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Equine fetal adrenal, gonadal and placental steroidogenesis

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Abstract

Equine fetuses have substantial circulating pregnenolone concentrations and thus have been postulated to provide significant substrate for placental 5α-reduced pregnane production, but the fetal site of pregnenolone synthesis remains unclear. The current studies investigated steroid concentrations in blood, adrenal glands, gonads and placenta from fetuses (4, 6, 9 and 10 months of gestational age (GA)), as well as tissue steroidogenic enzyme transcript levels. Pregnenolone and dehydroepiandrosterone (DHEA) were the most abundant steroids in fetal blood, pregnenolone was consistently higher but decreased progressively with GA. Tissue steroid concentrations generally paralleled those in serum with time. Adrenal and gonadal tissue pregnenolone concentrations were similar and 100-fold higher than those in allantochorion. DHEA was far higher in gonads than adrenals and progesterone was higher in adrenals than gonads. Androstenedione decreased with GA in adrenals but not in gonads. Transcript analysis generally supported these data. *CYP17A1* was higher in fetal gonads than adrenals or allantochorion, and *HSD3B1* was higher in fetal adrenals and allantochorion than gonads. *CYP11A1* transcript was also significantly higher in adrenals and gonads than allantochorion and *CYP19* and SRD5A1 transcripts were higher in allantochorion than either fetal adrenals or gonads. Given these data, and their much greater size, the fetal gonads are the source of DHEA and likely contribute more than fetal adrenal glands to circulating fetal pregnenolone concentrations. Low *CYP11A1* but high *HSD3B1* and *SRD5A1* transcript abundance in allantochorion, and low tissue pregnenolone, suggests that endogenous placental pregnenolone synthesis is low and likely contributes little to equine placental 5α-reduced pregnane secretion.

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Introduction

The endocrinology of pregnancy in the horse shares a remarkable number of similarities with human pregnancies, one facet of which is the placental synthesis of steroids utilizing fetal substrates that the placenta itself cannot produce efficiently (Conley 2016). The best known relates to the relative inability of the equine (Ainsworth & Ryan 1966) and primate (Ainsworth *et al*. 1969) placenta to synthesize androgens and thereby estrogens without substrates supplied by the fetus proper (Diczfalusy 1969, Raeside 1995). This is a strategy for estrogen synthesis during pregnancy known as a 'feto-placental unit' which, in primate pregnancies, involves androgen secreted by the fetal adrenal cortex (Conley *et al*. 2004) but in horses derives by way of androgens from the fetal gonads (MacArthur *et al*. 1967). Gonadectomy of equine fetuses *in utero* results in a rapid and marked reduction in maternal estrogen concentrations (Raeside *et al*. 1973, Pashen & Allen 1979, Pashen *et al*. 1982), and androgen synthesis by the equine fetal gonad has been extensively investigated and demonstrated convincingly (MacArthur *et al*. 1967, Raeside 1976, 1995, Raeside *et al*. 1979, 1982). Thus, androgen secretion by the fetal gonads plays a well-documented and significant role in providing substrate for placental estrogen synthesis in pregnant mares.

Despite the unarguable contribution of fetal gonadal androgens as substrates for placental estrogen secretion in horses, some believe that the equine fetal adrenal cortex also fuels the placental synthesis of progesterone and associated 5α-reduced metabolites. Specifically, it has been proposed that pregnenolone secretion by the fetal adrenal is the primary source of substrate for synthesis of placental 5α-reduced pregnanes (Thorburn 1993, Fowden *et al*. 2008) including 5α-dihydroprogesterone (DHP), which derives directly from metabolism of progesterone (Raeside *et al*. 2015, Corbin *et al*. 2016). In support of this possibility, pregnenolone was the highest among the measured steroids in equine umbilical artery from mid-gestation onward (Holtan *et al*. 1991, Ousey *et al*. 2003). In addition, pregnenolone is

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apparently taken up by the placenta and metabolized in considerable amounts as indicated by the very low concentrations in the umbilical vein compared to the umbilical artery and in view of the fact that little is found in either the uterine vein or artery either (Ousey *et al*. 2003). However, direct evidence for the contribution of the adrenal gland as the major source of pregnenolone in the equine fetus is lacking (Chavatte *et al*. 1995*a*). Some have proposed that the increase in placental secretion of 5α-reduced pregnanes stimulated by fetal administration of ACTH is evidence that the fetal adrenal is the principal source of pregnenolone in the fetal circulation. The same response to glucocorticoid administration (Rossdale *et al*. 1992, Ousey *et al*. 2011) argues otherwise (Conley 2016). Still, the apparent lack of effect of fetal gonadectomy on concentrations of maternal pregnanes (Pashen & Allen 1979) has long been cited as the most convincing evidence that the fetal gonads could not be a significant source of substrate for the synthesis of placental pregnanes (Thorburn 1993). The issue remains controversial, relatively poorly studied and ultimately unresolved (Conley 2016).

The proposed reliance of equine placental secretion of pregnanes on fetal pregnenolone pre-supposes that the allantochorion either lacks the ability to synthesize pregnenolone or contributes little. There appear to be little data that directly support this assumption. As noted previously, the placenta certainly removes considerable quantities of pregnenolone from the umbilical circulation while concentrations of pregnenolone in uterine veins and arteries are similar and low (Holtan *et al*. 1991, Ousey *et al*. 2003). Little of the pregnenolone that is delivered to the placenta by the fetus appears to escape metabolism based on low-to-undetectable concentrations in maternal systemic (Holtan *et al*. 1991, Legacki *et al*. 2016*b*) and uterine venous blood (Ousey *et al*. 2003). Thus, any synthesis of pregnenolone by the placenta itself due to the expression of *CYP11A1* in the allantochorion would likely be masked by utilization for the synthesis of 5α-reduced pregnanes. The expression of *CYP11A1* was detected in equine placenta by immunohistochemistry (Han *et al*. 1995*b*), but the intensity of staining was not compared to that in the fetal adrenal gland (Han *et al*. 1995*a*) as a gauge of relative levels of expression of the enzyme. In any case, these data give no indication as to the capacity for synthesis of pregnenolone by the placenta. Those who have examined the synthesis of pregnenes and pregnanes by the equine placenta have investigated only the metabolism of pregnenolone and/or progesterone to 5α-reduced pregnane, not the synthesis of pregnenolone itself (Ainsworth & Ryan 1966, Moss *et al*. 1979, Hamon *et al*. 1991, Chavatte *et al*. 1995*b*). Hence, to the best of the authors' knowledge, no attempts have been made to assess the capacity for the synthesis of pregnenolone by equine fetal and placental tissues by any quantitative means, such as activity of enzymes, abundance of transcript or expression of protein. Even if the synthesis of pregnenolone could be demonstrated, the shear size of the placenta compared with other fetal tissue compartments makes it difficult to estimate relative contributions on the scale of whole organs, particularly the placenta (Conley & Mason 1990).

The following studies were undertaken to address three issues deemed important in better understanding the potential source(s) of pregnenolone in the equine fetus. First, there remains a relative paucity of direct data from experiments conducted on the equine fetal adrenals themselves. The single most relevant study did not provide supportive evidence of a significant capacity for the synthesis of pregnenolone by equine fetal adrenal tissue (Chavatte *et al*. 1995*a*). No data were reported on the potential synthesis of pregnenolone by either the fetal gonad or the placenta. Second, to the best of the authors' knowledge, only one study has compared fetal gonads, adrenal and placental tissue together (Pashen *et al*. 1982) and none with placenta. Third, none to date have investigated the concentrations of pregnenes (pregnenolone and progesterone) and 5α-reduced pregnanes in fetal or placental tissues themselves as an indicator of synthesis or secretion. Therefore, equine fetal adrenal, gonadal and placental tissues were examined from mid-to-late gestation. Concentrations of pregnenolone, progesterone and 5α-reduced pregnanes among other steroids, along with analysis of transcripts encoding enzymes involved in steroid synthesis and metabolism, were determined. Different gestational ages (GA) were investigated based on the availability of specimens collected from untreated mares included in an unrelated study. This provided a better informed reassessment of the hypothesis that the equine fetal adrenal gland (but not the fetal gonad or placenta) is, (1) more likely to be the major fetal source of the high umbilical arterial concentrations of pregnenolone and (2) a likely determinant of the synthesis of placental 5α-reduced pregnanes.

Materials and methods

The experiments were approved by the Institutional Animal Use and Care Advisory Committee at the University of Kentucky, in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Tissue collection

Fetal blood and tissues were collected at necropsy from pregnant pony (*Equus caballus*) mares ($n=15$) at 4 months ($n=4$, 3 female, 1 male), 6 months ($n=4$, 3 males, 1 female), 9 months ($n=3$, 2 males, 1 female) and 10 months ($n=4$, 3 females, 1 male) of GA. The small number of samples at each fetal age precluded any reasonable analysis or assessment of data that might have been influenced by fetal sex, and these data are reported together as fetal gonads. Serum was harvested from blood collected by fetal cardiac puncture. Adrenal,

gonadal and fetal placental (allantochorionic) tissues were collected. Limited amounts of tissue were available, especially in the case of the adrenal glands of 4-month-old fetuses. No attempt was made to isolate specific regions of these glands, even though medulla is histologically recognizable in fetuses by 5 months of age (Han *et al*. 1995*a*). Allantochorion was collected from the region of the body of the uterus as a full-thickness section through all fetal layers. Tissues were divided for processing after collection. One 500–1000 mg portion was frozen on dry ice and stored at −80°C for organic extraction and steroid analysis, as described below. Another 400–500 mg portion of each tissue was kept on ice initially, then placed into RNAlater (Thermo Fisher Scientific), refrigerated in RNAlater overnight (at 4°C) and subsequently frozen and stored at −80°C until RNA was isolated for transcript analysis.

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

All fetal serum and tissue samples were analyzed by LC–MS/MS using a previously validated method (Legacki *et al*. 2016*a*,*b*). Standards were purchased from Steraloids (Newport, RI): 19-norandrostenedione (19-nor), pregnenolone (P5), progesterone (P4), 17α-hydroxyprogesterone (17OHP), 5α-dihydroprogesterone (DHP), allopregnanolone (3αDHP), 5α-pregnan-20α-ol-3-one (20αDHP), 5α-pregnan-3β, 20α-diol (3β,20αDHP), dehydroepiandrosterone (DHEA), androstenedione (A4), testosterone (T4), dihydrotestosterone (DHT), estrone, d_9-progesterone (P4-d_9), d_7-androstenedione (A4-d_7) and d_3-testosterone (T4-d_3). 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). Formic acid and methyl-tert butyl ether were of ACS grade and obtained from EMD.

Frozen tissues (250 mg) were put in a 7 mL tissue grinding tube (Precellys, Rockville, MD) with 3.2 mm stainless steel beads. Four mLs of a 50:50 mix of methanol and water were added to the grinding tube and shaken at 6500 rpm for 60 s using a Precellys tissue homogenizer. Samples were then stored for 20 min at −20°C and shaken again using the same parameters. The tissue homogenate and supernatant were removed from the grinding tube and put in a 13 × 100 glass screw top tube and dried to completion. One milliliter of water was added to the dried homogenate, followed by the addition of 100 µL of the internal standard mixture (A_4-d_7, T-d_3 and P_4-d_9) in 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 alongside samples by adding the standards to water and processing them in parallel. Five mLs of methyl-tert butyl ether was added to the reconstituted tissue homogenate, 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) at 45°C under N_2. Samples were reconstituted with 200 µL of a 50:50 mix of 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. Quantification of analytes in calibrators, QCs and unknown samples was determined by linear regression analysis of the ratio of analyte area to the ratio of area of designated internal standards. Internal standards were chosen for each analyte based on the closest chemical structure available and the analyte retention time. A detailed description has been reported previously (Legacki *et al*. 2016*a*,*b*). In brief, the analytic method utilized reverse-phase gradient separation performed on an Agilent Eclipse XDB-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 10.0–10.1 min, held at 90% B from 10.1 to 11.1 min, 90%–40% from 11.1 to 11.2 min and at 40% B until 13.10 min. Ionization was accomplished using an atmospheric-pressure chemical ionization (APCI) source in positive mode. Tandem mass spectral detection utilized an Adance UHPLC coupled with a Bruker EVOQ Elite triple quadrupole mass spectrometer. The assay was validated using the procedure defined in the FDA Guidelines for Industry Bioanalytical Method Validation. The responses for each analtye were linear and gave correlation coefficients (R^2) of at least 0.99. Intra-day and inter-day precision (% relative standard deviation) and accuracy (% of nominal concentration) were estimated from six replicate analyses of 1.5, 20 and 80 ng/mL QC samples to be <15%. The limit of quantitation (LOQ) was defined as the lowest concentration of each analyte quantified with a precision of at least 20% and an accuracy of ±20%.

Transcript analysis by quantitative polymerase chain reaction (qPCR)

Total cellular RNA was extracted from frozen fetal adrenal, fetal gonad and allantochorion samples using TRIzol Reagent (Thermo Fisher Scientific) as described previously (Ball *et al*. 2013). The purity and concentration of RNA were assessed using the Agilent bioanalyzer RNA 6000 Nano kit (Agilent Technologies) and via spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific) with 260/280 ratios above 1.95 and 260/230 ratios above 2.0 considered acceptable. Samples of RNA (n = 18) yielded RNA integrity values (RIN) above 8.0. Four samples of RNA yielded RIN values between 6.2 and 8.0. Two samples of 4-month fetal adrenals were dropped from this study due to poor RIN values (2.7 and 3.7). Samples of RNA (1 µg/reaction) were treated with rDNase I (Thermo Fisher Scientific) for 30 min at 37°C, followed by treatment with DNase Inactivation Reagent (room temperature for 2 min), and RNA was then reverse transcribed using the TaqMan Reverse Transcription Reagents.

The abundance of mRNA of cytochrome P450 family 11A1 (*CYP11A1*), hydroxy-Δ5-steroid dehydrogenase (*HSD3B1*), cytochrome P450 family 17A1 (*CYP17A1*), cytochrome P450 family 19A1 (*CYP19A1*) and 5α-reductase 1 (*SRD5A1*) were quantified by real-time quantitative PCR (qPCR) from fetal adrenal, gonad and allantochorionic extracts. Primers (Table 1) were designed as described for *HSD3B1*, *CYP17A1*, *CYP19A1* (Almeida *et al*. 2013) and *SRD5A1* (Corbin *et al*. 2016)

Table 1 Primer sequences used for qPCR analysis.

Gene	Accession no.	Product size (bp)	Sequence 5′-3′
CYP11A1	NM_001082521.3	98	Forward: GTCCCCATCCGGAACGATTT Reverse: CCAGGCGTCTGAGCTCTTAAA
HSD3B1	D89666.1	62	Forward: AGCAAATACCATGAGCACGA Reverse: TAACGTGGGCATCTTGTGAA
CYP17A1	D30688.1	60	Forward: GCATGCTGGACTTACTGATCC Reverse: CTGGGCCAGTGTTGTTATTG
CYP19A1	AF031520.1	60	Forward: CCACATCATGAAACACGATCA Reverse: TACTGCAACCCAAATGTGCT
SRD5A1	XM_014734978.1	75	Forward: GCTTTTTATTCACCAGAGCACA Reverse: TCCTGAACTTCGGATAATCTTCA
ACTB	NM_001081838	100	Forward: CGACATCCGTAAGGACCTGT Reverse: CAGGGCTGTGATCTCCTTCT

(*SRD5A1*) or using Primer-BLAST from the National Center for Biotechnology Information (Ye *et al*. 2012). Real-time qPCR of duplicate samples was performed using the ViiA-7 Real-Time PCR System (Thermo Fisher Scientific). Reactions contained a mixture of cDNA (5 ng), primers (25 ng each) and a SYBR Green Master Mix. Cycle parameters for PCR were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and then a dissociation step of 95°C for 15 s. Melting curves for each sample were assessed to evaluate the specificity of the reaction. Efficiencies of PCR were calculated using LinRegPCR (version 2013.0) (Ruijter *et al*. 2009). All reactions were pipetted using the epMotion Automated Pipetting Systems (Eppendorf; Hauppauge, NY).

The ΔCT for each gene of interest was calculated by subtracting the CT of the housekeeping gene from the CT of the gene of interest. Using Normfinder software (version 0.953), the most stable reference transcript across all tissue types proved to be the housekeeping gene encoding β-actin (*ACTB*) (Andersen *et al*. 2004). Gene expression data are presented as relative quantification values. Changes in relative abundance of specific transcripts were examined with the ΔCT method (Livak & Schmittgen 2001).

Statistical analysis

The concentration of each of the measurable steroids was subjected to ANOVA using the Proc Mixed function in SAS (SAS Statistical Software, SAS Institute Inc., Cary, NC) after log transformation when not normally distributed. Pearson's correlation coefficients were calculated among steroids in fetal serum, and differences among tissues and steroids were analyzed by orthogonal contrast, also using the Proc Mixed function in SAS. Untransformed means and standard errors were graphed. Abundance of select transcripts was analyzed as the ΔCT by subtracting the CT of ACTB from the CT of the transcript of interest (Livak & Schmittgen 2001). Differences in abundance of mRNA of steroidogenic enzymes among tissues and within tissue across time were examined using a Kruskal–Wallis test (JMP 12.0; SAS Institute). Data for abundance of transcripts are presented as ΔCT medians and ranges.

Results

The Δ5 steroids pregnenolone (200–2000 ng/mL) and DHEA (50–1500 ng/mL) were the highest of all measured steroids in fetal serum and were highly correlated ($r = +0.89$, $P < 0.001$). Serum concentrations of pregnenolone and DHEA were highest in 4-month-old fetuses but much lower thereafter (effect of GA, $P < 0.0001$, Fig. 1). The only other steroid found in significant concentrations in fetal serum was the 5α-reduced metabolite, 3β,20αDHP (50–250 ng/mL), which did not change significantly throughout gestation (not shown). Progesterone was detected in relatively low concentrations throughout (<5 ng/mL).

Broadly speaking, steroid concentrations in fetal gonadal and adrenal tissues expressed per gram of tissue wet weight reflected those seen in serum but at much higher concentrations. Pregnenolone was the highest observed steroid ($P < 0.001$) and was in relatively similar concentrations in the tissue profile of both organs

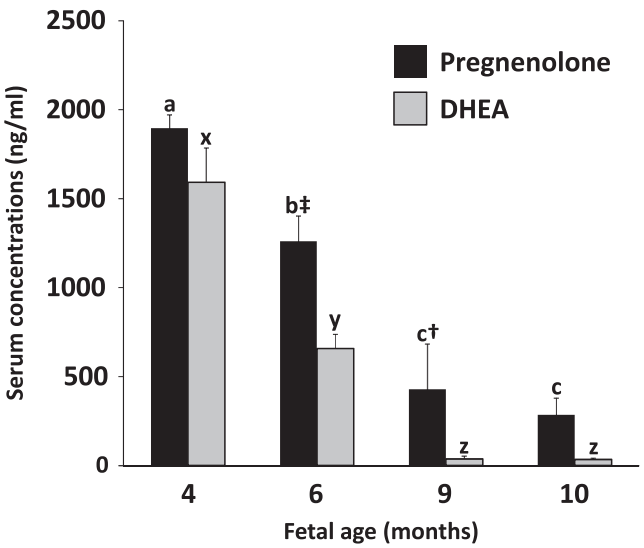

Figure 1 Serum concentrations of pregnenolone and dehydroepiandrosterone (DHEA) measured by LC–MS/MS in cardiac blood from equine fetuses at 4–10 months of gestational age. Comparisons among means within steroid across gestational ages are as indicated by superscripts for pregnenolone (a, b, c) and DHEA (x, y, z) where means with different superscripts differ, $P < 0.05$. Comparisons within gestational ages between pregnenolone and DHEA are as designated; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$.

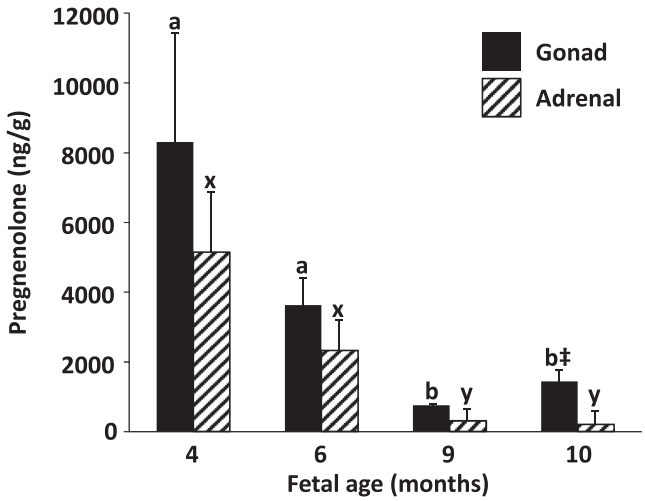

Figure 2 Tissue concentrations (ng/g) of pregnenolone measured by LC–MS/MS in gonads and adrenal glands from 4- to 10-month-old equine fetuses. Comparisons among means within tissues between gestational ages are as indicated by superscripts for gonads (a, b) and adrenals (x, y) where means with different superscripts differ, $P<0.05$. Comparisons within gestational ages between gonads and adrenals are as designated; ‡$P<0.01$.

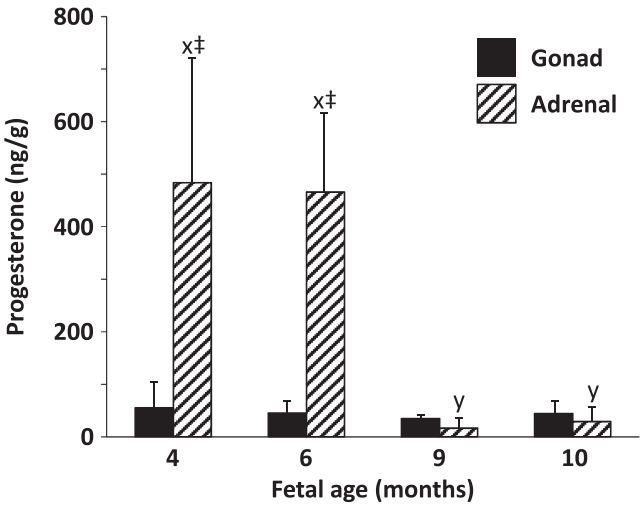

Figure 4 Tissue concentrations (ng/g) of progesterone measured by LC–MS/MS in gonads and adrenal glands from 4- to 10-month-old equine fetuses. Comparisons among means within tissues between gestational ages are as indicated by superscripts for adrenals (x, y) where means with different superscripts differ, $P<0.05$. Comparisons within gestational ages between gonads and adrenals are as designated; ‡$P<0.01$.

($P=0.07$), averaging >8000 ng/g in fetal gonads and >5000 ng/g in 4-month-old fetal adrenals (Fig. 2). DHEA was the next most abundant steroid in fetal gonads (ranging around 1200–3400 ng/g) but was comparatively low in fetal adrenal (and allantochorionic) tissues ($P<0.001$), decreasing in adrenals from a high of around 500 ng/g at 4 months GA to being almost undetectable by 9 and 10 months ($P\leq0.05$, Fig. 3). Progesterone was found in the fetal adrenal at 4- and 6-month GA (400–500 ng/g) but only trace amounts by comparison were found thereafter and was low in fetal gonads at all stages (Fig. 4). Interestingly, androstenedione concentrations in the adrenal gland decreased with fetal age ($P<0.05$) from 88.4 ng/mL at 4 to 0.85 ng/mL at 10 months GA, which tended to be different from the gonad (tissue*age interaction, $P=0.08$; Fig. 5) though variability was very high in gonads at 9-month GA. Gonads had 17OH-progesterone (20–60 ng/g, not shown), but none was detectable in fetal adrenal glands.

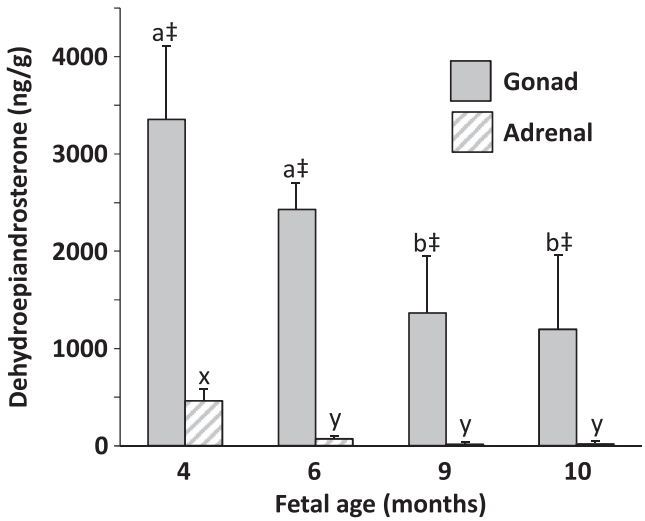

Figure 3 Tissue concentrations (ng/g) of dehydroepiandrosterone measured by LC–MS/MS in gonads and adrenal glands from 4- to 10-month-old equine fetuses. Comparisons among means within tissues between gestational ages are as indicated by superscripts for gonads (a, b) and adrenals (x, y) where means with different superscripts differ, $P<0.05$. Comparisons within gestational ages between gonads and adrenals are as designated; ‡$P<0.01$.

The profile of steroids detected in equine allantochorionic tissue was very different from that seen in the fetal adrenal glands and gonads. Notably, concentrations of both pregnenolone and progesterone in allantochorionic samples (Fig. 6) were orders of magnitude lower than those found in fetal gonads ($P<0.001$) and adrenal glands ($P<0.02$). Averaged over all fetal ages, pregnenolone concentrations in fetal adrenal and gonadal tissues were 100-fold higher than those in the allantochorion (2110.54 ± 709, 3719.07 ± 1102.04 and 22.63 ± 8.13 ng/g respectively). Similarly, when averaged across all GA, there was a 10-fold difference ($P<0.001$) in concentrations of DHEA in allantochorion (222.3 ± 50 ng/g) compared with fetal gonad (2136 ± 367 ng/g). In contrast to the fetal adrenal and gonads, which contained predominantly DHEA and pregnenolone, the predominant steroids found in allantochorionic tissues were 5α-reduced pregnanes (Fig. 6) that were largely absent from fetal adrenal glands and gonads. In general, concentrations of pregnenolone, progesesterone and 5α-reduced pregnanes were lower at

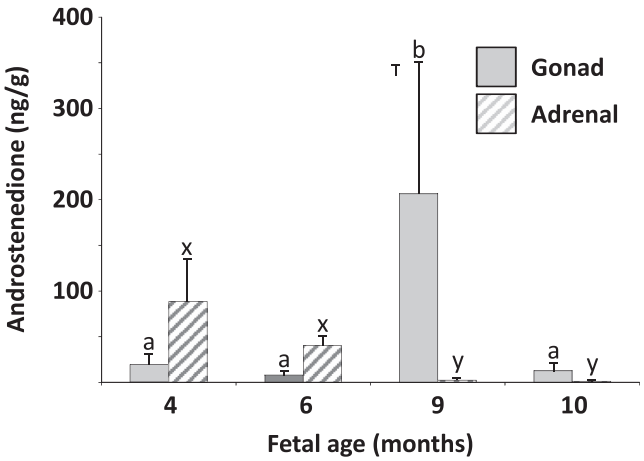

Figure 5 Tissue concentrations (ng/g) of androstenedione measured by LC–MS/MS in gonads and adrenal glands from 4- to 10-month-old equine fetuses. Comparisons among means within tissues between gestational ages are as indicated by superscripts for gonads (a, b) and adrenals (x, y) where means with different superscripts differ, $P<0.05$.

9 and 10 months than at earlier stages of gestation. The exceptions were 3β,20αDHP, which did not change with GA, and allopregnanolone (3αDHP), which was low at 9 months but had recovered ($P<0.05$) at 10 months of gestation.

Actin B was the most stable reference transcript to support comparisons made among tissues and ΔCt values were normalized to β-actin transcript accordingly. Thus, lower normalized values represent relatively higher abundance levels of transcripts. Data were analyzed by non-parametric methods and the median, minimum and maximum values were tabulated accordingly (Tables 2 and 3). Transcripts encoding *CYP11A1* and *CYP17A1* were consistently higher in the fetal gonad than the fetal adrenal, and both transcripts were consistently higher in adrenal and gonad than those in allantochorion (Table 2, $P<0.01$). Transcript levels of *CYP11A1* were lower in gonads at 10 months GA, but higher in the fetal adrenal, than at earlier stages (Table 3, $P<0.05$). *HSD3B1* was lower (higher ΔCt) in the fetal gonads than that in the fetal adrenals (Table 2, $P<0.01$) and higher in the allantochorion than either adrenal or gonad (Table 2, $P<0.01$). Transcript levels of *HSD3B1* in fetal adrenal were higher (ΔCt was lower) at 10 than at 6 or 4 months (Table 3, $P<0.05$). *SRD5A1* expression was relatively low in both fetal gonads and adrenal gland tissues but was consistently higher in allantochorion (Table 2, $P<0.01$) and higher in allantochorion at 6 and 10 months GA than at 4 months (Table 3, $P<0.05$). The expression of CYP19A1 was consistently higher in allantochorion than fetal adrenals or gonads (Table 2, $P<0.01$) and did not change with GA (Table 3).

Discussion

The current studies were the first to investigate the concentrations of multiple steroids (including pregnenolone, 5α-reduced pregnanes and DHEA among others) in equine fetal serum, adrenal and gonadal tissues and allantochorion at 4-, 6-, 9- and 10-month GA. Several previous studies using gas chromatography-mass spectrometry have detected (Marshall *et al*. 1999) and quantified (Holtan *et al*. 1991, Ousey *et al*. 2003) various steroids in equine umbilical arterial and venous blood. Additionally, previous investigators (Chavatte *et al*. 1995*b*) examined pregnenolone and corticoid synthesis by, and 3βHSD activity in, minced equine fetal adrenal tissue *in vitro*, but none have examined either steroid concentrations in equine fetal tissues or steroidogenic enzyme transcript abundance to the best of the authors' knowledge. Steroids were measured in fetal serum and tissues by LC–MS/MS using a recently developed method (Legacki *et al*. 2016*a*,*b*), and additional support for the steroid profiling was provided by the analysis of transcripts encoding key steroidogenic enzymes. The apparent, relative deficiency of HSD3B1 (3βHSD) transcript in fetal gonad compared with fetal adrenal (Table 2) was consistent with the predominance of Δ5 steroids (pregnenolone and

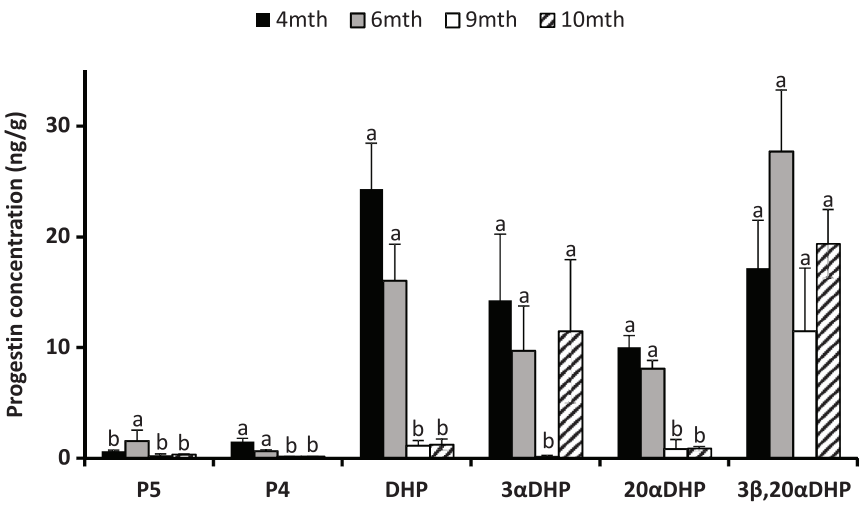

Figure 6 Equine allantochorionic concentrations of pregnenolone (P5), progesterone (P4) and 5α-reduced pregnanes (dihydroprogesterone, DHP; allopregnanolone, 3αDHP; 20α-hydroxy-DHP, 20αDHP ; 3β,20α-dihydroxyDHP, 3β,20αDHP; ng/g) measured by LC–MS/MS in tissue at 4–10 months of gestation (mth). Comparisons among means for each steroid across gestational ages are as indicated by superscripts (a, b) where means with different superscripts differ, $P<0.05$.

Table 2 Median and range ΔCT for transcript abundance of steroidogenic enzymes without regard to gestational ages.

	Fetal adrenal, *n* = 10		**Fetal gonad, *n* = 12**		**Allantochorion, *n* = 12**	
	Median	Range	Median	Range	Median	Range
CYP11A1	2.4[a]	−0.3, 4.3	−1.5[b]	−1.8, 0.0	7.1[c]	5.9, 9. 7
CYP17A1	0.6[a]	−3.7,2.4	−8.8[b]	−10.1, −5.4	2.5[c]	0.3, 3.9
CYP19A1	11.1[a]	2.3, 17.7	11.1[a]	4.4, 13.6	−5.8[b]	−7.2, −4.3
HSD3B1	0.4[a]	−3.4, 2.6	11.0[b]	4.5, 16.8	−1.9[a]	−3. 6, −1.0
SRD5A1	5.2[a]	4.1, 7.6	6.3[b]	4.7, 9.5	1.3[c]	−0.7, 4.9

[a,b,c]Values with different superscripts differ, $P < 0.01$.
CYP11A1, *CYP17A1* and *CYP19A1* (genes encoding cholesterol side chain cleavage, 17α-hydroxylase/17,20-lyase and aromatase cytochromes P450 respectively); *HSD3B1* and *SRD5A1* (genes encoding 3βhydroxysteroid dehydrogenase/Δ5–4 isomerase, and 5α-reductase type 1 respectively).

especially DHEA; Fig. 1) in fetal gonads and reported enzyme activity (Pashen *et al*. 1982).

Similarly, the higher abundance of transcripts encoding *CYP17A1* (together with low *HSD3B1*) in fetal gonads compared with fetal adrenal (Table 2) is equally consistent with the high concentrations of DHEA found in gonad compared with the adrenals (Fig. 3). The lower concentration of several steroids in fetal gonads at 10 months of age compared with earlier ages comported with lower levels of transcript encoding *CYP11A1* in gonads of fetuses at 10 months compared to earlier stages of gestation (Table 3). In general, more Δ4 steroids (e.g., progesterone and androstenedione) were detectable in fetal adrenals than gonads, which is consistent with greater *HSD3B1* transcript abundance detected in fetal adrenal glands compared to fetal gonads, most evident at 4 and 6 months GA. At the same time, not all fetal transcript data were consistent with tissue steroids. In fact, the increase in levels of adrenal *CYP11A1* and *HSD3B1* transcripts at 10 months of GA was associated with lower adrenal concentrations of progesterone (Fig. 4) and androstenedione (Fig. 5). It is possible that other steroids, corticoids most notably that were not measured by the method used, were increasing along with the expression of the requisite adrenocortical enzymes, *CYP21A1* and *CYP11B1*, also absent from this analysis. If so, the increasing abundance of transcripts encoding *CYP11A1* and HSD3B1 may reflect some level of early adrenal maturation, but there are insufficient data to do more than speculate at this time.

The pattern of abundance of transcript in allantochorion was distinct from the fetal adrenal and gonad, exhibiting very low levels of *CYP11A1* and *CYP17A1*, but relatively high *HSD3B1*, *SRD5A1* and *CYP19A1* (Table 2). These data are consistent with the production of predominantly 5α-reduced pregnanes and estrogens by the placenta. Concentrations of pregnenolone and DHEA in allantochorionic tissues were barely detectable and orders of magnitude lower than fetal adrenal and gonadal tissues. Compared to 4 and 6 months GA, concentrations of DHP, 20αDHP and allopregnanolone in allantochorion were lower at 9 months, but there were no concomitant changes in transcripts for *CYP11A1* or *HSD3B1*. This period corresponds with a distinct plateau, possibly even a dip, in systemic maternal concentrations of 5α-reduced pregnanes (Legacki *et al*. 2016*b*). In addition, it is a particularly dynamic period of placental development during which umbilical and uterine blood flows more than double (Ousey *et al*. 2003, Klewitz *et al*. 2015). Clearly, many factors including placental growth influence rates of secretion of some steroids and changes in concentrations of tissue steroids are not a reliable reflection of circulating concentrations. However, the general correspondence between concentrations of tissue steroids and, based on steady state abundance of transcripts, apparent expression of steroidogenic enzymes in fetal gonads, fetal adrenal and allantochorion adds confidence to the broad, tissue-related trends observed from both these analyses. Collectively, the observed relative deficiency in expression of *CYP11A1* and *CYP17A1* but high *HSD3B1*, *SRD5A1* and *CYP19A1* in equine allantochorion is consistent with the utilization of fetal pregnenolone for the synthesis of placental 5α-reduced pregnanes and fetal DHEA for placental synthesis of estrogen.

Previous studies reporting concentrations of steroids in fetal blood involved pregnancies over a slightly later gestational window than was available for investigation here. Specifically, the earliest samples taken by catheterization of umbilical arteries and veins (Ousey *et al*. 2003) were at 6–7 months of gestation and the latest were taken after 10 months (>300 days). The current data relate to fetuses starting from an earlier interval of development. More importantly, the samples analyzed here were taken by cardiac puncture and concentrations of steroids would necessarily differ from those in either umbilical arterial or venous blood. Nevertheless, in the current study, concentrations of steroids in the serum taken from 9- and 10-month-old equine fetuses were quite similar to those measured by others (Holtan *et al*. 1991) taken in samples from the umbilical artery between 250 and 300 days of gestation. In contrast, the fetal arterial concentrations reported previously (Ousey *et al*. 2003) were in the μg/ml range, 5-fold higher than those reported here for cardiac blood or by others (Holtan *et al*. 1991), but were collected from chronically implanted catheters in fetuses of pregnant pony mares. Concentrations of

Table 3 Median and range ΔCt for transcript abundance of steroidogenic enzymes in tissues by gestational ages in months mo.

	**Fetal adrenal			**Fetal gonad			**Allantochorion		
	4 months	6 months	10 months	4 months	6 months	10 months	4 months	6 months	10 months
CYP11A1									
Median	2.4[a,b]	3.7[a]	0.7[b]	−1.5[c,d]	−1.7[c]	−1.0[d]	7.0	7.5	7.4
Range	2.2, 2.6	3.0, 4.3	−0.3, 1.7	−1.8, −1.2	−1.7, −1.6	−1.4, 0.0	5.9, 8.1	6.7, 8.3	7.0, 9.7
CYP17A1									
Median	1.5	1.8	−2.9	−8.8	−9.2	−8.9	2.8	2.9	2.2
Range	1.2, 1.8	−0.2, 2.2	−3.7, 0.1	−9.4, −8.7	−10.1, −5.4	−9.1, −8.1	2.2, 3.9	1.3, 3.4	0.3, 2
CYP19A1									
Median	15.7	11.0	9.1	10.3	11.9	11.0	−5.7	−6.9	−5.3
Range	13.7, 17.7	2.3, 12.9	7.1, 12.1	9.9, 11.0	11.1, 12.8	4.3, 13.6	−6.3, −5.3	−7.2, −5.7	−5.9, −4.3
HSD3B1									
Median	1.3[a,b]	1.1[a]	−3.2[b]	11.5	8.0	12.0	−1.7	−2.0	−1.5
Range	0.8, 1.9	0.0, 2.6	−3.4, −0.6	5.3, 16.8	5.5, 12.8	4.5, 13.4	−2.3, −1.1	−2.8, −1.8	−3.6, −1.0
SRD5A1									
Median	5.3	5.6	4.5	5.8	6.3	7.1	3.3[e]	0.9[f]	1.2[f]
Range	4.8, 5.7	5.2, 6.1	4.1, 7.6	4.7, 6.4	4.9, 9.5	6.2, 7.3	2.2, 4.9	−0.7, 1.4	0.6, 1.8

**Within tissue fetal adrenal[a,b]; fetal gonad[c,d]; allantochorion[e,f]; different gestational ages with different superscripts differ, $P<0.05$.
CYP11A1, *CYP17A1* and *CYP19A1* genes encoding cholesterol side chain cleavage, 17α-hydroxylase/17,20-lyase and aromatase cytochromes P450 respectively; and *HSD3B1* and *SRD5A1* genes encoding 3β-hydroxysteroid dehydrogenase/Δ5–4 isomerase, and 5α-reductase type 1 respectively.

pregnenolone in 4- and 6-month-old (120–180 days) fetuses in this study were also significantly elevated above those seen in 9- and 10-month (270–300 days) fetuses. Previous investigators (Ousey *et al*. 2003) saw no significant change over the gestational intervals studied (180–220, 260–280 and >300 days). Consistent with the data reported herein, others (Chavatte *et al*. 1995*a*) have noted that corticoid synthesis from pregnenolone or progesterone decreased from a higher rate in tissues from 4- to 5-month-old fetuses to lower rate in 9- to 10-month and older fetuses. Although there were some discrepancies among the trends in tissue concentrations of steroids over time, the current data agree with previous reports (Holtan *et al*. 1991, Ousey *et al*. 2003) in terms of the relative abundance of steroids measured in fetal blood, gonad and adrenal tissues: concentrations of pregnenolone were higher than progesterone and other detectable 5α-reduced pregnanes.

Although the origin(s) of the very high concentrations of pregnenolone in the serum of equine fetuses was not entirely resolved, the results of the current study indicate that pregnenolone produced by the fetal gonads contributes significantly to the concentrations measured in fetal serum. Concentrations of pregnenolone in fetal gonads and adrenal glands were higher than all other steroids measured in these tissues (Fig. 2) and were 100–150 times higher than concentrations in the allantochorion (Fig. 6). These observations were consistent with the abundance of transcript encoding *CYP11A1*, which was far lower in allantochorion than either fetal adrenal or gonad (Table 2). Despite detection of *CYP11A1* protein in equine placenta by immunohistochemistry (Han *et al*. 1995*b*), the more quantitative data presented here argue against significant endogenous synthesis of pregnenolone by the equine placenta. Rather, they are consistent with an extra-placental, fetal origin. There was no clear distinction between fetal adrenal and gonad as to likely contributions of these organs based on tissue concentrations of pregnenolone or *CYP11A1* transcript data alone. However, DHEA is undoubtedly of fetal gonadal origin, and fetal serum concentrations of pregnenolone and DHEA were highly correlated with one another, which is consistent with having a common source. Moreover, the relative size of the fetal organs *in situ* would certainly influence (and perhaps determine to a significant degree) the extent of their contribution to circulating levels in the fetus. Previous reports demonstrate that the fetal gonads (Cole *et al*. 1933, Wesson & Ginther 1980) exceed the weight of the fetal adrenal glands by 10-fold or more at almost any point in gestation (Comline & Silver 1971, Yamauchi 1979). Therefore, despite similar tissue concentrations of pregnenolone, the high correlation between fetal serum concentrations of pregnenolone and DHEA and the greater gonadal than adrenal size (and probably blood flow) favors the gonads as the more likely, significant source of pregnenolone in the fetal circulation.

In contrast to the above considerations based on the greater size of the fetal gonads over the adrenal glands, experiments conducted previously (Pashen & Allen 1979) provide perhaps the most persuasive evidence that the fetal gonads do not make a major contribution to substrate supply for placental synthesis of pregnanes. In that elegant study, fetal gonadectomy did not influence maternal concentrations of pregnanes, although concentrations of estrogen declined dramatically. These latter data confirmed their role in the provision of androgens for placental aromatization but left doubt as to the importance of the gonads in also providing substrate for placental secretion of pregnanes. It would seem clear that, if fetal gonadal pregnenolone is the primary source of substrate driving placental synthesis of 5α-reduced pregnanes, fetal gonadectomy would have to significantly diminish maternal concentrations. However, maternal concentrations were measured by competitive binding in those studies, and the concentrations reported were already very low at a time when, by mass spectrometric analyses, levels of 5α-reduced pregnanes are in fact extremely high (Holtan *et al*. 1991, Legacki *et al*. 2016*b*). Cross-recognition of the multiple 5α-reduced pregnanes (present in very high concentrations) by the binding globulin used in that assay had to have been very low indeed. Under such circumstances, changes in relevant 5α-reduced pregnanes would not have been detectable by the competitive binding method used. Alternatively, it is possible that the fetal adrenal glands or the placenta itself increased the synthesis of pregnenolone to compensate for the loss of it after fetal gonadectomy. A compensatory increase in placental production of progesterone by, and conversion of pregnenolone to progesterone in, the placentomes of cows has been observed after induced regression of the corpus luteum (and thus loss of luteal production of progesterone) in mid-to-late gestation (Conley & Ford 1987). Perhaps fetal adrenal and/or placental synthesis of pregnenolone in equine pregnancies also increase after the removal of the fetal gonads and help to maintain synthesis of bioactive pregnanes and pregnancy, as previously proposed (Chavatte *et al*. 1997). Additional studies will be required to explore this possibility.

In summary, the data from the present studies demonstrate the broad agreement between concentrations of tissue steroids and levels of transcripts encoding steroidogenic enzymes among equine fetal adrenal glands, fetal gonads and allantochorion. The fetal gonads exhibit high abundance of transcripts encoding for *CYP11A1* and *CYP17A1*, but low abundance of *HSD3B1*, consistent with high tissue concentrations of pregnenolone and DHEA. Despite higher transcript abundance of *HSD3B1*, high *CYP11A1* in the fetal adrenal is equally consistent with high tissue concentrations of pregnenolone but also with detectable concentrations of progesterone. In contrast, allantochorion exhibits much lower expression of *CYP11A1* and *CYP17A1*, but higher *HSD3B1*, *SRD5A1* and *CYP19A1*. This pattern of transcript abundance in placenta is more consistent with the utilization of pregnenolone for the synthesis of 5α-reduced pregnanes, and of DHEA for estrogen production, than it is for the endogenous synthesis of either pregnenolone or DHEA by the placenta itself. Given similar tissue concentrations of pregnenolone, but considering their much greater comparative size, the fetal gonads likely contribute more significantly to circulating concentrations of fetal pregnenolone and thereby placental synthesis of 5α-reduced pregnanes than do the fetal adrenal glands.

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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