REPRODUCTION
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Progestin withdrawal at parturition in the mare

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Abstract

Mammalian pregnancies need progestogenic support and birth requires progestin withdrawal. The absence of progesterone in pregnant mares, and the progestogenic bioactivity of 5α-dihydroprogesterone (DHP), led us to reexamine progestin withdrawal at foaling. Systemic pregnane concentrations (DHP, allopregnanolone, pregnenolone, 5α-pregnane-3β, 20α-diol (3β,20αDHP), 20α-hydroxy-5α-dihydroprogesterone (20αDHP)) and progesterone) were monitored in mares for 10 days before foaling ($n=7$) by liquid chromatography–mass spectrometry. The biopotency of dominant metabolites was assessed using luciferase reporter assays. Stable transfected Chinese hamster ovarian cells expressing the equine progesterone receptor (ePGR) were transfected with an MMTV-luciferase expression plasmid responsive to steroid agonists. Cells were incubated with increasing concentrations (0–100 nM) of progesterone, 20αDHP and 3α,20βDHP. The concentrations of circulating pregnanes in periparturient mares were (highest to lowest) 3α,20βDHP and 20αDHP (800–400 ng/mL respectively), DHP and allopregnanolone (90 and 30 ng/mL respectively), and pregnenolone and progesterone (4–2 ng/mL). Concentrations of all measured pregnanes declined on average by 50% from prepartum peaks to the day before foaling. Maximum activation of the ePGR by progesterone occurred at 30 nM; 20αDHP and 3α,20βDHP were significantly less biopotent. At prepartum concentrations, both 20αDHP and 3α,20βDHP exhibited significant ePGR activation. Progestogenic support of pregnancy declines from 3 to 5 days before foaling. Prepartum peak concentrations indicate that DHP is the major progestin, but other pregnanes like 20αDHP are present in sufficient concentrations to play a physiological role in the absence of DHP. The authors conclude that progestin withdrawal associated with parturition in mares involves cessation of pregnane synthesis by the placenta.

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Introduction

Mammalian pregnancy is maintained by the physiological effects of progestins (Conley & Reynolds 2014) and parturition is believed to occur principally, or in large part, as a result of withdrawal of that support (Thorburn & Challis 1979, Smith 2007, Zakar & Hertelendy 2007, Renthal *et al*. 2015). In many species, the physiological withdrawal before parturition is due to a decline in available progesterone (Nathanielsz 1998, Norwitz 1999, Jenkin & Young 2004, Mitchell & Taggart 2009), the pregnane that is generally considered the most potent endogenous progestin and that decrease is apparent in systemic blood. Studies in women (Pieber *et al*. 2001) and mice (Condon *et al*. 2003, Mendelson & Condon 2005) revealed that birth is not invoked by a decline in systemic progesterone but involves a decrease in expression or function of the classic nuclear progesterone receptors (Kastner *et al*. 1990) in target tissues (Conneely *et al*. 2003, Mesiano *et al*. 2011) such as myometrium. In horses, the endocrinology of pregnancy is more complex (Conley 2016, Legacki *et al*. 2016) than other animals and the mechanisms initiating parturition are a matter of speculation (Thorburn 1993, Silver 1994, Conley & Neto 2008, Fowden *et al*. 2008). When rigorous methods were applied, progesterone disappeared from the circulation and was absent throughout much of the second half of gestation in mares (Short 1959). The hormone profile includes a variety of other 5α-reduced pregnanes, metabolites of progesterone (Moss *et al*. 1979, Hamon *et al*. 1991, Holtan *et al*. 1991). The immediate 5α-reduced metabolite of progesterone, 5α-dihydroprogesterone (DHP) is bioactive based on its competition binding with progesterone *in vitro* (Jewgenow & Meyer 1998, Chavatte-Palmer *et al*. 2000). In the first study on bioactivity in the horse, we showed that DHP stimulated endometrial growth and secretion, and that equine pregnancy could be maintained by

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DHP in the absence of progesterone (Scholtz *et al*. 2014). Further, we developed an *in vitro* bioassay and showed that DHP was as biopotent as progesterone in activating the equine progesterone receptor but not the human progesterone receptor (Scholtz *et al*. 2014). These data proved that DHP alone is sufficient to support equine pregnancies, but sampling was infrequent in late gestation and events around parturition were not well characterized (Scholtz *et al*. 2014, Legacki *et al*. 2016).

Previous studies investigating the changes in the concentration of progesterone and other pregnanes in periparturient mares have yielded conflicting data, depending on the pregnane measured. Discrepancies among studies are due to the differences in frequency of blood sampling as well as the analysis of steroids. Immunoassays are typically unable to distinguish progesterone from other, mainly 5α-reduced, pregnanes, whose levels increase in late equine gestation (Ganjam *et al*. 1975, Holtan *et al*. 1975*b*, 1991). Holtan and coworkers (1991) recognized the need for careful analysis of steroid profiles using frequent sampling around the time of parturition in mares. They were the first to perform mass spectrometry to monitor multiple pregnanes throughout pregnancy. Their data indicated a peak in pregnane concentrations 2 days before foaling, which was followed by a decline. They concluded that frequent sampling is required to confirm these patterns near term and it is not known whether these compounds are biologically active or are simply the metabolic end products (Holtan *et al*. 1991). The lack of progestogenic activity of the major 5α-reduced pregnane metabolites has only been inferred from competitive binding studies (Jewgenow & Meyer 1998, Chavatte-Palmer *et al*. 2000). Previous attempts to show bioactivity *in vitro* were unsuccessful (Ousey *et al*. 2000). Some recently developed methods measure multiple pregnanes and other steroids throughout pregnancy with accuracy and specificity (Legacki *et al*. 2016) using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The method included pregnenolone, the universal steroid substrate, which was predicted to provide information on changes in the overall rate of pregnane synthesis at parturition. Holtan and coworkers detected pregnenolone in samples taken from periparturient mares but did not measure (or report) its concentrations (Holtan *et al*. 1991). This study combines a detailed analysis of multiple pregnanes in samples taken daily until foaling with a direct assessment of the bioactivity of those present in the highest concentrations to more comprehensively investigate progestin withdrawal in parturient mares.

Materials and methods

Animal experiments were approved by the Institutional Animal Use and Care Advisory Committee at the University of California, Davis and the University of Kentucky, in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Blood samples were taken from pregnant mares stabled at the University of California, Davis (UCD) in the 2015 foaling season and the University of Kentucky, Lexington (UKL) in the 2014 season. Mares in Lexington ($n=3$) were mixed-breed light horse, aged 9.5 ± 2.2 years and gestation period was 330.0 ± 6.8 days. Mares sampled in Davis ($n=4$) were all quarter horses, aged 8.8 ± 0.9 years and gestation period was 344.8 ± 2.6 days. For analysis, day −1 was designated as the last sample taken before foaling. Thus, samples taken on the day of, but still before, foaling were designated as day −1 samples, but if taken after foaling on that day they were designated as day 0 samples. Only one blood sample per day was analyzed per mare. Plasma samples were stored at −20°C for analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Negative control plasma was charcoal-stripped to remove endogenous steroids and similarly stored at −20°C until use.

Steroid analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Standards and solutions

Standards were purchased from Steraloids (Newport, RI): 5α-dihydroprogesterone (DHP), allopregnanolone (3αDHP), pregnenolone (P5), 5α-pregnan-3β, 20α-diol (3β,20αDHP), 20α-hydroxy-5α-dihydroprogesterone (20αDHP), progesterone and d9-progesterone (P4-d9). A master mix of all reference standards was prepared and diluted in methanol (10, 1, 0.1 and 0.01 ng/mL). Methanol and water were of HPLC grade and obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid and methyl tert-butyl ether were of ACS grade and obtained from EMD (Gibbstown, NJ, USA).

Sample preparation

Samples were extracted according to the method developed and described by Legacki and coworkers (2016). Briefly, the P4-d9 internal standard was added to all plasma samples and the samples were extracted with methyl tert-butyl ether (1:5). Calibrators and quality control samples were prepared in charcoal-stripped plasma. Calibrators ranged from 0.1 to 100 ng/mL and four levels of quality control (QC) samples (0.6, 1.5. 20 and 80 ng/mL) were prepared along with the samples. The plasma samples were shaken for 15 min and centrifuged at 3000 ***g*** for 5 min. The resulting supernatant was transferred into a 12 × 75 glass tube and dried using a Zymark TurboVap concentrator (Hopkinton, MA, USA) at 45°C with N2. Samples were reconstituted with 200 µL of 50:50 water and methanol. Quantitation of analytes was determined by linear regression analysis of the ratio of analyte area to the area of designated internal standard. Tandem mass spectral detection was developed using a Bruker EVOQ Triple Quadrupole Mass Spectrometer (Billerica, MA, USA). Calculations were made using the Bruker software. A minimum of six-point calibration curve and a maximum of ten points were used depending on the concentration of each analyte.

Method

The LC–MS/MS method used was developed and described previously (Legacki *et al*. 2016). Briefly, reverse-phase gradient separation was performed on an Agilent UHPLC C18 analytical column (2.1 × 50 mm, 1.8 µm ps) with two mobile phases delivered at 0.4 mL/min, an injection volume of 20 µL and a column temperature of 40°C. Mobile phase A and B were water with 0.2% formic acid and methanol respectively. An elution gradient was held at 40% B for the first 0.2 min, 40–60% B from 0.2 to 1 min, 60–80% B from 1 to 10 min, 80–90% B from 10.0 to 10.1 min, 90% B from 10.1 to 11.1 min, 90–40% from 11.1 to 11.2 min and 40% B until 13.10 min. Ionization was achieved using an atmospheric-pressure chemical ionization (APCI) source. Tandem mass spectral detection was accomplished using a Bruker EVOQ. Detection and quantitation of all analytes were accomplished using multiple reaction monitoring with a minimum of two transitions per analyte. Inter- and intra-accuracy and precision were assessed at four QC concentrations for all analytes (six replicates). The analytes were measured with ≤15% deviation from the expected concentrations for the three highest QC concentrations (1.5, 20 and 80 ng/mL) and ≤15% coefficient of variation (%CV). For the lowest QC concentration (0.6 ng/mL), pregnenolone, 20αDHP and 3β,20αDHP had ≤20% deviation from expected concentrations. The analytes had a percent accuracy (%Acc) >90% and a precision <15%. The responses were linear and gave correlation coefficients (R^2) of >0.99.

In vitro *bioactivity assay*

The sequence of the ePGR was determined as described previously (Scholtz *et al*. 2014) from clones isolated from an equine endometrial expression library and by amplification and sequencing from genomic DNA. The full-length coding sequence was assembled in pcDNA3.1 for the construction and maintenance of a Chinese hamster ovarian (CHO, Eton Bioscience, San Diego, CA, USA) cell line stably expressing the construct under Geneticin selection (Corbin *et al*. 1999). Briefly, the CHO cells were transfected (Lipofectamine 2000, Invitrogen) with the linearized ePGR expression plasmid, and stably transfected cells were selected by antibiotic resistance (Geneticin, Gibco, 800 µg/mL) over a period of 3 weeks. Colonies formed by the surviving cells were lifted with filter paper and transferred to new plates for expansion. Real-time PCR was used to confirm the expression in one of the clonal lines in all the subsequent experiments. In the first experiment, this cell line was transfected transiently with an MMTV-luciferase expression plasmid responsive to steroid agonists. Cells were grown for 24 h and then incubated with increasing concentrations (0–300 nM) of progesterone and two of the most abundant pregnane metabolites present in high concentrations in late gestation mares, 20αOH-DHP (20αDHP) and 5α-pregnan-3β,20α-diol (3β,20αDHP) (Legacki *et al*. 2016). Luciferase expression was measured 48 h later to assess progestogenic bioactivity. A second experiment was conducted to measure progestogenic activity of the major pregnane metabolites at their measured peak and half-peak concentrations, consistent with the decline in preparturient concentration. Consequently, cells were incubated with two concentrations of 20αDHP (2 and 1 µM) or 3β,20αDHP (3 and 1.5 µM) respectively. The luciferase expression was measured 48 h later to assess progestogenic bioactivity of these two metabolites at physiologically relevant concentrations.

Statistical analysis

Analysis of variance was conducted using PROC MIXED with repeated measures in SAS (SAS Statistical Software, SAS Institute Inc, Cary, NC, USA). Location (UCD or UKL) was included as a main effect in the steroid analysis. The significance of differences in steroid concentrations between the designated time points was determined by linear contrasts. Changes in steroids over time were also analyzed with PROC REG to assess linear and quadratic regression models. The correlations among the steroids were determined with PROC CORR. To determine the differences between progesterone receptor responses to the three tested steroids, the data were subjected to PROC MIXED. The relationships between the values at designated concentrations were determined using the interaction between the type of steroid and concentration to perform linear contrasts. To determine linearity of responses, the biopotency data were analyzed using PROC REG procedure. Data that did not meet the standards for normality were log-transformed before analysis. The data were graphed using means and standard errors calculated for each steroid.

Results

There was no difference in age or gestation periods between the mares sampled at UCD and UKL ($P > 0.05$). The most abundant pregnanes were 3β,20αDHP (500–800 ng/mL) and 20αDHP 250–600 ng/mL), followed by DHP (50–100 ng/mL), allopregnanolone (10–30 ng/mL), pregnenolone (1.5–4.0 ng/mL) and progesterone (0.5–2.5 ng/mL). Steroid concentrations varied in the last few days of gestation. Although the steroid concentrations did not fit a simple linear regression model ($P > 0.05$), they fitted a significant quadratic regression model ($P < 0.02$; for all except allopregnanolone, $P = 0.05$). Thus, all the measured pregnanes reached a prepartum peak and then declined significantly over the last few days before foaling ($P \leq 0.05$). The peak was clearly on day −3 for progesterone (2.1 ± 0.6 ng/mL), or between days −5 and −3 for other pregnanes. Concentrations of DHP, 3β,20αDHP and allopregnanolone on day −5 were 89.0 ± 13.3, 771.9 ± 79.6 and 28.0 ± 4.5 ng/mL respectively. Concentrations of pregnenolone and 20αDHP were lower in mares at UCD than those at UKL ($P < 0.01$) – pregnenolone concentration peaked at 2.6 ± 0.5 ng/mL on day −4 and 4.3 ± 1.7 ng/mL on day −3 respectively. Concentrations of 20αDHP peaked in mares at UCD, 384.4 ± 29.1 ng/mL on day −7, and at UKL, 631.9 ± 157.1 ng/mL on day −3, respectively. The decrease in concentrations from peak to the last sample before foaling ranged from 37 to 71%. The concentrations of DHP, allopregnanolone, 3β,20αDHP and progesterone dropped on average by 63, 49, 54 and 55% respectively.

The concentration of pregnenolone dropped by 52 and 46% in mares at UCD and UKL respectively, whereas that of 20αDHP dropped by 28 and 63% in mares at UCD and UKL respectively. All pregnanes positively correlated with one another.

In vitro bioassay was used for assessing the activation of ePGR. It indicated that all three pregnanes had detectable bioactivity based on significant linear increases in response to the concentrations of progesterone ($P<0.01$), 20αDHP and 3β,20αDHP ($P<0.05$), albeit at different levels. The response to progesterone reached a maximum at 30 nM (<10 ng/mL) of 4.5 ± 0.9-fold induction. At the same concentration, 20αDHP and 3β,20αDHP averaged 2.0 ± 0.4- and 1.4 ± 0.2-fold activation respectively, which was significantly lower than that of progesterone ($P<0.01$) but not different from one another. Even at a concentration of 300 nM, the average induction of reporter activity was only 2.6 ± 0.3- and 2.0 ± 0.5-fold for 20αDHP and 3β,20αDHP respectively. At the prepartum peak and half-peak concentrations, the activation of ePGR by 20αDHP (2 and 1 μM induced 2.89 ± 0.30- and 2.73 ± 0.02-fold increases respectively) was higher ($P<0.001$) than that by 3β,20αDHP (3 and 1.5 μM induced 1.24 ± 0.05- and 1.33 ± 0.02-fold increases respectively).

Discussion

This study is the first to combine daily sampling of blood in periparturient mares with analysis of steroids by mass spectrometry and direct assessment of bioactivity of the dominant pregnanes *in vitro*. To the best of our knowledge, our steroid profiling includes pregnenolone, the immediate precursor of progesterone and the universal substrate for downstream steroids of all classes (Conley & Bird 1997, Conley *et al*. 2011), which has not been measured previously. The results of prior studies on the withdrawal of progestins preceding foaling have been mixed. Some of the earliest reports used chromatography to minimize distortions in steroid estimates due to cross-reaction between the immunoassays subsequently employed, but sampling was infrequent (Barnes *et al*. 1975, Ganjam *et al*. 1975, Holtan *et al*. 1975*a*,*b*). A decrease in immunoreactive progesterone (Lovell *et al*. 1975, Seren *et al*. 1981) and DHP (Hamon *et al*. 1991) concentrations was noted by those using daily sampling but without chromatography. Although not statistically significant, the data associated with more frequent sampling (followed by immunoassay without chromatography) indicated that concentrations may drop only during the last 12 (Pope *et al*. 1987) or 28 h (Haluska & Currie 1988). Progesterone was separated from DHP and hydroxyl-5α-pregnanones using chromatography by those who sampled mares twice each day, reporting that DHP decreased from 80 h before foaling but 'in no case did (progesterone) levels fall before parturition' (Seamans *et al*. 1979). The results of those studies are in accordance with the estimates of 5α-pregnanes reported here with respect to timing, indicating that concentrations peak at 3 days before foaling (day −3) and decline thereafter. Moreover, these data suggest that all pregnane concentrations decrease in mares in the immediate prepartum period.

The landmark studies of Holtan and coworkers provided foundational data on systemic pregnane concentrations throughout equine pregnancy – some of which are extraordinarily high during late gestation though their bioactivity and significance are unknown. Specifically, they reported that concentrations of individual DHP metabolites approach 1 μg/mL in some mares (Holtan *et al*. 1991). The concentrations of 5α-reduced pregnane exceeded 1 μg/mL, consistent with the total concentrations of immunoreactive pregnanes reported by others (Seamans *et al*. 1979). These concentrations are remarkable, even by the standards of human pregnancy (Hill *et al*. 2007). This study confirms that the major metabolites identified by Holtan and coworkers (1991) are 20αDHP and 3β,20αDHP, although the latter had the highest concentration in our analysis. Consistent with the lack of bioactivity suggested by competitive binding assays of progesterone in incubations with tissue extracts (Jewgenow & Meyer 1998, Chavatte-Palmer *et al*. 2000), our data confirm that 20αDHP and 3β,20αDHP are relatively inactive metabolites of DHP. Previous studies (Jewgenow & Meyer 1998, Chavatte-Palmer *et al*. 2000) did not expect 20αDHP or 3β,20αDHP to exhibit significant progestogenic bioactivity compared with progesterone. Many factors activate steroid receptors, which typically occurs in a tissue- and cell-selective fashion due to expression levels of receptor (Abd-Elnaeim *et al*. 2009) and coregulator (Knutti *et al*. 2000), chromatin accessibility (Wiench *et al*. 2011, Grontved & Hager 2012), as well as local steroid metabolism (Funder *et al*. 1988). The functions of steroid hormone response elements are influenced by both genetic sequence and chromatin structure. However, the MMTV promoter in a useful for modeling the steroid hormone response (Adom *et al*. 1991, Truss *et al*. 1992, McNally *et al*. 2000, Nagaich *et al*. 2004, Vicent *et al*. 2010, Wiench *et al*. 2011). Chinese hamster ovarian (CHO) cells are not the ideal vehicle to explore the bioactivation of progesterone receptors by potential agonists, although even yeast-based assays have been utilized successfully and extensively (McRobb *et al*. 2008). Primary cell cultures of equine endometrial epithelium, myometrium or mammary glandular epithelium could provide a better physiological cell model if they retained responsiveness *in vitro*. Previous attempts using equine tissues *in vitro* have not provided satisfactory results (Ousey *et al*. 2000). Despite their heterologous nature, with respect to species of cells and constructs (mix of hamster, mouse, equine and human), previous studies introducing constructs including MMTV-driven luciferase along with equine and human progesterone receptor sequences into HepG2 cells

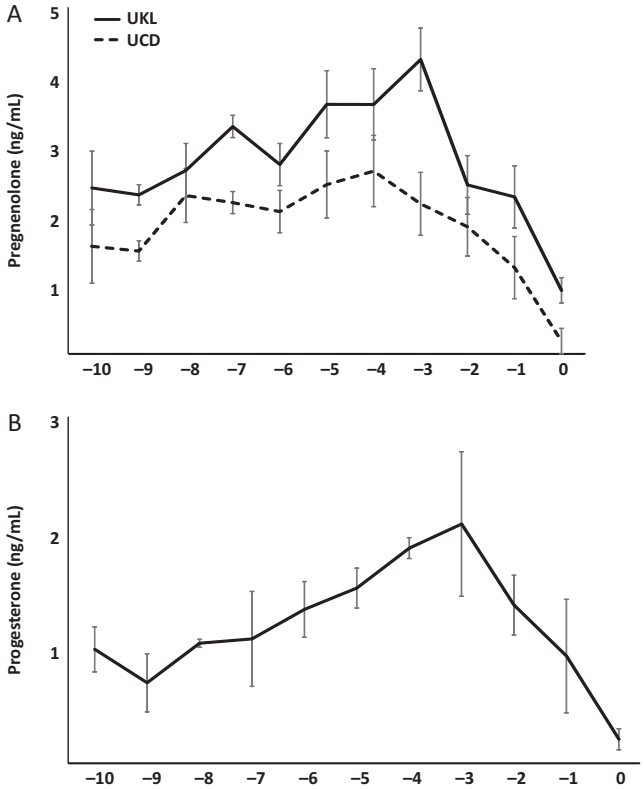

Figure 1 Concentrations (ng/mL) of pregnenolone (A) and progesterone (B) in serum of periparturient mares sampled at two different institutions (UKY, $n=3$, solid line; UCD, $n=4$, dotted line) over the last 10 days of gestation (day 0 is the day of foaling). (A) Pregnenolone concentrations were lower in mares at UCD ($n=4$) than those in mares at UKY ($n=3$; $P<0.01$). Pregnenolone concentrations peaked on day −4 at UCD or day −3 at UKY and significantly declined by day −1 ($P<0.01$) (B) Progesterone concentrations were similar among mares at UKY and UCD, so data were combined. Progesterone declined significantly from day −3 to −1 ($P=0.02$). Data represent mean ± S.E.M.

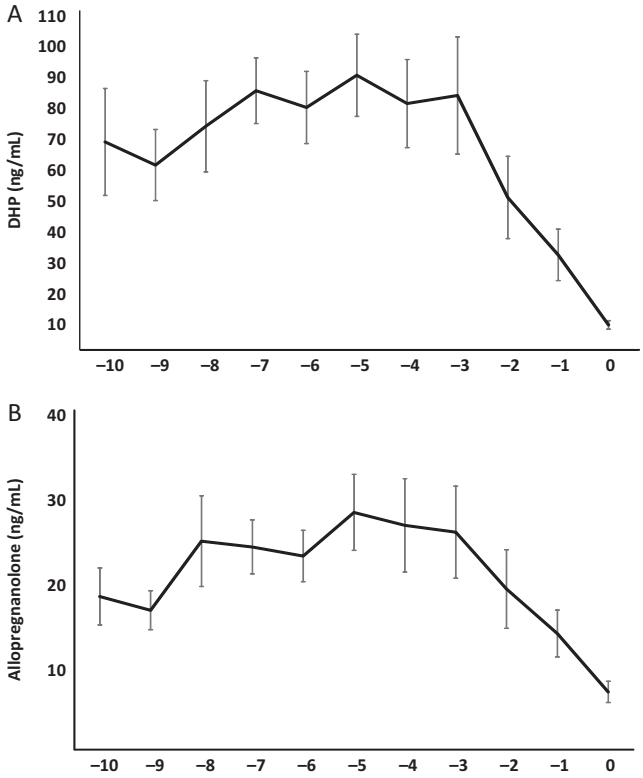

Figure 2 Concentrations (ng/mL) of 5α-reduced pregnanes, dihydroprogesterone (DHP, A) and allopregnanolone (B) in serum of periparturient mares sampled at two different institutions (UKY and UCD) over the last 10 days of gestation in mares ($n=7$, day 0 is day of foaling). DHP ($P<0.01$, A) and allopregnanolone ($P=0.05$, B) concentrations both declined significantly from day −5 to −1. Data represent mean ± S.E.M.

were successful in detecting species-specific differences in responses to DHP that accorded well with *in vivo* responses to DHP in mares (Scholtz *et al*. 2014). Thus, the bioactivity of the equine progesterone receptor in CHO cells likely represents a useful screening assay for progestogenic activity of pregnane metabolites circulating in mares in late gestation.

Given the above-mentioned caveats of assessing bioactivity *in vitro*, the results presented here extend our prior observations on biopotency of 5α-reduced metabolites, allowing an estimate of the potential contribution of all these pregnanes to ePGR activation at peak concentrations in prepartum mares. Progesterone peaked at 2 ng/mL (<6 nM), a concentration expected to induce a half-maximal ePGR activation based on the *in vitro* data. As previous studies in our laboratory demonstrated that DHP was equipotent with progesterone (Scholtz *et al*. 2014), prepartum peak DHP concentration (89.0 ng/mL or 28 nM) likely exerts maximal progestogenic activation of the ePGR.

Extrapolating from the maximal concentrations used *in vitro*, it is possible that 20αDHP may also exert some progestogenic influence, at least in the absence of DHP or progesterone. In this regard, the decline in 20αDHP may have relevance in terms of progestin withdrawal. Although the progestin withdrawal occurs systemically days before foaling, this may not be the sole mechanism that leads to parturition. That other mechanisms may operate to decrease progestogenic influence at parturition is suggested by the observation that DHP and 20α-DHP exhibited significant activation of the ePGR at concentrations approximating those seen the day before foaling. Although the concentrations decline steadily on the day preceding foaling, they were still high enough to exert progestogenic influence based on *in vitro* bioactivity. In addition, altrenogest administration can sustain pregnancy in ovariectomized embryo-recipient mares (Hinrichs *et al*. 1986, McKinnon *et al*. 1988). However, at twice the dose, it was not able to delay parturition (Neuhauser *et al*. 2008). This suggests that changes in ePGR expression or the ability of progestins to maintain progestogenic influence in the reproductive tract decrease around foaling regardless of changes in circulating pregnane concentrations. Thus, there is a

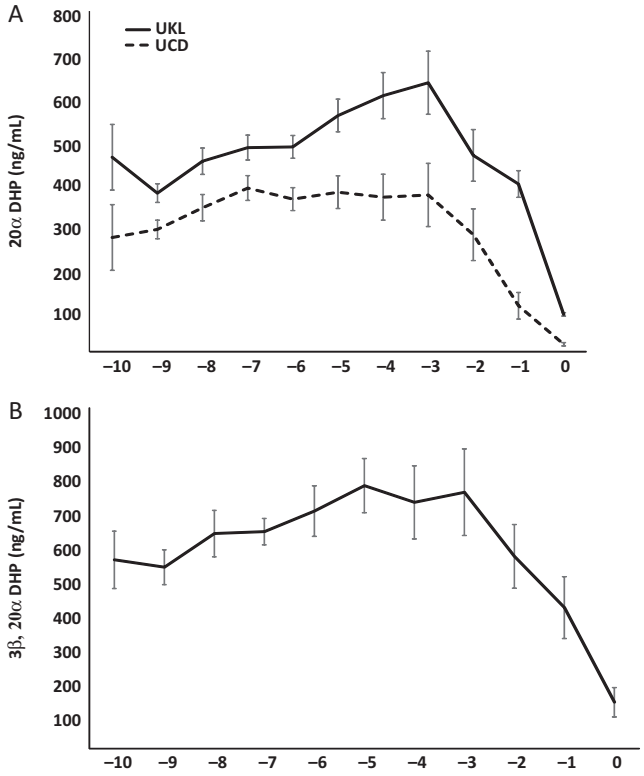

Figure 3 Concentrations (ng/mL) of metabolites of DHP, 20α-dihydroprogesterone (20αDHP, A) and 3β,20α-dihydroprogesterone (3β,20αDHP, B) measured in serum of periparturient mares sampled at two different institutions (UKY, $n=3$, solid line; UCD, $n=4$, dotted line) over the last 10 days of gestation (day 0 is the day of foaling). 20αDHP concentrations peaked on day −7 at UCD or day −3 at UKY and both significantly declined by day −1 ($P<0.01$) (A) Concentrations of 20αDHP in mares at UCD ($n=4$) were lower than those in mares at UKY ($n=3$; $P<0.01$). (B) Concentrations of 3β,20αDHP were similar among mares at UKY and UCD, so data were combined. Concentrations of 3β,20αDHP declined significantly from day −5 to −1 ($P<0.01$). Data represent mean ± S.E.M.

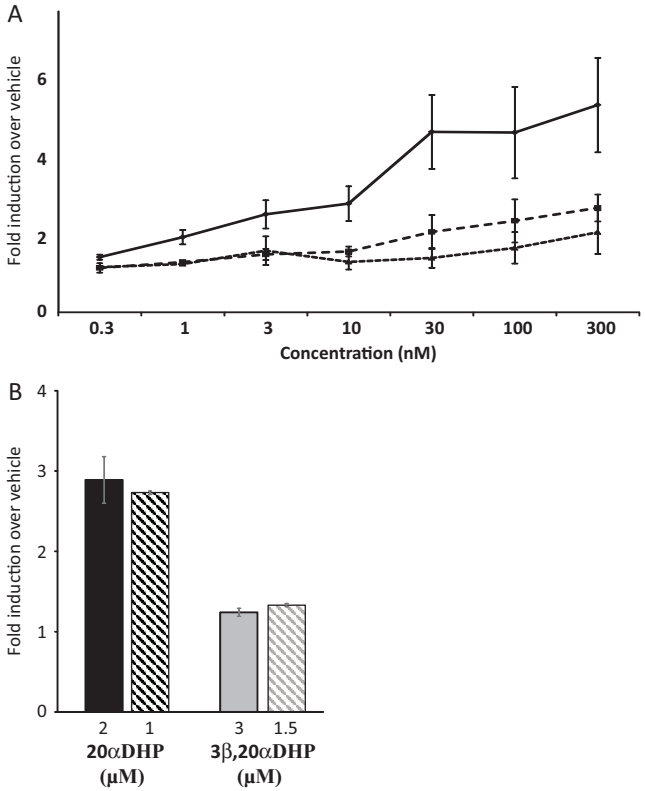

Figure 4 Concentration-dependent progestogenic bioactivation of the equine progesterone receptor (ePGR) by progesterone and the pregnane metabolites, 20α-dihydroprogesterone (20αDHP) and 3β,20α-dihydroprogesterone (3β,20αDHP), measured by stimulated (fold induction over vehicle) luciferase expression and activity driven by the mouse mammary tumor virus (MMTV) promoter. (A) Relative progestogenic bioactivity at increasing pregnane concentrations (0.3–300 nM). Progesterone (solid), 20αDHP (dashed) and 3β,20αDHP (dotted) exhibited detectable bioactivity with increasing concentrations of progesterone ($P<0.01$), and of 20αDHP and 3β,20αDHP ($P<0.05$), which is significantly less than that of progesterone. (B) Measured bioactivation of the ePGR by 20αDHP (2 and 1 μM) and 3β,20αDHP (3 and 1.5 μM) representing prepartum peak and half-peak concentrations respectively. Bioactivation of ePGR-stimulated luciferase activity was higher for 20αDHP than that for 3β,20αDHP at both peak and half-peak ($P<0.001$). Data represent mean ± S.E.M ($n=5$ replicates).

locally regulated mechanism of progestin withdrawal at parturition in mares (Conley 2016), even though pregnane concentrations decline prepartum as described here.

Despite their relative lack of biopotency compared with DHP and progesterone, the authors believe that the collapse of secretion of the most abundant of the 5α-reduced pregnanes late in equine gestation does have significance. As in sheep (Casida & Warwick 1945) and humans (Asdell 1928), some equine pregnancies can continue without luteal support (after ovariectomy) as early as day 55 of gestation, most mares after day 70 (Holtan *et al*. 1979). Hence, all circulating pregnanes in the second half of pregnancy in the mare are presumed to be of placental origin, consistent with the results of other *in vivo* and *in vitro* studies (Moss *et al*. 1979, Hamon *et al*. 1991). Thus, the decline in all pregnanes before parturition in the mare, no matter what the time frame, suggests that placental synthesis itself begins to decline. The present data clearly indicate for the first time that a decrease in pregnenolone concentrations in the maternal circulation plays an important role. There is convincing evidence that the equine placenta receives pregnenolone from the fetal foal (Ousey *et al*. 2003), although the site of synthesis in the fetus, adrenal cortex, gonad or other organ is unclear (Conley 2016). A peripartum decline in maternal pregnenolone concentrations implies that there may be a decrease in the supply of pregnenolone from the fetus or in the ability of the placenta itself to synthesize it, however small that contribution may be. Cortisol synthesis in the equine fetus increases at term (Silver & Fowden 1994, Cudd *et al*. 1995, Fowden *et al*. 2008) concomitant with the decrease in concentrations of pregnenolone and other pregnanes based on the

data presented here. It is difficult to reconcile these apparently opposing trends in steroid secretion if the fetal adrenal cortex were the source of pregnenolone. Chavatte and coworkers investigated 3βHSD in late gestation and term equine placentas, but the reported activities were highly variable and no difference was detected between them (Chavatte *et al*. 1995). No other studies have investigated the enzyme activities associated with pregnane synthesis in the equine placenta before and at parturition. Such studies are of great importance, especially in species like the horse, where progestin withdrawal involves a decrease in the most abundant circulating pregnanes as well pregnenolone before foaling.

The significantly lower concentrations of pregnenolone and 20αDHP in the serum of horses sampled at UCD compared with UKL was unexpected and warrants comment. These were the only location-dependent differences observed, which could not be explained by age or gestation period. These were similar in the mares at each site, although breeds and years of sample collection clearly differed. By immunoassay, Hamon and coworkers reported that DHP concentrations in thoroughbred mares were twice those seen in ponies in late gestation (Hamon *et al*. 1991). However, the authors of this study are unable to posit how breed might affect the concentrations of pregnenolone and 20αDHP but not those of other pregnanes. Pregnenolone, the lowest concentration of the measured pregnanes, and 20a[α]DHP, the highest, also represent the extremes of the metabolic cascade in terms initiating substrate (pregnenolone) and end product (20αDHP; Fig. 1). Yet, no other intermediate pregnane measured differed significantly or was even close to reaching significance. Further studies are necessary to verify the validity of this result, which might otherwise simply represent a type 1 statistical error. Regardless of the apparent difference in these two steroids in the two groups of mares, the pattern of prepartum decline was the same for all the examined pregnanes (Figs 1, 2, 3 and 4).

In summary, the data reported herein demonstrate that there is a general decline in the concentrations of pregnanes, including pregnenolone, 2 or 3 days before foaling in parturient mares. The authors theorize that progestin withdrawal associated with the initiation of parturition in horses involves an arrest of pregnane synthesis. Further studies are necessary to determine the molecular basis behind the apparent prepartum termination of pregnane synthesis in the mare.

Declaration of interest

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

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