REPRODUCTION
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The dynamic steroid landscape of equine pregnancy mapped by mass spectrometry

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Abstract

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) allowed comprehensive analysis of various steroids detectable in plasma throughout equine gestation. Mares ($n=9$) were bled serially until they foaled. Certain steroids dominated the profile at different stages of gestation, clearly defining key physiological and developmental transitions. The period (weeks 6–20) coincident with equine chorionic gonadotropic (eCG) stimulation of primary corpora lutea and subsequent formation of secondary luteal structures was defined by increased progesterone, 17OH-progesterone and androstenedione, all Δ4 steroids. The 5α-reduced metabolite of progesterone, dihydroprogesterone (DHP) paralleled progesterone secretion at less than half the concentration until week 12 of gestation when progesterone began to decline but DHP concentrations continued to increase. DHP exceeded progesterone concentrations by week 16, clearly defining the luteo-placental shift in pregnane synthesis from primarily ovarian to primarily placental. The period corresponding to the growth of fetal gonads was defined by increasing dehydroepiandrosterone and pregnenolone (Δ5 steroids) concentrations from week 14, peaking at week 34 and declining to term. Metabolites of DHP (including allopregnanolone) dominated the steroid profile in late gestation, some exceeding DHP by weeks 13 or 14 and near term by almost tenfold. Thus Δ4 steroids dominated during ovarian stimulation by eCG, inversion of the ratio of progesterone: DHP (increasing 5α-pregnanes) marked the luteo-placental shift, Δ5 steroids defined fetal gonadal growth and 5α-reduced metabolites of DHP dominated the steroid profile in mid- to late-gestation. Comprehensive LC–MS/MS steroid analysis provides opportunities to better monitor the physiology and the progress of equine pregnancies, including fetal development.

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Introduction

The endocrinology of equine pregnancy is complex with dramatic changes occurring in a suite of steroid hormones that include pregnanes, androgens and estrogens, appearing in (and many subsequently disappearing from) the maternal circulation at different times. The various patterns observed reflect developmental transitions in steroid synthesis and metabolism in tissue sites that include the maternal ovaries, fetal gonads and the placenta. The maternal ovary is the initial and the principal site of progesterone synthesis in early pregnancy (Squires *et al*. 1974*a*, Holtan *et al*. 1979). The primary corpus luteum synthesizes progesterone (Condon *et al*. 1979) but, under the influence of equine chorionic gonadotropin (eCG) secreted by the endometrial cups established in early pregnancy (Cole *et al*. 1931, Allen 1969, Squires *et al*. 1979), it also synthesizes estrone sulphate (Daels *et al*. 1990). Luteal tissue weight begins to decline as early as week 7 or 8 (Squires *et al*. 1979), coincident with regression of the endometrial cups and decreasing eCG (Day & Rowlands 1940), as the developing allantochorion gradually becomes the major site of progestogenic support for pregnancy (Squires & Ginther 1975, Holtan *et al*. 1979). The placenta also synthesizes increasing amounts of estrogen as pregnancy progresses through week 30 (Nett *et al*. 1973). This coincides with growth of the fetal gonads (Cole *et al*. 1933) secreting increasing amounts of androgens (MacArthur *et al*. 1967, Raeside *et al*. 1973, Raeside 1976, Pashen *et al*. 1982) that are utilized by the placenta as substrates for estrogen synthesis, functioning as an effective feto-placental unit (Pashen & Allen 1979, Raeside *et al*. 1979). The profiles of circulating steroids in pregnant mares are influenced

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by each of these developmental events and can be used to define them.

A variety of techniques have been used to measure steroid concentrations in the pregnant mare (Short 1959, van Niekerk *et al*. 1973, Smith 1974, Squires *et al*. 1974*a*, Burns & Fleeger 1975, Ganjam *et al*. 1975, Holtan *et al*. 1975*a*, Seren *et al*. 1981, Tsumagari *et al*. 1991, Hoffmann *et al*. 1996). Some investigators utilized assays validated in part by incorporating chromatography in sample preparation (van Niekerk *et al*. 1973, Barnes *et al*. 1975, Ganjam *et al*. 1975, Holtan *et al*. 1975*a*, Atkins *et al*. 1976) to reduce the potential interference from cross-reacting steroids but the resolution of separation was often limited compared with more contemporary chromatographic methods. The introduction and the use of gas chromatography coupled with mass spectrometry (GC–MS) provided both high resolution separation and greater specificity of steroid identification (Holtan *et al*. 1991, Houghton *et al*. 1991). To date however, most investigators using GC–MS in studies on steroid secretion during equine pregnancy focused on pregnanes, in particular those representing progesterone metabolites in mid- to late-stages, to better define the virtual disappearance of circulating progesterone in the second half of gestation (Holtan *et al*. 1991, Ousey *et al*. 2003). No single study has yet been conducted to investigate the changing profiles of sex steroids from all classes (pregnane, androstane and estranes) that might be predicted to clearly distinguish some of the major ongoing developmental events occurring in the dam and the fetus.

Refinements in, and the use of, mass spectrometry as a tool to analyze multiple steroids with specificity and sensitivity in a single method (Moeller & Stanley 2012) provides opportunities to better explore the relationships among maternal and fetal steroid hormones. We previously developed a method using liquid chromatography–tandem mass spectrometry (LC–MS/MS) to quantify progesterone and its 5α-reduced metabolite dihydroprogesterone (DHP) in pregnant mares. These studies also defined their relative progestogenic bioactivities, firmly establishing the physiological significance of DHP in the maintenance of equine pregnancies (Scholtz *et al*. 2014). The present study was designed to use the same longitudinally-collected series of samples from some of those mares but to expand markedly the number of steroids measured by LC–MS/MS, directed in part by the results of past studies (Holtan *et al*. 1991, Ousey *et al*. 2003). The method also included allopregnanolone, a potent neurosteroid of potential importance during fetal development (Brunton *et al*. 2014), the concentrations of which have been measured in neonatal foals (Aleman *et al*. 2013) but not previously in pregnant mares to the best of our knowledge. Among those detected with this method, the patterns of secretion of seven different pregnanes and two androgens were established and correlations among their relative concentrations were calculated. Other steroids previously reported to circulate at concentrations that should have been quantifiable with the methods used herein were not found. Specifically, testosterone has been reported to circulate in mares in both early (Daels *et al*. 1996) and late-gestation (Silberzahn *et al*. 1984) but was not detected at quantifiable concentrations in the present study, and this also is an observation worthy of note. In all, a comprehensive picture of endocrinology of equine pregnancy emerges to a degree not previously achieved.

Materials and methods

Animal experiments were approved by the Institutional Animal Use and Care Advisory Committee at the University of California, Davis, in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Thoroughbred mares ($n=9$; 10.9 ± 1.4 years of age, 500–600 kg estimated body weight, parity 4.6 ± 1.2) were bred to Thoroughbred stallions by natural cover. All pregnancies were confirmed as singletons by trans-rectal ultrasound at 14–17 days post-service and heart beats were confirmed at GD24. Jugular blood samples were collected into heparinized tubes every second day for the first 2 months of gestation, then weekly to the sixth month and reduced to once every 4 weeks until parturition. These mares represent a subset of those for which progesterone and DHP have already been reported (Scholtz *et al*. 2014). Plasma was collected and stored at −20 °C for analysis by LC–MS/MS. Negative control plasma was charcoal stripped to remove endogenous steroids and similarly stored at −20 °C until used. One mare aborted at gestation day 262. No etiology was ascertained; her pregnancy had been uneventful and apparently healthy until then.

Standards and solutions

Standards for the analytes examined in this study (see Table 1 and Fig. 1) were purchased from Steraloids (Newport, RI, USA): 17α-hydroxyprogesterone (17OH-progesterone), 5α-DHP, allopregnanolone (3αDHP), androstenedione, DHEA, d_7-androstendione (A_4–d_7), estrone, pregnenolone, 5α-pregnan-3β, 20α-diol (3β,20α-dihydroxy-dihydroprogesterone (3β,20α-DHP)), 20α-hydroxy-5α-DHP (20α-hydroxy-dihydroprogesterone (20αDHP)), progesterone and d_9-progesterone (P_4-d_9). The remaining analytes were purchased from Cerilliant (Round Rock, TX, USA): 19-norandrostenedione, 5α-dihydrotestosterone (DHT), d_3-testosterone (T-d_3), and testosterone. 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 buytl ether were of ACS grade and obtained from EMD (Gibbstown, NJ, USA).

Sample preparation

One milliliter of sample plasma was added to 13×100 glass screw top tubes followed by the addition of 100 µl of the internal standard (IS) mixture (A_4-d_7, T-d_3 and P_4-d_9) in

Table 1 Steroids investigated and listed by class (pregnanes or C21, androstanes or C19, estranes or C18 steroids) with chemical names and abbreviations used in the text. Note, metabolites of DHP, with the exception of allopregnanolone (3αDHP), are abbreviated so as to identify them as such (see Fig. 1).

Steroids analyzed	Chemical name	Abbreviations
Pregnanes		
Pregnenolone	Pregn-5-ene-3β-ol,20-one	
Progesterone	Pregn-4-ene-3,20-dione	
Dihydroprogesterone	5α-pregnan-3,20-dione	DHP
17α-hydroxyprogesterone	Pregn-4-ene-17-ol-3,20-dione	
Allopregnanolone	5α-pregnan-3α-ol-20-dione	(3αDHP)
20α-hydroxy DHP	5α-pregnan-20α-ol-3one	20αDHP
3β,20α-dihydroxy DHP	5α-pregnan-3β, 20α-diol	3β,20αDHP
Androstanes		
Dehydroepiandrosterone	Androst-5-ene-3β-ol-20-one	DHEA
Androstenedione	Androst-4-ene-3,20-dione	
Testosterone	Androst-4-ene-17β-ol-20-one	
Dihydrotestosterone	5α-androstan-17β-ol-20-one	DHT
Estranes		
Estrone	estra-1,3,5(10)-trien-17-one	
19-norandrostenedione	estr-4-ene-3,17-dione	

methanol. Calibrators ranged from 0.1–100 ng/ml and four levels of quality control (QC) samples (0.6, 1.5. 20 and 80 ng/ml) were prepared simultaneously as the samples by adding the standards to charcoal stripped control plasma and processed along with the samples. Five milliliters of methyl-tert buytl ether was added to the 1 ml of plasma and mixed 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 N_2. Samples were reconstituted with 200 µl of 50:50 water and methanol and shaken for 1 min. Calibration standards were run at the beginning and at the end of each sample set with QC samples run daily. Quantitation of analytes was determined by linear regression analysis of the ratio of analyte area to the ratio of area of designated IS. ISs were chosen for each analyte based on closest chemical structure and the analyte retention time. The IS for 17-OH-progesterone, 19-norandrostenedione, DHT, testosterone and estrone was T-d_3, the IS for androstenedione and DHEA was A_4-d_7, and the IS for DHP, 3αDHP, pregnenolone, 3β,20αDHP, 20αDHP and progesterone was P_4-d_9 Two methods were used to analyze the extracted samples. Method I measured 17OH-progesterone, 19-norandrostenedione, DHT, estrone, androstenedione, DHEA, DHP, 3αDHP, pregnenolone, 3β,20αDHP, 20αDHP and progesterone. The second method measured only testosterone. Tandem mass spectral detection was developed using a Bruker EVOQ Triple Quadrupole Mass Spectrometer (Billerica, MA, USA) for both methods. Calculations were made using the Bruker Software. A minimum of a six-point calibration curve and a maximum of ten points were used depending on the concentration range of each analyte. Method I was developed to assess a broad range of steroids of all classes. Method II was developed for testosterone alone to optimize the sensitivity of detection over that achieved with the multiple analyte method.

LC–MS/MS analysis

LC–MS/MS Method I: 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 consisted of water with 0.2% formic acid. Mobile phase B was methanol. An elution gradient was held at 40% B for the first 0.2 min, 40–60% B from

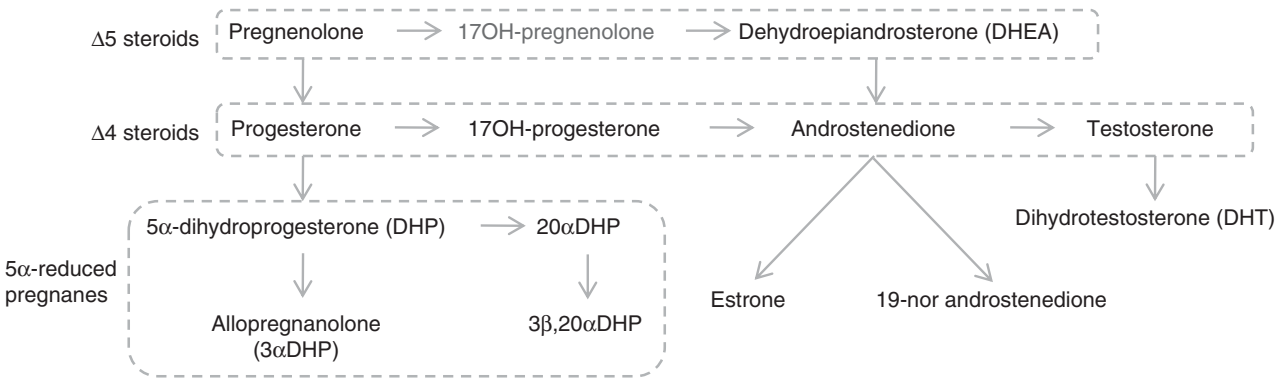

Figure 1 Schematic showing the metabolic relationships among the measured steroids and their chemical similarities; those with a double bond at C-5 (Δ5 steroids), a double bond at C-4 (Δ4 steroids) and those pregnanes in which the double bond has been reduced (5α-reduced pregnanes). Estrone and 19-norandrostenedione (estranes) are also shown as metabolites of androstenedione.

0.2–1 min, 60–80% B from 1–10 min, 80–90% B from 10.0–10.1 min, held at 90% B from 10.1 to 11.1 min, 90–40% from 11.1 to 11.2 min and held at 40% B until 13.10 min. Ionization achieved utilized an atmospheric-pressure chemical ionization (APCI) source. APCI source conditions for cone gas flow, probe and nebulizer gas flows were held at 25, 25 and 50 arbitrary units of dry nitrogen. The cone and heated probe temperatures were 300 and 450 °C, respectively.

LC–MS/MS Method II: reverse phase gradient separation was performed using an Agilent eclipse XDB-phenyl analytical column (2.1×150 mm, 5 µm ps) with two mobile phases delivered at 0.6 ml/min, an injection volume of 20 µl and a column temperature of 40 °C. Mobile phase A was water with 5 mM ammonium fluoride. Mobile phase B was methanol. An elution gradient was held at 50% B for the first 0.1 min, 50–95% B from 0.1 to 1 min, held at 95% B from 1.0 to 2.0 min, 95–50% from 2.0 to 2.1 min and held at 50% B until 5.0 min. Ionization utilized a heated-electrospray ionization (HESI) source with cone, probe and nebulizer gas flows held at 30, 40 and 65 arbitrary units of dry nitrogen. The cone and heated probe temperatures were 350 and 400 °C.

Tandem mass spectral detection was accomplished using a Bruker EVOQ. Both methods used argon as the collision gas, set to 1.5 arbitrary units. Resolution parameters were set with Q1 and Q3 both at 0.7 m/z. Detection and quantitation of all analytes were accomplished using multiple reaction monitoring with a minimum of two transitions per analyte. Both methods were validated using assay linearity and inter/intra-assay accuracy and precision (Table 2). Both inter and intra accuracy and precision were assessed at four QC concentrations for all analytes (six replicates). All analytes measured with ≤15% deviation from 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) estrone, DHEA, DHT, pregnenolone, 20αDHP and 3αDHP had ≤20% deviation from expected concentrations. All analytes measured had a percent accuracy (%Acc) >90% and a precision <15%. Accuracy was reported as percent nominal concentration and precision was reported as percent relative S.D. Extraction efficiency (% EE) was assessed by comparing analyte peak areas from injections of equivalent amounts of analyte with and without liquid–liquid extraction. The efficiency was >80% for all of the analytes measured (Table 2). The responses for all analytes were linear and gave correlation coefficients (R^2) of 0.99 or better.

Statistical analysis

Steroid concentrations from multiple samples per mare within each week of pregnancy were averaged for the week, up to 6 months of gestation and were similarly averaged within each remaining month to term. Weekly and monthly means and standard errors were calculated from these averages across mares. The data from the mare that aborted her pregnancy at GD262 showed no unusual deviation from those of the other mares to that point in pregnancy and were therefore included in the analysis. The 42 and 46 week samples from a second mare had greater than tenfold higher concentrations of pregnenolone than the highest concentrations observed in samples at any stage of gestation from any other mare and all steroid data from these two plasma samples were eliminated from all further analyses. Weekly/monthly concentration of each of the nine measurable steroids was subjected to ANOVA using Proc Mixed in SAS (SAS Statistical Software, SAS Institute Inc., Cary,

Table 2 Method validation: retention times (Rt, min), precursor and product ions are shown for the analytes examined (see Table 1 for abbreviations). The transition ions used for quantification are underlined and the most abundant ions are bolded. Parameters listed for each analyte include the limits of detection (LOD, ng/ml) and limits of quantification (LOQ, ng/ml), % Acc and precision (% CV) determined at QC 3 (20 ng/ml). The inter assay % Acc and % CV were assessed at four QC levels for all analytes; 0.6, 1.5, 20 and 80 ng/ml. All analytes measured with ≤15% deviation from expected concentrations for the three highest QC levels (1.5, 20 and 80 ng/ml) and ≤15% coefficient of variation (%CV). For the lowest QC level (0.6 ng/ml) estrone, DHEA, DHT, pregnenolone, 20αDHP and 3αDHP had ≤20% deviation from expected concentrations. The average and %CV for extraction efficiency (% EE) was determined for each analyte at each of the QC levels (six replicates) within each compounds quantitation range. % EE = (analyte peak area with extraction)/(analyte peak area without extraction)×100.

							Avg % Acc % CV				
Compound	**Rt**	**Precursor ion**	**Production**	**LOD**	**LOQ**	**% CV**	QC 3–20 ng/ml			**% EE**	**% CV**
19-norandrostenedione	1.94	273.1	**197**, 109, 83.1	0.025	0. 1	7. 2	19.6	98.0	5.4	96.5	5.2
Androstenedione	2.61	287.1	**97.1**, 109,	0.025	0. 1	3.4	20.8	104.0	7. 1	95.3	5.8
A4-d7	2.61	294.1	**100.1**, 113.1	NA	NA	NA	NA	NA	NA	NA	NA
Estrone	2.79	271.1	253, 133, 157	0.2	0. 5	4. 6	19.0	94.8	7. 1	100.0	7. 1
T-d3	2.95	292.1	**109**, 97.1	NA	NA	NA	NA	NA	NA	NA	NA
DHEA	3.04	289.1	**271.1**, 253. 1, 91. 1	0.2	0.5	5. 8	18.2	91.1	8. 3	96.1	5.7
17-OH progesterone	3.25	331.2	**97.1**, 109, 313.1	0.05	0. 1	4. 6	19.0	95.2	10.1	100.0	6. 1
DHT	3.81	291.1	**255.1**, 105.1, 159	0.2	0. 5	3. 5	18.6	93.2	8. 3	89.1	4.2
Progesterone	4.61	315.1	**97.1**, 109, 79.2	0.05	0. 1	2. 9	20.1	100.1	5.4	91.9	4.1
P4-d9	4.66	324.2	**100.2**, 113.2	NA	NA	NA	NA	NA	NA	NA	NA
Pregnenolone	5.44	299.1	**85.2**, 159.1, 131.1	0.2	0. 5	4. 7	20.3	101.4	7. 2	100.0	4. 3
3β,20α DHP	5.46	285.2	**135.2**, 175. 2, 95. 2	0.05	0. 2	8.0	19.3	96.6	14.4	98.2	4.9
20α DHP	5.78	319.2	**283.3**, 105.2	0.2	0. 5	9. 2	19.8	99.2	15.1	86.9	7.3
DHP	6.02	317.1	**281.2**, 105.2, 119.1	0.1	0. 2	4. 6	19.3	96.3	8. 7	87.6	3.7
Allopregnanolone	6.14	319.1	**283.1**, 257.1	0.2	0. 5	6. 4	19.7	98.3	9. 7	88.1	7.0
T-d3[a]	1.87	292.1	**97.1**, 109.1	NA	NA	NA	NA	NA	NA	NA	NA
Testosterone[a]	1.87	289.1	**97.1**, 109, 79.2	0.05	0. 1	6. 4	20.3	101.5	8. 5	99.1	4.7

[a]Validation parameters for Method II.

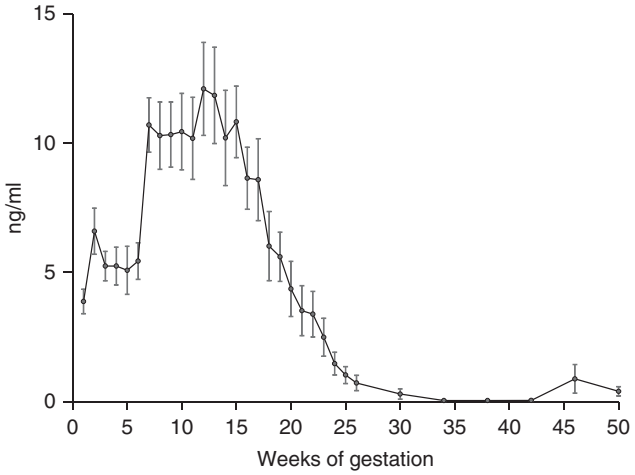

Figure 2 Progesterone concentrations (ng/ml) measured in maternal serum sampled longitudinally throughout gestation in mares ($n=9$). Data represent means ± S.E.M.

NC, USA) with time as a repeated measure. Relationships between values at designated time points were determined with linear contrasts. The correlations among the nine measurable steroids within each mare and the overall correlations for all nine mares were determined with the Proc Corr procedure. The data were graphed using the means and standard errors calculated for each week/month of gestation sampled.

Results

Δ4 steroids (progesterone, 17OH-progesterone, androstenedione and testosterone)

Progesterone concentrations peaked first in the second week post-ovulation at around 7 ng/ml, decreased transiently before rising precipitously from 5.44 ± 0.71 to 10.70 ± 1.05 ng/ml from week 6 to 7 ($P<0.01$, Fig. 2). Concentrations peaked a second time at almost 12 ng/ml in week 12 then slowly declined to values that were undetectable in some mares by week 30, reappearing at ≈1 ng/ml in week 46. 17OH-progesterone concentrations followed a similar, if less well-defined pattern (Fig. 3), increasing from 0.53 ± 0.15 in week 5 to 3.19 ± 0.75 ng/ml in week 7 ($P<0.01$), peaking at 4.12 ± 0.86 ng/ml in week 8. Thereafter, concentrations decreased transiently to 2.94 ± 0.70 ng/ml in week 10 ($P<0.05$) before increasing to a second peak of 4.28 ± 1.28 in week 12 ($P<0.05$), then declining steadily to <0.5 ng/ml in week 23 and <0.1 ng/ml after week 34. Androstenedione concentrations were one tenth those of 17OH-progesterone (Fig. 3), increasing from <0.10 ng/ml in week 6 to peak at 0.43 ± 0.09 ng/ml in week 8, maintaining these concentrations until week 13, then declining steadily thereafter to 0.05 ng/ml or less after week 20. Testosterone was below the limits of quantitation (LOQ, 0.10 ng/ml, Table 2) throughout most of gestation. Testosterone appeared at concentrations of 0.10–0.34 ng/ml between weeks 6 to 14 in 3 of 5 mares analyzed, coincident with peak concentrations of 17OH-progesterone and androstenedione but was undetectable for the remainder of gestation. All of the three Δ4 steroids that were readily quantifiable were positively correlated with one another across all of gestation (+0.35 to +0.80, $P<0.001$), and progesterone was most highly correlated with 17OH-progesterone (+0.8, $P<0.05$) when both peaked in week 8.

Δ5 steroids (pregnenolone and DHEA)

Pregnenolone concentrations remained <1 ng/ml through week 20. Concentrations began to rise steadily from 0.56 ± 0.12 to 3.41 ± 0.67 ng/ml ($P<0.01$) from week 18 to 30, decreasing slowly thereafter. DHEA concentrations were much higher, fluctuating between 1 and 3 ng/ml in early pregnancy before increasing steadily from 1.19 ± 0.31 to a peak of 17.12 ± 5.61 ng/ml ($P<0.01$) between weeks 13 and 34. DHEA concentrations declined steadily thereafter to week 50. Concentrations of pregnenolone and DHEA were positively correlated (+0.52, $P<0.01$; Fig. 4).

5α-reduced pregnanes (DHP, allopregnanolone, 20αDHP and 3β,20α-DHP)

DHP concentrations paralleled progesterone at less than half the concentration until week 12 when progesterone began to decline but DHP continued to increase and numerically exceeded progesterone by week 16. DHP concentrations rose from 1.49 ± 0.21 ng/ml in the first week of pregnancy to a peak of 37.26 ± 7.83 in week

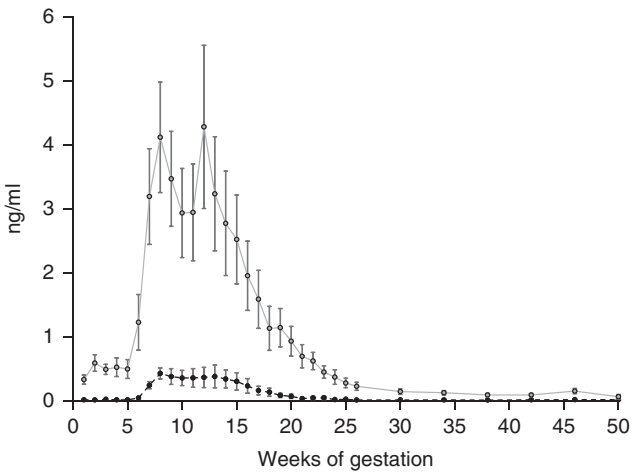

Figure 3 17OH-progesterone (solid line) and androstenedione (broken line) concentrations (ng/ml) measured in maternal serum sampled longitudinally throughout gestation in mares ($n=9$). Data represent means ± S.E.M.

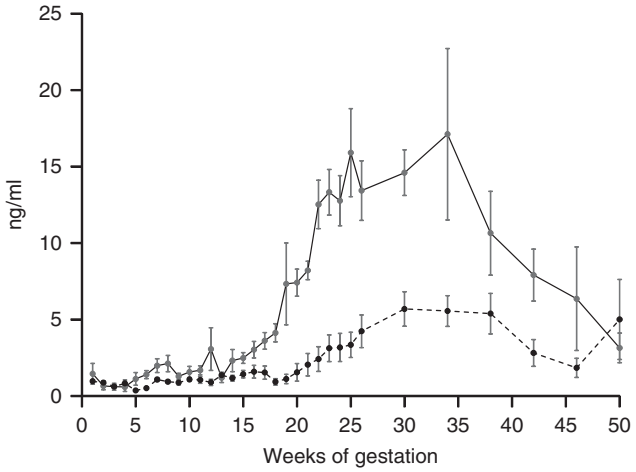

Figure 4 Pregnenolone (broken line) and DHEA (solid line) concentrations (ng/ml) measured in maternal serum sampled longitudinally throughout gestation in mares ($n=9$). Data represent means ± S.E.M.

50 ($P<0.01$). The increase was continuous from week 7 to 30 (4.31 ± 0.39–31.23 ± 5.28, $P<0.01$) and, after a transient drop to 25.38 ± 3.70 ng/ml at week 34, increased consistently to term. Allopregnanolone concentrations (Fig. 5) rose from 1.05 ± 0.23 to 16.02 ± 4.02 ($P<0.01$) from week 9 to 30, paralleling DHP ($r=+0.87$, $P<0.01$) at slightly less than half the concentration with a pause between weeks 30 and 38, before reaching a peak of almost 20 ng/ml in week 50. 20αDHP was <1 ng/ml through week 10, increased steadily from 5.03 ± 1.82 to 69.42 ± 8.92 ng/ml ($P<0.01$) between weeks 13 and 30, with a secondary rise from 62.22 ± 6.66 to 300.18 ± 100.54 ng/ml ($P<0.01$) between weeks 42 and 50 (Fig. 6). The most abundant of the steroids measured in the second half of gestation was 3β,20αDHP (Fig. 6). Like 20αDHP, it remained at low concentrations until week 10 (1.83 ± 0.60 ng/ml), increased from 8.56 ± 2.75 to 148.42 ± 24.28 ng/ml ($P<0.01$) between weeks 13 and 30. Concentrations experienced a second increase from 158.66 ± 23.96 to 484.19 ± 121.17 ($P<0.01$) between weeks 42 and 50, exceeding DHP concentrations by greater than tenfold. DHP and allopregnanolone were highly correlated (+0.87, $P<0.01$) as were 20αDHP and 3β,20αDHP (+0.93, $P<0.01$). DHP and allopregnanolone ($r=+0.80$) and 20αDHP and 3β,20αDHP ($r=+0.76$) were also correlated within mares on week 38 but DHP and allopregnanolone were not correlated with 20αDHP or 3β,20αDHP at that stage of pregnancy. The Δ4 steroids were negatively correlated with both the Δ5 steroids and the 5α-reduced pregnanes ($P<0.05$).

Estranes (C18)

Neither unconjugated estrone nor 19-norandrostenedione were found in any samples at the limits of detection of the analytical method used (200 and 25 pg/ml respectively, Table 2).

Discussion

The concentrations and variable patterns of multiple steroid hormones secreted throughout equine pregnancy, as reported here, provides an unusually complete picture of the physiological transitions occurring from luteal formation to placental steroidogenesis near term. Specifically, the appearance of different steroids earlier and later in gestation help to define distinct developmental phenomena including endometrial cup formation, the luteo-placental shift and the equine feto-placenta unit. For instance, the increase in 17OH-progesterone and androstenedione, together with progesterone, coincided with the expected stimulation of the primary CL and induction of accessory luteal structures by eCG from the endometrial cups (Allen 1969, Squires *et al*. 1974*a*). Previous studies have found strong positive correlations between progesterone and eCG concentrations between weeks 5 and 20 of gestation in mares (Squires, *et al*. 1979, Hoffmann *et al*. 1996). The decline in progesterone after week 12 corresponded with the period during which endometrial cups regress and eCG concentrations decline (Allen 1969, Squires *et al*. 1979). This is also the point at which progesterone and DHP start to diverge. Week 13 was the first week progesterone concentrations began to decline but DHP continued to rise. The rise in DHP as progesterone falls effectively marks the shift from luteal to placental progestogenic support of the pregnancy (the luteo-placental shift) (Holtan *et al*. 1979). DHP and allopregnanolone continued to increase to term and were highly correlated with increasing 20αDHP and

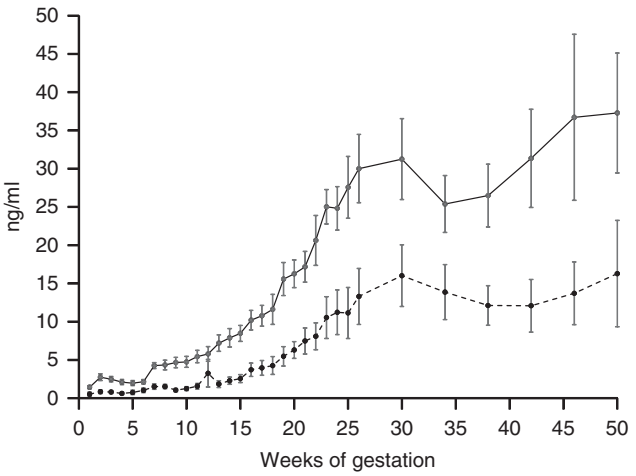

Figure 5 DHP (solid line) and allopregnanolone (broken line) concentrations (ng/ml) measured in maternal serum sampled longitudinally throughout gestation in mares ($n=9$). Data represent means ± S.E.M.

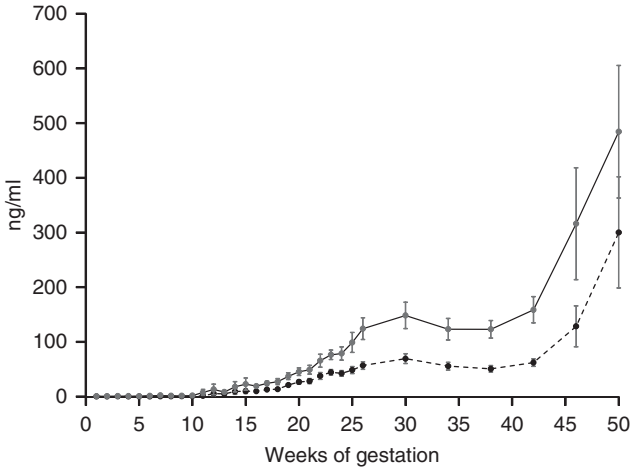

Figure 6 20α-DHP (broken line) and 3β20αDHP (solid line) concentrations (ng/ml) measured in maternal serum sampled longitudinally throughout gestation in mares ($n=9$). Data represent means ± S.E.M.

3β,20αDHP in late gestation, as previously shown (Holtan *et al*. 1991). Lastly, DHEA and pregnenolone concentrations peaked during the period when the fetal gonads are expected to reach maximum size (≈30 weeks) before beginning to regress (Wesson & Ginther 1980). Fetal gonadal androgens fuel placental estrogen synthesis (Pashen & Allen 1979, Pashen *et al*. 1982, Raeside 1995), establishing the equine feto-placental unit (Raeside *et al*. 1979, Mostl 1994). Interestingly, in each case, the steroids involved share a relatively similar basic chemical structure and concentration. Progesterone, 17OH-progesterone and androstenedione are all of the Δ4 series, and DHEA and pregnenolone are both of the Δ5 series of steroids (Fig. 1). DHP, allopregnanolone, 20αDHP and 3β,20αDHP are all 5α-reduced pregnanes (Fig. 1) and reach concentrations in late-gestation that are orders of magnitude higher in some cases than the Δ4 and Δ5 steroids and the magnitude of differences even in peak concentrations is remarkable (androstenedione, <0.5 ng/ml; 3β,20αDHP, >500 ng/ml). From this perspective, ovarian stimulation by eCG early in gestation is characterized by increased Δ4 steroids, fetal gonadal growth in mid-gestation by increased Δ5 steroids and placental growth and function from mid- to late-gestation by 5α-reduced pregnanes, all detectable in maternal blood.

Progesterone was the major focus of initial studies on steroid concentrations in pregnant mares, the earliest reports utilizing chemical methods (Short 1959), competitive protein binding assays (van Niekerk *et al*. 1973, Smith 1974) and RIA (Squires, *et al*. 1974*b*). Discrepancies in results, among those using more careful separation (alongside pregnane standards) (Ganjam *et al*. 1975, Holtan *et al*. 1975*a*) than others (Squires *et al*. 1974*a*, Barnes *et al*. 1975), led to the recognition that at least two cross-reacting steroids caused a gross over-estimate of progesterone concentrations measured in late-gestation (Ganjam *et al*. 1975, Holtan *et al*. 1975*a*,*b*). Progesterone is in fact extremely low in mid- to late-gestation in mares, as first proposed by Short (1959). One of the cross-reacting steroids was identified subsequently as DHP in pooled samples taken from late pregnant mares that were analyzed by GC–MS (Atkins *et al*. 1976). This was confirmed, and additional 5α-reduced pregnanes were identified thereafter, by a more comprehensive GC–MS analysis of longitudinal samples from pregnant mares (Holtan *et al*. 1991). No studies have reported utilizing the specificity afforded by mass spectrometry to analyze pregnanes, androgens and estrogens longitudinally in mares during pregnancy from the time of accessory CL formation through growth and regression of the fetal gonads that are known to synthesize androgens in considerable amounts (Tait *et al*. 1983, 1985, Raeside & Renaud 1985).

The establishment of the endometrial cups around GD37, secretion of eCG and the subsequent stimulation of luteal tissue formation and steroid secretion that follow are well known (Cole *et al*. 1931, Allen 1969, Squires *et al*. 1979). However, most studies on steroid secretion examined a limited number analytes and most used immunoassays only. The results presented here confirm the presence and pattern of secretion of 17OH-progesterone in pregnant mares (van Niekerk *et al*. 1973, Holtan *et al*. 1975*a*, Seren *et al*. 1981), which increases by week 7 with formation of the endometrial cups (Holtan *et al*. 1975*a*, Seren *et al*. 1981), reaching peak concentrations of around 4 ng/ml by week 8 returning to baseline concentrations after week 20. This is consistent with ovarian expression of 17α-hydroxylase/17,20-lyase cytochrome P450 (P450c17), the enzyme directly responsible for 17OH-progesterone and androgen synthesis (Conley & Bird 1997, Neto *et al*. 2010). P450c17 increases in equine CL at this time (Albrecht *et al*. 2001) and its expression is stimulated by eCG (Daels *et al*. 1998). Interestingly, the increase in 17OH-progesterone concentrations appears to precede that of progesterone by a week but peak at concentrations one third those of progesterone. The current data also confirm a concomitant increase in androstenedione (Daels *et al*. 1998) which, though one tenth the concentration of 17OH-progesterone, is highly correlated with it and likely provides substrate for the increased secretion of estrone sulphate seen at this point in pregnancy (Evans *et al*. 1984, Hyland *et al*. 1984). The detection of testosterone in some mares during this interval is also consistent with the stimulation of Δ4 steroids in general. These data are equally consistent with the low concentrations of testosterone measured by immuno-assay (Daels *et al*. 1996) and suggest that the equine CL expresses little 17β-hydroxy-steroid dehydrogenase, the enzyme required for testosterone synthesis. In any case, 17OH-progesterone and androstenedione are more readily measurable,

providing a more accurate and reliable index of the ovarian response to eCG secretion early in equine pregnancy.

A second major physiological transition is marked by a sustained increase in Δ5 steroids, namely DHEA and at much lower concentrations pregnenolone, as well as B-ring unsaturated androgens (Tait *et al*. 1983, 1985, Raeside & Renaud 1985, Marshall *et al*. 1999). The increase in DHEA detected in the maternal circulation begins around weeks 13–14 which corresponds well with initiation of growth of the fetal gonads (Walt *et al*. 1979, Wesson & Ginther 1980). DHEA is a major product of the fetal gonads (MacArthur *et al*. 1967, Raeside 1976), and although it fuels estrogen synthesis by the placenta (Bhavnani *et al*. 1969, Raeside *et al*. 1979), a surprising amount appears to escape that fate. The concentrations measured by immunoassay in previous studies (Rance & Park 1978, Pashen *et al*. 1982) compare favorably to those reported here in terms of both patterns and peak concentrations. Testosterone has been reported to increase during pregnancy, reaching as much as 250 pg/ml around the time the fetal gonads reach their maximum size (Silberzahn *et al*. 1984). However, those data were not confirmed by the present results even though 250 pg/ml was well within the LOQ of the method employed here. Based on the analytical sensitivity of our method, if present, testosterone must remain below 50–100 pg/ml after regression of the endometrial cups and the decline in eCG secretion. This would suggest that most of what has been detected previously by immuno-assay in the second half of gestation likely represents cross-reacting steroids including DHEA and perhaps B-ring unsaturated androgens, the concentrations of which have not yet been carefully quantified (Tait *et al*. 1983, Raeside 1995). Similarly, we were unable to detect estrone in maternal plasma at any stage of gestation with limits of detection and quantitation of 0.2 and 0.5 ng/ml respectively. Others have reported unconjugated estrone concentrations well within this range (Nett *et al*. 1973, Park *et al*. 1976, Rance & Park 1978). However, estrone eluted with equilin and equilenin in the chromatography used by Nett *et al*. (1973). Additionally, estrone is an expected product of DHEA metabolism, yet estrone and DHEA were not well correlated (Rance & Park 1978). Thus, present results suggest that immuno-assays for unconjugated estrone are likely confounded by other cross-reacting estrogens also. The current data indicate that gonadal growth and endocrine function in the developing fetal foal is characterized by secretion of Δ5 steroids including DHEA but not testosterone in maternal blood.

The pregnane concentrations reported here include several of the most abundant of those first identified in pregnant mares by Holtan *et al*. (1991), confirming and comparing favorably with those data, with the exception that 20αDHP was consistently less than 3β,20αDHP in our study. The expected time of endometrial cup formation corresponds with increases in luteal secretion of Δ4 steroids (progesterone, 17OH-progesterone and androstenedione) in response to eCG. The subsequent shift from luteal to placental support of pregnancy is marked clearly by the decrease in these Δ4 steroids and an increase in 5α-reduced pregnanes. This is a gradual process but DHP concentrations exceed those of progesterone around week 15, as reported previously (Scholtz *et al*. 2014), and this may be a useful clinical milestone to end progestin treatment of mares suspected of having luteal insufficiency (Allen 2001). The 5α-reduced pregnanes rise rapidly thereafter. This includes, and to the best of our knowledge is the first report of, allopregnanolone concentrations in mares. DHP and allopregnanolone reached concentrations comparable with those seen in the third trimester of human pregnancy (Milewich *et al*. 1975, Stoa & Bessesen 1975, Hill *et al*. 2007). Though 6- to 20-fold higher concentrations (120 to >400 ng/ml) may be required to induce signs of anesthesia (Madigan *et al*. 2012), those observed in pregnant mares are well within concentrations observed in patients reacting to panic-inducing stimuli (Eser *et al*. 2006). It is unclear whether or not allopregnanolone reached concentrations needed to alter neural behavior in these pregnant mares. In any case, allopregnanolone and DHP were among the most highly correlated of any of the steroids measured and the significant negative correlations with Δ4 pregnanes suggests an increase in 5α-reductase expression and activity in the placenta (Scholtz *et al*. 2014) as the allanto-chorion develops and matures. The increase in other 5α-reduced metabolites of DHP, 20αDHP and 3β,20αDHP, accelerates toward term and suggests increased expression of additional oxidoreductases in the placenta before parturition. The developmental changes in expression of steroid metabolizing enzymes in the equine placenta merits investigation. The comprehensive analysis of changes in steroid profiles during equine gestation presented here establishes a better understanding of the underlying physiology of pregnancy, and perhaps suggests diagnostic opportunities for better assessing it.

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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