was significantly higher ($P < 0.001$) and reached > 25 ng/ml by as early as day 200. Thereafter, progestagen concentrations continued to rise steadily to reach values of ≥ 30 ng/ml during the last 20 days pre-partum (Fig. 5). The mean serum progestagen concentrations exhibited by the two control groups of mares were relatively similar and midway between those of the two experimental groups. Mean progestagen concentrations in the Tb-in-Tb group tended to be higher than in the P-in-P group. The latter group

had an earlier pre-partum rise but higher mean values were achieved by the Tb-in-Tb mares during the last 20 days pre-partum.

Discussion

The amounts and patterns of secretion of the 3 major fetoplacental unit hormones measured in the 4 types of pregnancy studied in this experiment showed some interesting differences. In the case of the chorionic gonadotrophin, eCG, differences between individual mares within each group were very marked, both in the peak concentration reached and in the duration of eCG activity in the blood. As highlighted previously (Allen 1969*a*), this variability was more marked in the pony mares than the Thoroughbred mares, irrespective of the breed of foal being carried. For example, in the Tb-in-P group, one mare showed a peak serum eCG concentration of only 9 i.u./ml on day 46 of gestation and eCG had become undetectable again by as early as day 65. By contrast, in another mare in the same group, a peak of 218 i.u. eCG/ml was recorded on day 69 and gonadotrophin was still measurable in her blood as late as day 128 (Fig. 3).

Figure 4 Comparison of the mean total oestrogens profiles measured in the four types of pregnancy between day 80 of gestation and term. □ = Tb-in-Tb; ○ = P-in-P; ■ = Tb-in-P; ● = P-in-Tb.

Figure 5 Comparison of the mean serum progesterone profiles measured in the four types of pregnancy during the second half of gestation. □ = Tb-in-Tb; ○ = P-in-P; ■ = Tb-in-P; ● = P-in-Tb. Note the big difference in levels between the Tb-in-P and P-in-Tb experimental pregnancies and the earlier and more pronounced rise in the former.

Even in the Tb-in-Tb and P-in-Tb groups, in which the degree of individual variation was less pronounced, peak serum eCG concentrations ranged from 12 to 86 i.u./ml and the time of disappearance of gonadotrophic activity ranged from days 78 to 142 of gestation (Fig. 3).

The amount of eCG produced by an individual mare depends upon the total volume of endometrial cup tissue that develops in her uterus (Allen 1975) and this, in turn,

is controlled by the width and general development of the progenitor chorionic girdle and how much of this tissue manages to invade the maternal endometrium at day 36 to form the endometrial cups (Allen & Stewart 1992, Enders *et al.* 1996). Mare parity and age (Day & Rowlands 1947), fetal genotype (Bielanski *et al.* 1955, Allen 1969b) and uterine environment (Allen *et al.* 1993) have all been implicated as exerting marked effects upon the develop-

ment and productivity of the endometrial cups and, in the mares in the present study, it is likely that an interaction between the genotype (breed) of the fetus and the degree of folding and rugae formation in the endometrium of the genetic or surrogate mare would have exerted the biggest influence on the amount of endometrial cup that could develop and secrete its hormone into the maternal circulation (Allen & Stewart 1992).

Peripheral serum total oestrogen concentrations in all the mares rose steadily after day 100 of gestation to reach a well defined, but individually variable, peak between days 138 and 205. Although analysis of the mean curves showed no significant differences in peak concentration between the 4 types of pregnancy, measurement of the area under each curve did show significant differences, with the amount of oestrogen being produced by the two types of pregnancy in which a Thoroughbred fetus was being carried (i.e. Tb-in-Tb and Tb-in-P) being greater than in the pregnancies with a pony fetus (P-in-P and P-in-Tb). This difference might have had some basis in the smaller blood volumes of the pony compared with the Thoroughbred, at least with regard to the higher levels of oestrogens in the Tb-in-P compared with the P-in-Tb group. However, it is more appealing to suppose that it may have been related to differences in size and/or synthetic capacity of either the fetal gonads or the allanto-chorion, the two essential components of the fetoplacental unit that are involved in oestrogen synthesis (Bhavnani & Short 1973, Pashen & Allen 1979). It seems reasonable to suggest that the bigger Thoroughbred fetus may develop bigger gonads than its smaller pony counterpart. Furthermore, the fact that the placenta was smaller in every respect in the Tb-in-P pregnancies than in the P-in-Tb equivalents (Allen *et al.* 2001), despite the higher mean plasma oestrogen concentrations in the former group, supports the speculation that it was the size and precursor productivity of the fetal gonads, rather than the size of the placenta or volume of maternal blood, that accounted for the higher mean oestrogen levels measured in the Tb-in-Tb and Tb-in-P pregnancies (Fig. 4).

Even more marked differences in mean peripheral serum progesterone profiles were exhibited by the 4 types of pregnancy. In the P-in-Tb group, in which the genetically small pony fetus in the large Thoroughbred surrogate uterus could reasonably be assumed to have been the least stressed of the 4 types of foals, progesterone concentrations were significantly lower ($P < 0.01$) throughout the second half of pregnancy than in the other 3 types and the pre-parturient rise occurred latest in gestation. In contrast, in the Tb-in-P group, in which there was likely to be substantial fetal stress due to restricted placental development and fetal growth (Allen *et al.* 2001), mean progesterone concentrations began to rise as early as day 180 of gestation and reached higher values than all the other groups (>25 ng/ml) for the remainder of gestation (Fig. 5). The profiles of the two control groups remained

between these two experimental extremes, and, as demonstrated previously by Rossdale *et al.* (1991), concentrations reached higher values during the pre-parturient period in the Tb-in-Tb than in the P-in-P pregnancies (Fig. 5).

The experiments of Bhavnani *et al.* (1969, 1971) involving the infusion of labelled steroid precursors into the umbilical circulation of pregnant mares, showed that the allantochorion utilises maternal sources of cholesterol to synthesise progestagens between days 100 and 300 of gestation. More recently, Holtan *et al.* (1991) and Hamon *et al.* (1991) demonstrated that pregnenolone produced from cholesterol is secreted by the fetus and converted to progesterone and 5α -reduced progestagens by the allanto-chorion and maternal endometrium. Pregnenolone is thought to be produced by the fetal adrenal gland rather than the fetal gonads because the adrenal tissue exhibits P450 SCC enzyme activity (Han *et al.* 1995) and because fetal gonadectomy does not affect maternal progestagen concentrations (Pashen & Allen 1979). The pre-partum rise in maternal progestagens is also thought to be due to increased fetal pregnenolone production (J C Ousey and A L Fowden, unpublished observations). Fetal stress caused by adrenocorticotrophin administration, or as a result of conditions affecting the placenta (e.g. placentitis) causes a precocious increase in maternal progestagen concentrations and delivery of premature foals which have low body weight but normal adrenocortical activity (Rossdale *et al.* 1991, Ousey *et al.* 1998). Thus, fetal adrenal maturation increases maternal plasma progestagen concentrations in mares in which the fetus is stressed. In the present study, therefore, it is entirely logical that maternal progestagen concentrations in mid-pregnancy were lowest, and the pre-parturient rise least pronounced, in the P-in-Tb pregnancies in which the fetuses were least stressed and in which fetal growth requirements could be easily met in terms of uterine space and placental function. Conversely, the Tb-in-P fetuses were likely to be highly stressed because the genetic drive for fetal growth and development would have exceeded the capacity of the uterus and the exchange potential of the smaller placenta from early in gestation. Consequently, this fetal stress, in turn, probably stimulated increased pregnenolone production, resulting in high maternal serum progestagen levels in the surrogate pony mares.

These marked differences in intrauterine stress experienced by the Tb-in-P versus the P-in-Tb fetuses no doubt contributed appreciably to the significant differences in mean gestation length between the 4 types of pregnancy. Gestation length is remarkably variable in the horse such that anywhere from 315 to 360 days is widely considered to be within normal limits for this species (Rossdale & Short 1967). It is influenced by season to the extent that pregnancy tends to last longer in mares that conceive early in the breeding season and, *vice versa*, to be shorter in mares mated later in the year (Hammond 1940). Breed also has

an effect and Rossdale *et al.* (1991) recorded the mean gestation period as being shorter at around 331 days in pony mares compared with the mean period of 341 days in larger Thoroughbred mares. This effect of breed was evident in the present results, with the mean $\pm$ s.e.m. of 324.6 ± 3.0 days in the 7 P-in-P pregnancies being significantly shorter ($P < 0.001$) than the mean $\pm$ s.e.m. of 338 ± 6.9 days exhibited by the 7 Tb-in-Tb pregnancies (Allen *et al.* 2001). It was interesting that the mean gestation length of the two experimental groups, the 'deprived' Tb-in-P pregnancies at 331.6 ± 2.8 days and the 'luxurious' P-in-Tb pregnancies at 333.1 ± 3.0 days, were similar to each other and were shorter and longer respectively than the gestational ages of the respective control groups. It may be hypothesised that the increased stress of spatial and nutritional limitations suffered by the Tb-in-P foals initiated earlier maturation of the fetal adrenal gland, as reflected by the substantially higher maternal serum progestagen concentrations, with the consequential shortening of the gestation period compared with that of the Tb-in-Tb controls, and the birth of weaker, slightly dysmature foals. In contrast, the relative lack of spatial and nutritional stress experienced by the P-in-Tb foals, as reflected by lower maternal serum progestagen levels, may have diminished the fetal signals for readiness for birth and adrenal maturation with the result that gestation was prolonged compared with the P-in-P controls.

In summary, the results of this experiment portrayed convincingly the complex relationships that exist between the uterus, placenta and fetus in the synthesis and secretion of the hormones necessary to maintain the pregnancy state in equids. Fetal, and possibly also maternal, nutritional stress can exert a strong influence on endocrine profiles but it is nonetheless biologically fascinating how both the maternal and fetal organisms, through alterations in placental development, can circumvent or accommodate such stresses to produce a viable offspring at or close to term.

Acknowledgements

This study was kindly sponsored by The Horserace Betting Levy Board (project no. 653) and the Thoroughbred Breeders' Association. The authors are grateful to Dr Alison Forhead for help and advice with the statistical analyses and to Miss Caroline Turnbull and Mr Edward Sear for expert assistance with the management of the mares and their foalings.

References

- Allen WR 1969a A quantitative immunological assay for pregnant mare serum gonadotrophin. *Journal of Endocrinology* **43** 581–591.
- Allen WR 1969b Factors influencing pregnant mare serum gonadotrophin production. *Nature* **223** 64–66.
- Allen WR 1970 Equine gonadotrophins. PhD thesis. University of Cambridge.
- Allen WR 1975 The influence of fetal genotype upon endometrial cup development and PMSG and progestagen production in equids. *Journal of Reproduction and Fertility* **23** (Suppl) 405–413.
- Allen WR 1982 Embryo transfer in the horse. In *Mammalian Egg Transfer*, pp 135–154. Ed. CE Adams. Florida: CRC Press.
- Allen WR 2001 Fetomaternal interactions and influences during equine pregnancy. *Reproduction* **121** 513–527.
- Allen WR & Moor RM 1972 The origin of the equine endometrial cups. I. Production of PMSG by fetal trophoblast cells. *Journal of Reproduction and Fertility* **29** 313–316.
- Allen WR & Sanderson MW 1987 The value of a rapid progesterone assay (AELIA) in equine stud veterinary medicine and management. *Proceedings of the 9th Bain-Fallon Memorial Lectures*, pp 75–82. Sydney: Australian Equine Veterinary Association.
- Allen WR & Stewart F 1992 Equine chorionic gonadotrophins. In *Equine Reproduction*, pp 81–96. Eds AO McKinnon & JL Voss. Philadelphia: Lea and Febiger.
- Allen WR, Hamilton DW & Moor RM 1973 The origin of the equine endometrial cups. II. Invasion of the endometrium by trophoblast. *Anatomical Record* **177** 485–502.
- Allen WR, Skidmore JA, Stewart F & Antczak DF 1993 Effects of fetal genotype and uterine environment on placental development in equids. *Journal of Reproduction and Fertility* **97** 55–60.
- Allen WR, Wilsher S, Turnbull C, Stewart F, Ousey J, Rossdale PD & Fowden A 2001 The influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development *in utero*. (In Press).
- Amoroso EC 1955 Endocrinology of pregnancy. *British Medical Bulletin* **11** 117–125.
- Amoroso EC, Hancock JL & Rowlands IW 1948 Ovarian activity in the pregnant mare. *Nature* **161** 355–356.
- Bangham DR & Woodward PM 1966 The second international standard for serum gonadotrophin. *Bulletin of the World Health Organisation* **35** 761–773.
- Bhavnani BR & Short RV 1973 Formation of steroids by the pregnant mare. IV. Metabolism of ^{14}C -mevalonic acid and ^3H -dehydro-isoandrosterone injected into the fetal circulation. *Endocrinology* **92** 1397–1404.
- Bhavnani BR, Short RV & Solomon S 1969 Formation of oestrogens by the pregnant mare. I. Metabolism of $7\text{-}^3\text{H}$ -dehydro-isoandrosterone and $4\text{-}^{14}\text{C}$ -androstenedione injected into the umbilical vein. *Endocrinology* **85** 1172–1179.
- Bhavnani BR, Short RV & Solomon S 1971 Formation of estrogens by the pregnant mare. II. Metabolism of ^{14}C -acetate and ^3H -cholesterol injected into the fetal circulation. *Endocrinology* **89** 1152–1157.
- Bielanski W, Ewy Z & Pigoniowa H 1955 Differences in endocrine secretion of mares pregnant with stallion or jack. *Bulletin of the Polish Academy of Sciences Class II Biological Science Series* **3** 37–39.
- Clegg MT, Boda JM & Cole HH 1954 The endometrial cups and allanto-chorionic pouches in the mare with emphasis on the source of equine gonadotrophin. *Endocrinology* **54** 448–463.
- Cole HH & Hart GH 1930 The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. *American Journal of Physiology* **93** 57–68.
- Cole HH & Goss H 1943 The source of equine gonadotropin. In *Essays in Honor of Herbert M Evans*, pp 107–119. Berkeley: University of California Press.
- Cole HH, Howell CE & Hart GH 1931 The changes occurring in the ovary of the mare during pregnancy. *The Anatomical Record* **49** 199–209.
- Cox JE 1975 Oestrone and equilin in the plasma of the pregnant mare. *Journal of Reproduction and Fertility* **23** (Suppl) 463–468.
- Day FT & Rowlands IW 1947 Serum gonadotrophins in Welsh and Shetland ponies. *Journal of Endocrinology* **5** 1–8.

- Enders AC, Meadows S, Stewart F & Allen WR 1996 Failure of endometrial cup development in the donkey-in-horse model of equine abortion. *Journal of Anatomy* **188** 575–589.
- Evans MJ & Irvine CHG 1975 Serum concentrations of FSH, LH and progesterone during the oestrous cycle and early pregnancy in the mare. *Journal of Reproduction and Fertility* **23** (Suppl) 193–200.
- Ewart JC 1897 *A Critical Period in the Development of the Horse*. London: Adam and Charles Black.
- Gaudry R & Glen WL 1958 Sur la fraction stéroïde de l'urine de la jument gravide. *Etude Belgique* **2** (Suppl) 435–440.
- Gospodorowicz D 1972 Purification and physicochemical properties of the pregnant mare serum gonadotrophin (PMSG). *Endocrinology* **91** 101–106.
- Hammond J 1940 Fertility and growth: Horses. In *Farm Animals. Their Breeding, Growth and Inheritance*, pp 16–35. London: Edward Arnold and Co.
- Hamon M, Clarke SW, Houghton E, Fowden AL, Silver M, Rossdale PD, Ousey JC & Heap RB 1991 Production of 5 α -dihydroprogesterone during late pregnancy in the mare. *Journal of Reproduction and Fertility* **44** (Suppl) 529–535.
- Han X, Fowden AL, Silver M, Holdstock N, McGladdery AJ, Ousey JC, Allen WR, Rossdale PD & Challis JRG 1995 Immunohistochemical localisation of the steroidogenic enzymes phenylethanolamine-n-methyl-transferase (PNMT) in the adrenal gland of the fetal and newborn foal. *Equine Veterinary Journal* **27** 140–146.
- Hay MF & Allen WR 1975 An ultrastructural and histochemical study of the interstitial cells in the gonads of the fetal horse. *Journal of Reproduction and Fertility* **23** (Suppl) 557–561.
- Heap RB & Hamon M 1979 Oestrone sulphate in milk as an indication of a viable conceptus in cows. *British Veterinary Journal* **135** 355–365.
- Holtan DW, Squires EL, Lapin DR & Ginther OJ 1979 Effect of ovariectomy on pregnancy in mares. *Journal of Reproduction and Fertility* **27** (Suppl) 457–463.
- Holtan DW, Houghton E, Silver M, Fowden AL, Ousey J & Rossdale PD 1991 Plasma progesterone in the mare fetus and newborn foal. *Journal of Reproduction and Fertility* **44** (Suppl) 517–528.
- van Niekerk CH 1965 The early diagnosis of pregnancy, the development of foetal membranes and nidation in the mare. *Journal of South African Veterinary Medical Association* **36** 483–488.
- Ousey JC, Rossdale PD, Dudan FE & Fowden AL 1998 The effects of intrafetal ACTH administration on the outcome of pregnancy in the mare. *Reproduction, Fertility and Development* **10** 359–367.
- Pashen RL & Allen WR 1979 The role of the fetal gonads and placenta in steroid production, maintenance of pregnancy and parturition in the mare. *Journal of Reproduction and Fertility* **27** (Suppl) 499–509.
- Pashen RL, Sheldrick EL, Allen WR & Flint APF 1982 Dehydro-epiandrosterone synthesis by the fetal foal and its importance as an oestrogen precursor. *Journal of Reproduction and Fertility* **32** (Suppl) 389–397.
- Pupkin MJ, Schomberg DW, Nagey DA & Crenshaw C 1975 Effects of exogenous dehydroepiandrosterone upon fetoplacental biosynthesis of estrogens and its effect upon uterine blood flow in the term pregnant ewe. *American Journal of Obstetrics and Gynecology* **121** 227–232.
- Raesside JI & Liptrap RM 1975 Patterns of urinary oestrogen excretion in individual pregnant mares. *Journal of Reproduction and Fertility* **23** (Suppl) 469–475.
- Resnik R, Killam AP, Battaglia FC, Makowski EL & Meschia G 1974 The stimulation of uterine blood flow by various oestrogens. *Endocrinology* **94** 1192–1196.
- Rossdale PD & Short RV 1967 The time of foaling in thoroughbred mares. *Journal of Reproduction and Fertility* **13** 341–343.
- Rossdale PD & Silver M 1982 The concept of readiness for birth. *Journal of Reproduction and Fertility* **32** (Suppl) 507–510.
- Rossdale PD, Ousey JC, Cottrill J, Chavatte P, Allen WR & McGladdery 1991 Effects of placental pathology on maternal plasma progesterone and mammary secretion calcium concentrations and on neonatal adrenocortical function in the horse. *Journal of Reproduction and Fertility* **44** (Suppl) 579–590.
- Schauder W 1912 Untersuchungen über die Eithäute und Embryotrophie des Pferdes. *Archives of Anatomie und Physiology* 259–302.
- Simpson DJ, Greenwood RES, Ricketts SW, Rossdale PD, Sanderson M & Allen WR 1982 Use of ultrasound echography for early diagnosis of single and twin pregnancy in the mare. *Journal of Reproduction and Fertility* **32** (Suppl) 431–439.
- Squires EL & Ginther OJ 1975 Follicular and luteal development in pregnant mares. *Journal of Reproduction and Fertility* **23** (Suppl) 429–433.
- Stanley CJ, Johannsson A & Self CH 1985 Enzyme amplification can enhance both the speed and the sensitivity of immunoassays. *Journal of Immunological Methods* **83** 89–95.
- Stanley CJ, Paris F, Webb AE, Heap RB, Ellis ST, Hamon M, Worsfield A & Booth JM 1986 Use of a new and rapid milk progesterone assay to monitor reproductive activity in the cow. *Veterinary Record* **118** 664–667.
- Stewart F, Allen WR & Moor RM 1976 Pregnant mare serum gonadotrophin: ratio of follicle-stimulating hormone and luteinizing hormone activities measured by radioreceptor assay. *Journal of Endocrinology* **71** 371–382.
- Stewart F, Allen WR & Moor RM 1977 Influence of foetal genotype on the follicle-stimulating hormone:luteinizing hormone ratio of pregnant mare serum gonadotrophin. *Journal of Endocrinology* **73** 419–425.
- Tait AD, Santikarn LC & Allen WR 1983 Identification of 3 β -hydroxy-5,7 pregnandien-20-one and 3 β -hydroxy-5,7 androstadien-17-one as endogenous steroids in the foetal horse gonad. *Journal of Endocrinology* **99** 87–92.
- Taussig MJ, Deverson E, Ellis S, Brown M, Wang M-W, Heap RB & Fenistein A 1987 Antiprogesterone antibodies: specificity, structure, idiotypes and anti-idiotypic manipulation. *Monographs in Allergy* **22** 89–98.
- Thorburn GD 1993 A speculative view of parturition in the mare. *Equine Veterinary Journal* **14** (Suppl) 41–49.
- Urwinn VE & Allen WR 1982 Pituitary and chorionic gonadotrophin control of ovarian function during early pregnancy in equids. *Journal of Reproduction and Fertility* **32** (Suppl) 371–382.
- Walt ML, Stabenfeldt JP, Hughes DP, Neely DP & Bradbury R 1979 Development of the equine ovary and ovulation fossa. *Journal of Reproduction and Fertility* **27** (Suppl) 471–477.

Received 30 July 2001

Accepted 4 October 2001