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Steroids in the establishment and maintenance of pregnancy and at parturition in the mare

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Abstract

Historically, studies on the endocrinology of pregnancy and parturition in horses have made major contributions of relevance to mammals in general. Recent use of liquid chromatography mass spectrometry, measuring multiple steroid hormones simultaneously in blood, foetal and placental tissues throughout normal gestation, and in mares with experimentally induced placentitis, has advanced our current understanding of many of the unusual strategies seen during gestation and at foaling. This includes the stimulation of luteal steroidogeneisis by equine chorionic gonadotropin (eCG) from the endometrial cups, resulting in additional androgen and oestrogen secretion. Progesterone declines as the endometrial cups and eCG disappears, replaced by 5α-dihydroprogesterone (DHP), a potent equine progesterone receptor (PR) agonist, as the chorioallantoic placenta develops. Placental steroidogenesis thereafter is influenced by foetal pregnenolone and dehydroepiandrosterone secretion, providing substrate for 5α-pregnane and oestrogen synthesis, an unusual example of a 'foeto-placental unit'. Foetal gonadal dehydroepiandrosterone fuels placental oestrone sulphate secretion, peaking at higher concentrations in mares than any other species known, declining steadily thereafter to term. Additional 5α-reduced (DHP) metabolites increase from mid-gestation to peak concentrations 3–5 days before foaling, declining prepartum, most likely as a result of selective loss of placental SRD5A1 (5α-reductase) expression and activity. Similar changes occur in mares with experimentally induced placentitis, which is also associated with a decreased ratio of equine PR-B:PR-A in myometrium, suggesting that progestin withdrawal is both systemic (pregnanes) and local (receptor-dependent) in mares. In addition, some steroids detected during equine pregnancy by immuno-assay are not detected by mass spectrometry, further illustrating the immense value of this technology.

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Introduction

By some estimates, our knowledge and understanding of the endocrinology of mammalian pregnancy and parturition has been barely 100 years in process but, however calculated, studies in horses have figured centrally in the progress made from the very beginning. The endocrinology of pregnancy in the horse is complex and often regarded as exhibiting characteristics that are unique to the equids. Perhaps, it is more correct to consider that horses utilize many strategies employed by other species, but few of them utilize as many as are found in horses (Conley 2016). Recent studies have generated a far more detailed picture of the endocrinology of pregnancy in horses, perhaps more than any other species and these events are reflected in dynamic changes in steroid profiles that can be traced in systemic blood (Holtan *et al.* 1991, Legacki *et al.* 2016*b*). The endocrinology of pregnancy and parturition in the mare was recently reviewed (Conley 2016). However, additional data in the past few years have added to existing literature particularly with respect to the contributions of the foetal adrenal glands, foetal gonads and placenta to steroid secretion in mid-to-late gestation and parturition. The opportunity will be taken to integrate these most recent publications into a more contemporary understanding of the unusual endocrinology of pregnancy and parturition in horses. In addition, progress has been made in the endocrine changes that precede the disruption of pregnancy in studies using experimental induction of ascending placentitis in mares and these findings will also be included in the current review.

Historical background

In the second edition of the classic text 'The Physiology of Reproduction' by Marshall, published in 1922 (Marshall 1922) he writes, 'What constitutes the original

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stimulus for the changes that occur in pregnancy remains still outside our ken … At least the influence of the cerebrum is not all-important …. We are thus forced to conclude that the phenomena of pregnancy and parturition are brought about by chemical stimuli acting through the blood-stream'. Endocrinology was in its adolescence, much would change in the ensuing decade and a half (Parkes 1966), and studies in the horse played a prominent role at a time when hormones were identified by their bioactivity (Frank *et al.* 1925, Smith & Engle 1927, Corner & Allen 1929). It was logical then that the discovery of a gonadotrophic activity in the urine of pregnant women (Aschheim & Zondek 1927) would lead to similar studies in other species. Indeed, Cole and Hart reported finding gonadotrophic activity in serum from pregnant mares that first appeared between days 37 and 42 of gestation and remained detectable until about day 100, stimulating an increase in ovarian weight when injected into immature rats and mice (Cole & Hart 1930*a*). Later in pregnancy, sera were found to stimulate uterine and vaginal responses but to inhibit ovarian growth (Cole & Hart 1930*b*) and the urine of pregnant mares became a major source for hormone purification and characterization. In itself, this suggests similarities in the endocrinology of equine and human pregnancy, as discussed previously (Conley 2016).

Progesterone and 5α-dihydroprogesterone (DHP), bioactive progestins

Progesterone is the hormone required to establish pregnancy in mammals (Davies & Ryan 1972), but it is special among steroids for a variety of other reasons. It is generally recognized as being the only endogenous bioactive steroid in the class known as progestins. For instance, among other steroid classes, oestrogens (oestradiol, oestrone, oestriol), androgens (testosterone, dihydrotestosterone, 19nor-androstenedione) and corticoids (cortisol, corticosterone, 11-deoxycortisol), multiple endogenous steroids exist within each class, all having varying biopotencies at their various classical nuclear receptors, the oestrogen, androgen and glucocorticoid receptors, respectively. In contrast, the nuclear progesterone receptor (PR) is named not after the steroid class, but it is named after progesterone as the only recognized bioactive member of the progestins. Progesterone (pregn-4-ene,3,20-dione) is unusual in being one of the few bioactive steroids that lacks a hydroxyl group. While steroids in all other classes require a hydroxyl group to be bioactive, hydroxylation of progesterone essentially eliminates bioactivity (Kontula *et al.* 1975). Lacking a hydroxyl group, progesterone cannot be sulphated (physiologically) nor can it be esterified (chemically) to both enhance solubility for injection and increase serum half-life as a therapeutic agent. The advent of methods to analyse progesterone chemically (Short 1956) rather than biologically led to studies investigating concentrations in tissues including blood (Short 1959). Though progesterone could be detected in equine placenta, it could not be detected in equine blood in mid-to-late gestation (Short 1959). Appearing to be unique among species studied in this regard, the possible existence in horses of an alternative progestin other than progesterone was proposed (Short 1960).

Hormone assays continued to be refined, and the introduction of protein-binding and immune-based methods in particular allowed estimates to be made with greater sensitivity in even smaller sample volumes (Behrman 1988). Both approaches however suffer from potential cross-reactivity with multiple analytes, depending entirely on the relative specificity of the binding protein or primary antisera used and the concentrations of cross-reactants in samples of relevance. This can be a significant problem, progesterone representing a case in point (Wynn *et al.* 2018*c*), requiring chromatography or other means to minimize the potential error introduced by cross-reacting metabolites or other related steroids. This proved to be a particular problem when trying to measure progesterone in pregnant mares. Careful analysis employing chromatography before immunoassay was therefore required to confirm the earlier observations (Short 1959) concerning the relative loss of progesterone in the serum of pregnant mares from mid-to-late gestation (Holtan *et al.* 1975*b*,*c*). The cross-reacting steroids were identified as 5α-reduced metabolites of progesterone (Holtan *et al.* 1975*a*), including 5α-dihydroprogesterone (DHP; Atkins *et al.* 1976) A more complete analysis of multiple 5α-reduced pregnanes in mares sampled longitudinally throughout gestation was conducted using gas chromatography mass spectrometry in what remains a landmark paper (Holtan *et al.* 1991). Among the multiple 5α-reduced pregnanes, some present in exceedingly high concentrations, DHP has been presumed to be the most physiologically relevant based on results of competitive binding assays. Using equine endometrium (Jewgenow & Meyer 1998), uterus and mammary gland (Chavatte-Palmer *et al.* 2000) as presumptive sources of equine PR, DHP was shown to displace radio-labelled progesterone from extracts of these tissues at almost equal concentrations. But attempts to demonstrate actual bioactivity have not been successful. Neither progesterone nor DHP affected the contractile activity of equine myometrial strips *in vitro* (Ousey *et al.* 2000*a*) for instance. Therefore, despite progress in identifying multiple metabolites of progesterone, the physiological relevance of DHP as a progestin of importance in horses remained a matter of speculation.

Resolution of the role of DHP as a physiologically relevant progestin, perhaps the only endogenous progestin with significant bioactivity other than progesterone itself, was confirmed in a series of *in vivo*

and *in vitro* experiments (Scholtz *et al.* 2014). DHP was shown to stimulate endometrial gland development and the expression of progesterone responsive genes in ovariectomized mares after 10 days of administration of a preparation that achieved less than 4 ng/mL in systemic blood. The real test of progestagenic activity is the maintenance of pregnancy (Glasser 1975). Pregnancy was maintained during DHP treatment for almost 2 weeks after luteolysis was induced at 14 days of gestation (Scholtz *et al.* 2014). The relative biopotency of progesterone compared to DHP was demonstrated *in vitro* using a steroid-responsive luciferase reporter plasmid co-transfected with an equine PR expression plasmid into HepG2 cells which were subsequently treated with increasing concentrations of the pregnanes. Parallel experiments were conducted with a human PR expression construct. Progesterone and DHP were equipotent agonists of the equine PR but progesterone was five-fold more potent than DHP in assays with the human PR. Moreover, increased responsiveness of the equine PR to DHP appeared to be associated with decreased biopotency of progesterone at the equine PR compared to the human PR (Scholtz *et al.* 2014). The increased binding of DHP in the ligand-binding domain of the equine PR appears to be due to a G→A substitution at amino acid 722 which is also shared by the elephant PR (Wierer *et al.* 2012). Whether or not this single amino acid substitution is sufficient and predictive of the physiological relevance of DHP in other species in unknown at this time. Although the equine placenta exhibits high levels of 5α-reductase activity (Legacki *et al.* 2018) and the ability to synthesize DHP and other 5α-reduced metabolites (Moss *et al.* 1979, Hamon *et al.* 1991), DHP is present in cyclic mares and progesterone is rapidly 5α-reduced systemically (Conley *et al.* 2018*b*) and, contrary to conclusions drawn from previous observations (Schutzer & Holtan 1996), cannot be synthesized directly from pregnenolone (Raeside *et al.* 2015, Corbin *et al.* 2016). In any case, DHP is not just a progestin of significance during pregnancy but also during the luteal phase in cyclic mares.

Stimulation of steroid secretion by chorionic gonadotropin

The ability to measure multiple steroids from all classes in a single method for simultaneous quantification by liquid chromatography tandem mass spectrometry (LC-MS/MS) provides an unprecedented opportunity to illustrate the complexity of the endocrinology of equine gestation (Legacki *et al.* 2016*b*). The rise in equine chorionic gonadotropin (eCG) that begins around 37 days of gestation (Cole & Hart 1930*a*, Allen 1969, Nett *et al.* 1975, Squires *et al.* 1979, Hoffmann *et al.* 1996) with formation of the endometrial cups (Clegg *et al.* 1954, Murphy & Martinuk 1991, Antczak *et al.* 2013) stimulates

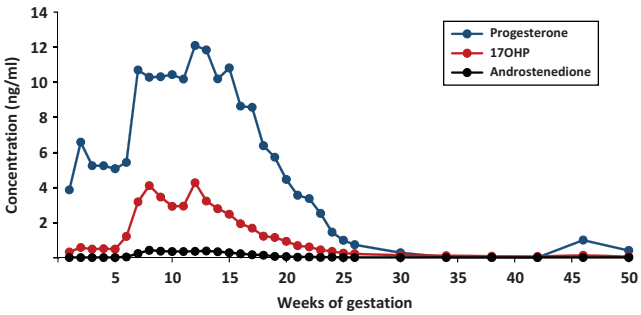

Figure 1 Progesterone, 17OH-progesterone (17OHP) and androstenedione concentrations (ng/mL) in mares throughout gestation, shown in weeks. The abrupt increase in concentrations of all three steroids that is evident from 6 weeks of pregnancy is stimulated by equine chorionic gonadotropin (eCG) secreted by the endometrial cups forming in the endometrium in the preceding week. Their involution is evident from the decline in concentrations of progesterone, 17OH-progesterone and androstenedione from week 12 or 13. Adapted from Legacki *et al.* (2016*b*).

an increase in progesterone and DHP in maternal blood, as well as increases in 17OH-progesterone, androstenedione (Fig. 1) and testosterone (Daels *et al.* 1996, Legacki *et al.* 2016*b*) from the primary corpus luteum. The parallel secretion of DHP and progesterone, which is seen prior to the establishment of the chorioallantois, probably represents 5α-reduction of progesterone in the peripheral circulation as occurs during the luteal phase of cyclic mares (Conley *et al.* 2018*b*). The increased secretion of these unconjugated steroids from the primary corpus luteum of pregnancy (Squires *et al.* 1979, Albrecht & Daels 1997, Albrecht *et al.* 1997, 2001, Daels *et al.* 1998, Boeta & Zarco 2012), and subsequent secondary luteal structures, also provides substrates for an increase in oestrone sulphate secretion (Terqui & Palmer 1979, Daels *et al.* 1990, 1991, Hyland & Langstrom 1990). Even dehydroepiandrosterone sulphate concentrations increase transiently in maternal blood during this period (Legacki *et al.* 2019). The peak of eCG secretion around 60–70 days of gestation is highly variable among mares (Murphy & Martinuk 1991, Antczak *et al.* 2013) but is correlated with progesterone secretion (Squires *et al.* 1979, Hoffmann *et al.* 1996, Boeta & Zarco 2012). The subsequent decrease in eCG from its peak is accompanied by a decline in progesterone, 17OH-progesterone and androstenedione that continues progressively (Fig. 1) and progesterone becomes undetectable in some mares as early as 180 days of gestation (Holtan *et al.* 1991, Scholtz *et al.* 2014, Legacki *et al.* 2016*b*). In contrast, DHP concentrations begin to steadily increase from around gestation day 70 as the chorioallantoic placenta develops (Steven 1982), becomes steroidogenically competent (Squires & Ginther 1975), and the ovaries become dispensable as a source of progestagenic support (Holtan *et al.* 1979). This represents the luteo-placental shift in progestin synthesis

and support of pregnancy (Legacki *et al.* 2016*b*) such that DHP synthesis shifts from the ovary to the placenta (Legacki *et al.* 2017) for the rest of gestation. The point at which DHP concentrations exceed those of progesterone (days 105–110) is an indication that the shift is essentially complete, and this may be of clinical importance for mares on supplementary progestin therapy that would not be necessary thereafter (Conley 2016).

The foetoplacental unit

An unusual feature of equine foetal development is the remarkable growth of the foetal gonads (Cole *et al.* 1933, Wesson & Ginther 1980), the initiation of which coincides with a secondary and dramatic increase in secreted oestrogen (Cole & Saunders 1935, Nett *et al.* 1973, Cox 1975, Hoffmann *et al.* 1996, Legacki *et al.* 2019), particularly oestrone sulphate (Fig. 2). At the peak of development, the foetal gonads can exceed the size of the maternal ovaries (see insert, Fig. 2). The equine placenta has demonstrated the ability for aromatization of androgens to oestrogens (Marshall *et al.* 1989, 1996) if provided androgen substrate which the foetal gonads can provide. The quantitative capacity for aromatization in the equine placenta has only recently been reported (Legacki *et al.* 2018), along with transcript abundance of the *CYP19A1* gene examined at various stages of gestation in foetal and placental tissues (Legacki *et al.* 2017). This was coupled with similar analyses of other enzyme activities and transcript abundance of the genes encoding them. These included studies into the activity of 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase (*HSD3B1* transcript) and 17α-hydroxylase/17,20-lyase cytochrome P450 activity (*CYP17A1* transcript) (Legacki *et al.* 2017, 2018) in equine chorioallantois. Like the human placenta, the equine placenta has little capacity to synthesize androgen, lacking in both 17α-hydroxylase/17,20-lyase activity (Legacki *et al.* 2018) and the requisite expression of *CYP17A1* compared with foetal adrenal glands and gonads (Legacki *et al.* 2017). Hence, androgen substrates for oestrogen synthesis, in the form of DHEA, must be supplied by the foetus, most from the foetal gonads (MacArthur *et al.* 1967, Raeside 1976, Raeside *et al.* 1979, 1982, Legacki *et al.* 2017), regardless of whether they are testes or ovaries (Legacki *et al.* 2017). Both have abundant interstitial tissue (Cole *et al.* 1933, Gonzalez-Angulo *et al.* 1971, Hay & Allen 1975, Barreto *et al.* 2018) that accounts for their unusual growth *in utero*. Equine foetal gonads contained high concentrations of DHEA (Legacki *et al.* 2017), consistent with high levels of *CYP11A1* and *CYP17A1*, and lower levels of expression of *HSD3B1* (Legacki *et al.* 2017), and 3βHSD enzyme expression (Hay & Allen 1975) and activity (Pashen *et al.* 1982), than either foetal adrenal or placental tissue (Chavatte *et al.* 1995, Legacki *et al.* 2017). Foetal adrenal glands have equal amounts of pregnenolone, despite lower *CYP11A1* and *CYP17A1*

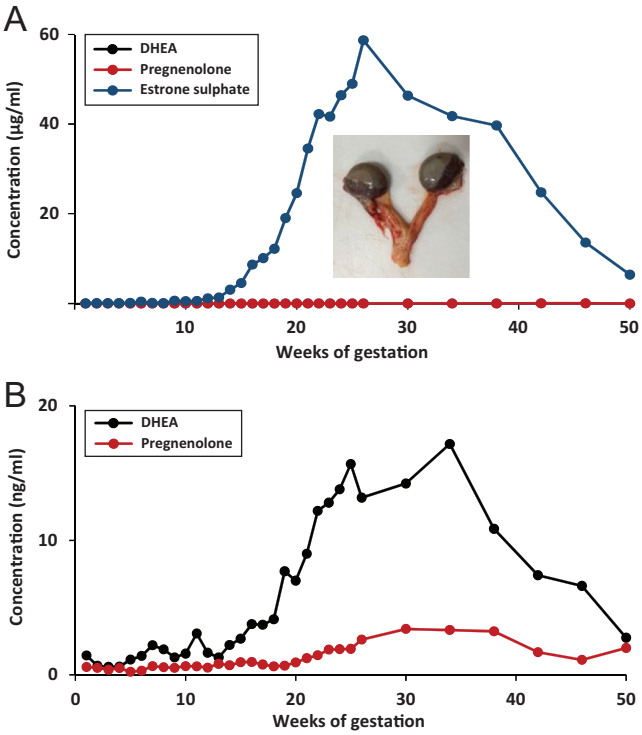

Figure 2 Oestrone sulphate (A; µg/mL), DHEA and pregnenolone (B; ng/mL) concentrations in mares throughout gestation, shown in weeks. (A) Note that oestrone sulphate concentrations (µg/ml) are several orders of magnitude greater than those of DHEA and pregnenolone (ng/ml) and cannot be seen on the same scale. The inserted image is of a foetal reproductive tract at about 6 months of gestation, illustrating the enormous size of the foetal ovaries compared to the uterus at this stage of development. (B) Despite the enormous difference in concentrations, DHEA and pregnenolone secretion is highly correlated with oestrone sulphate because pregnenolone and DHEA are both secreted by the foetal gonads and serve as substrates for synthesis of oestrone by the chorioallantois which is subsequently sulphated largely in the endometrium. This represents the foeto–placental unit in pregnant mares. DHEA secretion declines as the foetal gonads regress in last few months of gestation. Adapted from Legacki *et al.* (2016*b*, 2019).

transcript abundance than the foetal gonads (Legacki *et al.* 2017). Coupled with the smaller size of the foetal adrenal glands (Comline & Silver 1971, Yamauchi 1979), foetal gonads are the predominant source of DHEA found in foetal and maternal blood (Fig. 2) and the principal source of substrate for equine placental oestrogen synthesis. The most definitive evidence remains the immediate decline in maternal oestrogen concentrations following foetal gonadectomy (Raeside *et al.* 1973, Pashen & Allen 1979*b*, Pashen *et al.* 1982) and foetal death (Kasman *et al.* 1988, Hyland & Langstrom 1990, Hoffmann *et al.* 1996). Thus, oestrogen synthesis during equine pregnancy is achieved by the foetal supply of androgen substrate which is converted to oestrogens in the placenta, a true foeto-placental unit (Mostl 1994, Raeside 2017) as the concept was first conceived (Diczfalusy 1969).

The role of the equine foetus in placental steroidogenesis extends beyond oestrogen to pregnane synthesis because of the high concentrations of pregnenolone in foetal blood (Holtan *et al.* 1991, Ousey *et al.* 2003, Legacki *et al.* 2017) as well as uptake and utilization by the placenta (Holtan *et al.* 1991, Ousey *et al.* 2003). There is little information on the ability of the placenta for the synthesis of pregnenolone *de novo*, though foetal testis readily utilized acetate for steroid synthesis (MacArthur *et al.* 1967). However, recent studies have demonstrated that placental tissue has very low concentrations of pregnenolone, <1% that of either foetal adrenal gland or testes. Foetal adrenal and gonadal tissues had similar pregnenolone concentrations that decreased from 4 to 10 months of gestational age (Legacki *et al.* 2017). The low concentrations of placental pregnenolone were consistent with extremely low *CYP11A1* transcript abundance in placenta compared to foetal adrenal and gonadal tissues at all gestational ages studied. This was true even at 10 months of gestation when foetal adrenal and gonadal *CYP11A1* transcripts were lower than at 4 or 6 months (Legacki *et al.* 2017). The debate over where pregnenolone originates in the foetus has been dominated by the results of the same experiments that so clearly define the contribution of the foetal gonads to DHEA and oestrogen secretion, foetal gonadectomy. In contrast to maternal oestrogen concentrations, there was no significant change in maternal progestagen concentrations in mares following removal of the foetal gonads (Pashen & Allen 1979*b*) between 197 and 253 days of gestation. Progestagen concentrations were measured using a competitive protein-binding assay which indicated concentrations were reportedly 4–6 ng/mL at the time of surgery. These reported concentrations are surprisingly low. At this stage in pregnancy, progesterone becomes undetectable and 5α-reduced pregnanes including 5α-pregnan-20α-ol-3,20-dione (20αDHP) and 5α-pregnan-3β,20α-diol-3,20-dione (3β,20αDHP) increasingly dominate the progestin profile as pregnancy progresses (Fig. 3) in levels exceeding 100 ng/mL many times (Legacki *et al.* 2016*b*). Canine plasma was the source of binding protein used to assay progestagen concentrations (Pashen & Allen 1979*b*) but how it cross-reacts with DHP and other 5α-reduced metabolites is unknown. Therefore, what was measured is questionable and whether or not pregnane concentrations were affected following foetal gonadectomy remains unclear, especially in light of the size of, and pregnenolone concentration in, foetal gonads. It is equally possible that placental pregnane synthesis could increase after ablation of the principal source, as demonstrated in cows (Conley & Ford 1987) and suggested by others (Chavatte *et al.* 1997). Further studies are necessary to ascertain the tissue origin of pregnenolone in the equine foetus that seems to provide a substantial amount of substrate for placental pregnane secretion during equine gestation.

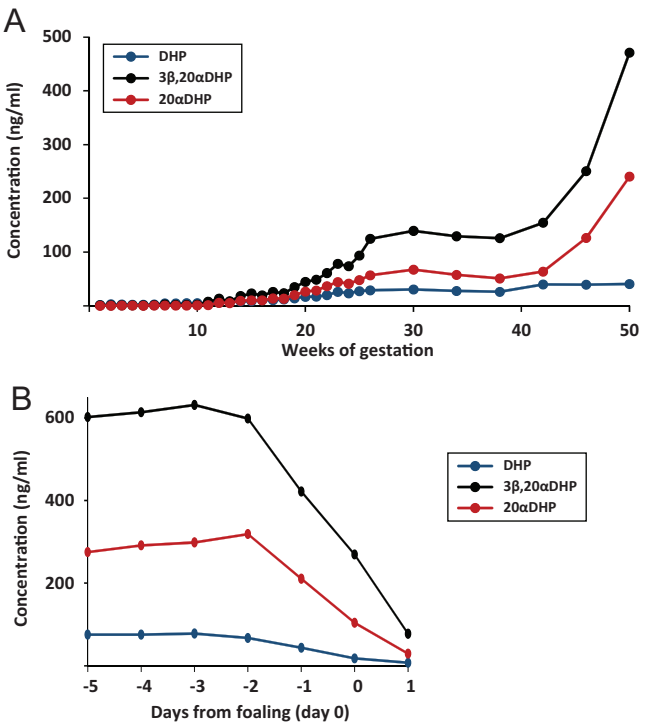

Figure 3 Concentrations (ng/mL) of 5α-dihydroprogesterone (DHP) and its hydroxylated metabolites 5α-pregnan-3β,20α-diol (3β,20αDHP) and 5α-pregnan-20α-ol-3-one (20αDHP) in mares throughout gestation, shown in weeks (A), and in the days before to 1 day after foaling (B) which is designated as day 0. Adapted from Legacki *et al.* (2016*a*,*b*)).

Steroidogenesis in late gestation and at parturition

Historically, data documenting progesterone and 5α-reduced pregnane concentrations in the final days and hours of equine gestation have been mixed. Some reported progestagen concentrations remaining elevated until the time of parturition (Barnes *et al.* 1975, Ganjam *et al.* 1975, Holtan *et al.* 1975*c*, Lovell *et al.* 1975), others noted decreases, but within hours of parturition (Seamans *et al.* 1979, Pope *et al.* 1987, Haluska & Currie 1988). Both the frequency of sampling and the use or not of chromatography are likely to have influenced the results of these studies. Studies employing mass spectrometry are consistent in detecting a peak in pregnane concentrations rise just days before foaling (Holtan *et al.* 1991, Legacki *et al.* 2016*a*). This pre-parturient peak includes pregnenolone, the transient reappearance of progesterone and additional metabolites of DHP, 20αDHP and 3β,20αDHP (Fig. 3), reaching concentrations that, in the case of 20αDHP, exhibit significant progestagenic activity *in vitro* (Legacki *et al.* 2016*a*). In fact, once daily sampling was adequate to define the slow rise and subsequent decline 3–5 days prior to parturition (Fig. 3; Legacki *et al.* 2016*a*), confirming earlier reports of declining DHP, if not progesterone itself (Seamans *et al.* 1979).

The pre-parturient decline in 5α-reduced pregnanes is associated with a complete loss of 5α-reductase activity observed in the postpartum placentas compared with placentas at 300 days of gestation, despite no observable change in 3βHSD and aromatase activities (Legacki *et al.* 2018). This suggests that the loss of 5α-reductase is selective and not due to a loss of placental viability. Logically, the apparent loss of 5α-reductase activity in the placenta at foaling would be expected to decrease metabolism of progesterone overall. Indeed, inhibition of 5α-reductase activity in the late pregnant mare is associated with an increase in maternal and foetal progesterone concentrations (Wynn *et al.* 2018*a*). Yet, at term, progesterone reappears in maternal blood (Fig. 1) and declines in parallel with all 5α-reduced pregnanes. However, pregnenolone concentrations decline prepartum also (Legacki *et al.* 2016*a*), suggesting that placental pregnane synthesis itself must decrease. How, is unknown.

The role of oestrogens and foetal adrenal activation in what triggers parturition in mares is less clear than it is for livestock species. The sustained activity of placental aromatase (Legacki *et al.* 2018) is consistent with the maintenance of high oestrogen in late gestation (Barnes *et al.* 1975, Cox 1975, Nett *et al.* 1975, Esteller-Vico *et al.* 2017) until after parturition (Haluska & Currie 1988, O'Donnell *et al.* 2003). There is no prepartum increase of maternal oestrogens in mares as is seen in sheep and cattle. Therefore, although evidence suggests that foetal levels of ACTH and cortisol increase just before parturition (Silver & Fowden 1994, Cudd *et al.* 1995), it appears that this is associated with a steroid hormone response that differs from that seen in other livestock (Conley & Reynolds 2014). Dexamethasone initiates a decline in progesterone in both sheep (Anderson *et al.* 1975, Thorburn & Challis 1979) and cattle (Hoffmann *et al.* 1979, Shenavai *et al.* 2012, Conley *et al.* 2019) even though the response involves different tissues (a placental response in sheep, a luteal response in cows). In contrast, glucocorticoid and ACTH administration to the equine foetus or the mare will also induce parturition but stimulates an increase in pregnane secretion in maternal blood (Rossdale *et al.* 1992, Ousey *et al.* 2000*b*, 2011). This seems unlikely to be of foetal adrenal origin (Han *et al.* 1995, Chavatte *et al.* 1997, Weng *et al.* 2007, Legacki *et al.* 2017). In summary then, there are several unusual features of equine parturition compared with other livestock species. This includes the loss of placental 5α-reductase activity, the placental response to glucocorticoids, as well as the lack of an increase in placental aromatase activity and oestrogen concentrations in the immediate peri-parturient period in mares. However, this might not necessarily preclude there being common underlying mechanisms ensuring physiological progestin withdrawal that have not been adequately explored in horses and other livestock where the focus has remained on systemic hormones rather than receptor expression in target tissues.

Lessons from pregnancy loss

Many issues remain to be resolved concerning the endocrinology and mechanisms of parturition in mares and other species, not least of which is how the influence of progestins ceases and includes both the myriad ways in which progesterone is metabolized among species and what it signifies. Although the late, systemic decline in concentrations of bioactive pregnanes preceding foaling is consistent with other signs of progestin withdrawal, including changes in milk electrolytes (Ousey *et al.* 1984, Rossdale *et al.* 1991), it does not mean that it is the only, or perhaps even the major, mechanism of progestin withdrawal. It is noteworthy that parturition in mares is not delayed and may even be hastened by treatment with the potent synthetic progestin altrenogest (Neuhauser *et al.* 2008) at doses adequate to otherwise maintain pregnancies (Hinrichs *et al.* 1986, McKinnon *et al.* 1988). This may be of particular relevance in circumstances where pregnancy is disrupted. As noted earlier, progestational support of pregnancy in mares is complex, and the progestins that appear responsible for maintenance of pregnancy during the second half of gestation are products of the foeto–placental unit. Monitoring peripheral concentrations of progestins in the late pregnant mare has been used for many years as a potential method to assess the well-being of the equine foetus (Rossdale *et al.* 1991, Ousey 2006, Morris *et al.* 2007, Shikichi *et al.* 2017). This effort has been confounded by the use of progesterone immunoassays with varying cross-reactivity across the spectrum of pregnanes, some in very high concentrations, present in the pregnant mare (Wynn *et al.* 2018*b*). The use of mass spectrometry has helped resolve changes in specific pregnanes associated with foetal well-being (Holtan *et al.* 1991, Houghton *et al.* 1991, Ousey *et al.* 2005, Wynn *et al.* 2018*b*). Placental pathology associated with diseases such as chronic ascending placentitis is often accompanied by a marked increase in pregnanes in maternal circulation with peak concentrations of some pregnanes (20αDHP and 3β,20αDHP) three- to four-fold higher than those of gestationally age-matched control mares (Wynn *et al.* 2018*b*). These changes associated with placental pathology are more readily identified in mares prior to approximately 300 days of gestation when a prominent increase in maternal pregnanes is normally seen in the weeks preceding foetal delivery (Holtan *et al.* 1991, Legacki *et al.* 2016*a*,*b*). The underlying mechanism responsible for the elevation in pregnanes associated with placental pathology is uncertain. Some authors have suggested that this upturn is associated with increased foetal synthesis of pregnenolone as a substrate for increased pregnane synthesis by the placenta

(Rossdale *et al.* 1992, Ousey *et al.* 2005, Fowden *et al.* 2008), possibly as a consequence of activation of the foetal hypothalamic–pituitary–adrenal axis. Because both the foetal adrenal and foetal gonad appear able to synthesize pregnenolone (Legacki *et al.* 2017), the relative importance of each tissue under conditions of foetal stress remains unknown. The increases in maternal pregnane concentrations associated with chronic placental disease (Wynn *et al.* 2018*b*) are generally comparable to those noted during the last 30 days or so of normal equine gestation and, moreover, are accompanied by a decline in pregnane concentrations in the last 2–3 days prior to pregnancy loss, similar to normal-term mares (Legacki *et al.* 2016*a*). This suggests that changes in pregnanes associated with chronic placental disease and normal term pregnancies may share a common mechanism regulating these changes.

Myometrial quiescence is critical to the maintenance of pregnancy, and results of numerous studies increasingly link physiological signs of myometrial activation with a shift in expression of anti-inflammatory to pro-inflammatory signalling pathways in both preterm and normal term labour (Romero *et al.* 2014, Renthal *et al.* 2015). These events are related to changes in systemic progesterone, but may relate to progesterone responsiveness by changes in PR activation instead (Mesiano *et al.* 2011). Although changes in peripheral concentrations of pregnanes in mares with abnormal pregnancies have received considerable study, little information is available concerning ligand–receptor (progestin–PR) interactions at the level of the myometrium. Recently, we have shown that myometrial concentrations of DHP, allopregnanolone (3αDHP) and 20αDHP are reduced in mares with acute, experimentally induced placentitis compared to gestationally age-matched control tissues (El-Sheikh Ali *et al.* 2019). This local reduction in DHP and its downstream metabolites is accompanied by a significant decline in expression of *SRD5A1* and *AKR1C23*, which encode enzymes responsible for synthesis of these pregnanes (El-Sheikh Ali *et al.* 2019). Further, expression of the nuclear PR is downregulated, and there is a decrease in the ratio of PR-B/PR-A protein isoforms of the receptor, which in pregnant myometrium from women is associated with a functional progesterone withdrawal (Merlino *et al.* 2007). Together, these findings suggest that placental inflammation results in a local reduction of both ligand (DHP) and active forms of the PR in association with activation of myometrial contractility. The decline in progestin-PR receptor signalling is in turn associated with a localized inflammation (Lyle 2014) with activation of the NF-κB pathway, upregulation of the pro-inflammatory cytokine, IL-1β and infiltration of the myometrium with neutrophils and macrophage (El-Sheikh Ali *et al.* 2019). This inflammation, in turn, is associated with increased expression of myometrial contractile-associated genes. Although there are no reports characterizing normal prepartum changes in the equine myometrium at foaling, it appears likely that a similar pro-inflammatory signalling pathway is important in normal equine parturition as has been shown in women (Romero *et al.* 2014, Renthal *et al.* 2015).

The role of oestrogens

Oestrogens are also products of the foeto-placental unit in equine pregnancy and thus maternal concentrations of various oestrogens and their sulfoconjugates have been measured as a means of assessing foetal well-being or foetal loss (Kasman *et al.* 1988, Hyland & Langstrom 1990, Canisso *et al.* 2017, Shikichi *et al.* 2017). Changes in concentrations of oestrone sulphate in maternal circulation may be less predictive of foetal loss later in gestation (Canisso *et al.* 2017) possibly because of their high circulating concentration at this stage (Legacki *et al.* 2019) and because of their relatively longer half-life. However, in general, a decline in 17β-oestradiol in maternal circulation has been associated with mares at risk of abortion in clinical studies (Shikichi *et al.* 2017) as well as in mares with experimentally induced placentitis (Canisso *et al.* 2017) during mid-to-late pregnancy. This is consistent with recent observations in mares with experimentally induced placentitis in which concentrations of oestrone and expression of *CYP19A1* in the chorioallantois are both reduced, suggesting that placental synthesis of oestrogens may be negatively impacted by infection or the associated inflammation (El-Sheikh Ali & Ball unpublished data). However, a decrease in both maternal 17β-oestradiol concentrations and placental *CYP19A1* expression in mares with placentitis are notable differences in events around normal parturition, where there is no change in either maternal oestrogen concentrations (O'Donnell *et al.* 2003) or placental *CYP19A1* expression (Legacki *et al.* 2018).

As noted earlier, pregnancy in mares is characterized by a remarkably high circulating concentration of oestrogens, and a recent study indicates that concentrations of the predominant sulfoconjugate, oestrone sulphate, as measured by LC-MS/MS peak around 50 μg/mL at about 7 months of gestation (Legacki *et al.* 2019). The biological role for the high circulating concentration of oestrogens in the pregnant mare remains somewhat uncertain, principally because inhibition of oestrogen synthesis during gestation was not accompanied by either a demonstrable decrease in uterine blood flow, placental weight or neonatal viability at term (Esteller-Vico *et al.* 2017). Similarly, removal of the foetal gonads resulted in a precipitous decline in circulating oestrogen concentrations in pregnant mares, and the authors concluded that placental oestrogens were not required for maintenance of pregnancy, lactogenesis or initiation of parturition in the mare (Pashen & Allen 1979*b*,*a*). However, foetal growth, development and viability, along

with labour at term, were abnormal in the gonadectomized foetuses. Suppression of aromatase activity in mares with the inhibitor, letrozole, from 8 months of gestation until term also suppressed maternal oestrogen concentrations by approximately 90% compared to control mares (Esteller-Vico *et al.* 2017). There were no effects on gestation length, and parturition proceeded normally in letrozole-treated mares. Foals from treated mares were, however, approximately 15% lighter in weight at term compared to controls. Uterine blood flow was assessed as part of this study, and there were no differences between letrozole-treated and control mares in either resistance or pulsatility indices in uterine arteries as measured by Doppler ultrasonography (Esteller-Vico *et al.* 2017). Studies in other species suggest that oestrogens stimulate placental angiogenesis during early gestation (Reynolds & Redmer 2001, Albrecht *et al.* 2004). Indeed, treatment of mares during early gestation (gestation days 30–120) with letrozole decreased the expression of a number of angiogenic factors in the endometrium (Haneda & Ball unpublished data), consistent with effects of oestrogen on early placental angiogenesis in mares. Therefore, inadequate oestrogen synthesis during early placental development may be more consequential in terms of detrimental effects on subsequent blood flow and foetal well-being than if experienced later in gestation when angiogenesis is no longer as active.

The potential and limits of mass spectrometry

The results of recent studies have added significantly to those of past investigations on the endocrinology of equine pregnancy and parturition. This is certainly true with respect to the impact of mass spectrometry on the ability to measure simultaneously, multiple steroids with accuracy and superior specificity (Wudy *et al.* 2018). Methods to separate steroids before they are quantified is an essential benefit of approaches that use mass spectrometry but chromatography has limits that are only realized when consideration is given to the almost limitless array of steroids that can possibly exist. The potential that steroids co-elute or do not separate enough always exists, as does the possibility of quantification using common transition ions that result from ionization during mass spectrometry. Without establishing complete mass spectra of all possible steroids, no steroid can be ruled out with absolute certainty, though it is convincing enough when standards exist and those analytes are not found. So it is that despite finding an increase in DHEAS coincident with the stimulation of ovarian steroid secretion by eCG, none was found in mid-gestation when DHEA increases (Legacki *et al.* 2016*b*), even though immunoassay results suggest otherwise (Canisso *et al.* 2017, Esteller-Vico *et al.* 2017). Similarly, there is a concomitant increase in testosterone secretion during the same period of ovarian stimulation by eCG but again none detected coincident with the mid-gestational increase in DHEA (Legacki *et al.* 2016*b*), contrary to earlier reports (Silberzahn *et al.* 1984). The recent development of methods to detect sulphated steroids during equine pregnancy (Legacki *et al.* 2019) yielded an additional surprise. Previous reports of oestrone sulphate concentrations in equine pregnancy measured by immunoassay, with or without acid or enzymatic de-conjugation, organic extraction, chromatography or sample preparation otherwise, never peaked at over 1–2 µg/mL in systemic blood at any point during gestation (Cox 1975, Terqui & Palmer 1979, Kindahl *et al.* 1982, Hyland *et al.* 1984, Pashen 1984, Kasman *et al.* 1988, Daels *et al.* 1991, Stabenfeldt *et al.* 1991, Hoffmann *et al.* 1996, Henderson *et al.* 1998, Allen *et al.* 2002, Esteller-Vico *et al.* 2017). In contrast, and as noted above, results of analysis using LC-MS/MS detected peaks of 50–60 µg/mL (Legacki *et al.* 2019). These are concentrations of sulpho-conjugated steroids that are matched only by those of DHEAS at the peak of adrenal secretion in neonatal rhesus macaques (Conley *et al.* 2011). It seems unlikely to be explained by an over-estimate due to B-ring unsaturated oestrogens, even if B-ring unsaturated androgens might help to explain the peaks of immunodetectable DHEAS in mares treated with aromatase inhibitor (Esteller-Vico *et al.* 2017) as has been suggested (Legacki *et al.* 2019). In any case, it remains unclear if LC-MS/MS grossly overestimates oestrone sulphate concentrations at their peak in pregnant mares or other methods grossly underestimate them, but at least the patterns are consistent across studies in various laboratories.

Conclusion

This review of the literature, and the choice of citations included above, is not exhaustive and many seminal contributions have been omitted that are cited in other reviews (Pashen 1984, Thorburn 1993, Silver 1994, Ousey 2004, Fowden *et al.* 2008, Conley 2016). The authors have focused here on steroid hormones and the processes that influence their secretion during equine pregnancy. This has necessarily precluded a discussion of protein hormones including inhibins (Nambo *et al.* 1996), as well as inhibins-A and -B (Conley *et al.* 2018*a*) and anti-Müllerian hormone (Almeida *et al.* 2011). These are all of practical importance in aiding the diagnosis of disease processes occurring during pregnancy in mares (Conley & Ball 2018). As reviewed previously, equine pregnancy utilizes a number of unusual strategies adopted by other species (Conley 2016) and may provide lessons, or broadly relevant insight, that few other species can. The authors hope that this review might continue to promote studies in equine reproduction.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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