Steroidogenic enzyme activities in the pre- and post-parturient equine placenta

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Abstract

Steroidogenic enzymes in placentas shape steroid hormone profiles in the maternal circulation of each mammalian species. These include 3 β -hydroxysteroid dehydrogenase/ Δ 5-4 isomerase (3 β HSD) and 17 α -hydroxylase/17,20-lyase cytochrome P450 (P450c17) crucial for progesterone and androgen synthesis, respectively, as well as aromatase cytochrome P450 (P450arom) that converts Δ 4-androgens to estrogens. 5 α -reductase is another important enzyme in equine placentas because 5 α -dihydroprogesterone (DHP) sustains pregnancy in the absence of progesterone in the second half of equine pregnancy. DHP and its metabolites decline dramatically days before foaling, but few studies have investigated placental enzyme activity before or at parturition in mares. Thus, key enzyme activities and transcript abundance were investigated in equine placentas at 300 days of gestation (GD300) and post-partum (term). Equine testis was used as a positive control for P450c17 activity. Substrates were incubated with microsomal preparations, together with enzyme inhibitors, and products were measured by liquid chromatography tandem mass spectrometry or radiometric methods (aromatase). Equine placenta expressed high levels of 3 β HSD, 5 α -reductase and aromatase, and minimal P450c17 activity at GD300 compared with testis (600-fold higher). At foaling, 3 β HSD and aromatase activities and transcript abundance were unchanged but 5 α -reductase (and P450c17) was no longer detectable ($P < 0.05$) and transcript was decreased. Trilostane inhibited 3 β HSD significantly more in testis than placenta, suggesting possible existence of different 3 β HSD isoforms. Equine placentas have significant capacity for steroid metabolism by 5 α -reductase, 3 β HSD and aromatase but little for androgen synthesis lacking P450c17. Declining pre-partum 5 α -reduced pregnane concentrations coincide with selective loss of placental 5 α -reductase activity and expression at parturition in horses.

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Introduction

The mammalian placenta expresses an array of steroidogenic enzymes that ultimately shapes the unique profile of steroid hormones identifiable in the maternal circulation for each species. Those enzymes most crucial for placental sex steroid synthesis include 3 β -hydroxysteroid dehydrogenase/ Δ 5-4 isomerase (3 β HSD) and 17 α -hydroxylase/17,20-lyase cytochrome P450 (P450c17) that are involved in progesterone and androgen synthesis, respectively, and aromatase cytochrome P450 (P450arom) that converts Δ 4-androgens to estrogens (Conley & Mason 1990, 1994, Conley & Hinshelwood 2001). 5 α -reductase is another important enzyme especially in the equine placenta because 5 α -reduced pregnanes dominate the steroid profile in the second half gestation (Holtan *et al.* 1991, Scholtz *et al.* 2014, Legacki *et al.* 2016a,b). Progesterone itself is undetectable after approximately

200 days of gestation, and the maintenance of pregnancy relies on the synthesis of 5 α -dihydroprogesterone (DHP) that activates the equine progesterone receptor with a biopotency equal to progesterone itself (Scholtz *et al.* 2014). Clearly, however, major differences exist in the types, patterns and concentrations of steroids secreted during gestation, even among closely related species, that reflect variations in the expression of placental steroidogenic enzymes (Geisert & Conley 1998, Jenkin & Young 2004, Mitchell & Taggart 2009). For example, unlike humans, the placentas of rats and mice express P450c17, but they lack P450arom expression and thus androgens not estrogens are the major steroids secreted by the rodent placenta (Geisert & Conley 1998). In contrast, the human and non-human primate placenta expresses high levels of P450arom but lacks expression of P450c17 and as a result must utilize fetal androgens for placental estrogen synthesis

(Simpson & MacDonald 1981, Conley *et al.* 2004). As in human pregnancies, estrogen synthesis in pregnant mares is fueled by fetal androgens, because the placenta is also believed to lack P450c17 expression and androgen synthetic capacity (Conley 2016). To date, this has not been thoroughly documented in the literature.

The endocrinology of equine pregnancy differs markedly from most other mammals (Conley 2016). During the second trimester of gestation in the mare, the concentration of maternal blood progesterone gradually decreases to the point that it becomes undetectable (Short 1959, Holtan *et al.* 1991, Legacki *et al.* 2016b). As progesterone concentrations decrease, there is a concomitant increase in 5 α -reduced pregnanes including 5 α -dihydroprogesterone (DHP) and associated metabolites (Holtan *et al.* 1991, Scholtz *et al.* 2014, Legacki *et al.* 2016b). The concentrations of DHP and multiple other 5 α -reduced metabolites increase in the second half of pregnancy, reaching peaks a few days before foaling, when concentrations suddenly fall (Seamans *et al.* 1979, Legacki *et al.* 2016a). This contrasts with estrogens, the concentrations of which do not change detectably until after parturition (Haluska & Currie 1988, O'Donnell *et al.* 2003). The contrasting patterns of 5 α -reduced pregnane and estrogen concentrations around the time of parturition presumably reflect dynamic changes in enzyme activities in the placenta. To date, few studies have been conducted to characterize enzyme activities in the equine placenta (Ainsworth & Ryan 1966, Pashen *et al.* 1982, Numazawa & Osawa 1987, Marshall *et al.* 1989, 1996, 1999, Chavatte *et al.* 1995). None have reported quantitative estimates of activity, compared the activities of different enzymes or investigated the potential changes in them, before and at the time of parturition. Though transcript abundance (and immunoblot analyses) can provide insight into what might be regulating expression of specific proteins (Legacki *et al.* 2017), reactions are dependent on multiple protein components including redox partners and accessory proteins (Conley & Bird 1997, Corbin *et al.* 2001, Nguyen *et al.* 2009). Thus, transcript analysis of steroidogenic enzymes cannot accurately predict (or allow a comparison of) catalytic capacities, which is what ultimately determines steroid synthesis and the patterns of secretion that are unique to each species.

Therefore, the following studies were conducted to investigate the activities and transcript abundance of several key steroidogenic enzymes in equine placentas. The relative levels of activity of placental 3 β HSD, aromatase and 5 α -reductase were examined at day 300 of gestation (GD300) and at the time of parturition. The interval from GD300 to term represents a period during which the concentrations of 5 α -reduced pregnanes decrease dramatically (Legacki *et al.* 2016a). It was hypothesized that measuring placental enzyme activities prior to and at foaling would provide insight into the

mechanisms regulating the dramatic changes in steroid concentrations seen over the last few days of gestation. In addition, we sought to confirm the lack of P450c17 enzyme activity in the equine placenta, published data for which is surprisingly scant (Mason *et al.* 1993), including information from a single placenta only in one published report (Ainsworth & Ryan 1966). Equine testes were included in experiments as a positive control based on these previous studies and our recent observations indicating a relative lack of transcript abundance for P450c17 in equine placentas (Legacki *et al.* 2017).

Materials and methods

Animals

Experiments were approved by the Institutional Animal Use and Care Advisory Committee at the University of California, Davis (UCD) and the University of Kentucky, Lexington (UK), in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Placental samples were recovered from normal mares killed at 300 days of gestation (GD300, $n=5$, 11.6 ± 2.1 years of age) and from term mares ($n=4$, 9.5 ± 2.2 years of age) after they were released. All mares were controls for unrelated studies conducted at the United Kingdom, as described previously (Legacki *et al.* 2017). Additional samples were collected from term placentas passed at foaling by mares stabled at UCD ($n=9$) specifically for measuring aromatase activity. These were all quarter horses, aged 8.8 ± 0.9 years. Gestation lengths were normal ranging from around 330 to 343 days. All foals were alive and healthy at birth without any veterinary intervention or assistance. Testes were obtained at routine clinical castration from stallions ($n=4$) of mixed breed and of unknown ages. All tissues were collected on ice until processed. Aliquots of tissues were frozen rapidly and stored until analyzed for steroidogenic enzyme activity. Additional aliquots were preserved in RNeasy lysis buffer (Thermo Fisher Scientific), refrigerated overnight (4°C) and subsequently frozen at -80°C until RNA isolation.

Microsomal enrichment

Equine placental and testicular tissues were homogenized in buffer (0.1 M K_3PO_4 , pH 7.4, 20% glycerol, 5 mM β -mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride and 1 $\mu\text{g}/\mu\text{L}$ aprotinin) and then briefly sonicated. Homogenates were centrifuged at 15,000 g for 10 min and supernatant was transferred to a new tube and centrifuged again at 100,000 g for 1 h. The resulting microsomal pellet was recovered and resuspended. The concentrations of crude protein were determined using the Pierce BCA Protein Reagent (Thermo Scientific). Microsomal preparations were stored in aliquots at -80°C .

Standards and solutions

The following standards were purchased from Steraloids (Newport, RI, USA): 5 α -dihydroprogesterone (DHP),

allopregnanolone (3 α DHP), pregnenolone, 5 α -pregnan-3 β ,20 α -diol (3 β ,20 α DHP), 20 α -hydroxy-5 α -dihydroprogesterone (20 α DHP), progesterone and d9-progesterone. 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).

Enzyme activities

All reactions were conducted with 10–12 μ g of microsomal protein in phosphate buffer (50 mM K₃PO₄, 1 mM EDTA, pH 7.4) with appropriate substrates. Parallel reactions were conducted with addition of enzyme inhibitors to verify that the steroids detected after incubation resulted from active synthesis. Placental 5 α -reductase activity in microsomes from GD300 and term placentas (as well as equine testes) were incubated with 3 μ M progesterone with and without finasteride (100 μ M; specific 5 α -reductase inhibitor) for 2 h at 37°C essentially as described previously for equine epididymis (Corbin *et al.* 2016). The activity of 17 α -hydroxylase/17,20-lyase cytochrome P450 (P450c17) was assessed similarly by measuring the accumulation of 17 α OH-progesterone and androstenedione from progesterone (3 μ M) substrate. Finasteride (100 μ M) was included as a control in 5 α -reductase assays to verify active synthesis. Finasteride also was included in all P450c17 assays to limit 5 α -reduced metabolism of progesterone. Ketoconazole (100 μ M), an inhibitor of cytochromes P450, was also added in the P450c17 reactions to confirm active synthesis. Reactions for both 5 α -reductase and P450c17 activities were supported by co-incubation with NADPH and a generating system (1 mM NADP⁺, 10 mM glucose-6-phosphate and 1.25 U glucose-6-phosphate dehydrogenase). The activity of 3 β HSD was examined essentially as described earlier (Conley *et al.* 2011), with minor modifications, from the accumulation of androstenedione from the added substrate, dehydroepiandrosterone (DHEA; 3 μ M). Incubations were conducted in the presence of 1 mM β NAD. Trilostane (30 μ M), a specific inhibitor of 3 β HSD, was used to verify active synthesis.

Steroid product analysis

The products of reactions for 5 α -reductase, P450c17 and 3 β HSD were extracted and analyzed by liquid chromatography tandem mass spectrometry as described in the following sections. This method detects progesterone, 17 α OH-progesterone, androstenedione, DHEA, dihydrotestosterone (DHT), testosterone, DHP and several of its metabolites including allopregnanolone, 20 α -hydroxy-5 α -dihydroprogesterone (20 α DHP), 3 β -hydroxy-5 α -dihydroprogesterone (3 β ,20 α DHP), as well as estrone. Samples were extracted and prepared as previously described (Legacki *et al.* 2016b). Briefly, the progesterone-d9 internal standard was added to all samples that were extracted subsequently with methyl-tert butyl ether (1:5). Calibrators ranged from 0.1 to 100 ng/mL and four levels of quality controls (QC; 0.6, 1.5, 20 and 80 ng/mL) were prepared alongside the samples, transferred into 12 $\times$ 75

glass tubes and dried in a Zymark TurboVap concentrator (Hopkinton, MA, USA) at 45°C under N₂. Samples were reconstituted with 200 μ L of 50:50 mix of water and methanol.

The LC–MS/MS method used has been validated as described in detail (Legacki *et al.* 2016b). In brief, however, reverse-phase gradient separation was performed on an Agilent UHPLC C18 analytical column (2.1 $\times$ 50 mm, 1.8 μ m) with two mobile phases delivered at 0.4 mL/min, an injection volume of 20 μ L 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, 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. 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 2 transitions per analyte. Inter- and intra-accuracy and precision were assessed at four QC concentrations for all analytes (six replicates). All analytes were measured with $\leq$ 15% deviation from expected concentrations for the three highest QC concentrations (1.5, 20 and 80 ng/mL) and $\leq$ 15% coefficient of variation (%CV). For the lowest QC concentration (0.6 ng/mL) pregnenolone, 20 α DHP and 3 β ,20 α DHP had $\leq$ 20% deviation from expected concentrations. All analytes measured had a percent accuracy (%Acc) $>$ 90% and a precision $<$ 15%. The responses for all analytes were linear and gave correlation coefficients (R^2) of $>$ 0.99.

Aromatase activity

Aromatase activity was measured radiometrically as validated and reported previously (Corbin *et al.* 2001) by monitoring the incorporation of tritium from [1 β -³H]-androstenedione (Perkin-Elmer) into ³H₂O. All reactions were conducted with 10 μ g of microsomal protein and were incubated for 2 h at 37°C in the presence of 150 nM androstenedione (10% labeled, 90% cold; Steraloids, Wilton, NH, USA) with a generating system (1 mM NADP⁺, 10 mM glucose-6-phosphate and 1.25 U glucose-6-phosphate dehydrogenase). The assay was stopped with 30% cold trichloroacetic acid, extracted with chloroform and mixed with a suspension of 5% charcoal and 0.5% dextran. After centrifugation at 2000 g for 30 min, the amount of ³H₂O produced was quantified by scintillation counting. Letrozole (1 μ M) was used in parallel reactions to verify active synthesis.

Transcript analysis by quantitative polymerase chain reaction (qPCR)

Total cellular RNA was extracted from frozen allantochorion using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA purity and concentration were assessed using the Agilent bioanalyzer RNA 6000 Nano kit (Agilent Technologies) with RNA integrity values (RIN) above 8.8. Samples of RNA (1 μ g/reaction) were treated with rDNase I (Thermo Fisher Scientific) and then reverse transcribed using the TaqMan Reverse Transcription Reagents

as described previously (Legacki *et al.* 2017). The mRNA expression of hydroxy- Δ^5 -steroid dehydrogenase (*HSD3B1*), cytochrome P450 family 19A1 (*CYP19A1*) and 5 α -reductase 1 (*SRD5A1*) were quantified by real-time quantitative PCR (qPCR) using primers described and validated as previously reported (Legacki *et al.* 2017). PCR efficiencies were calculated using LinRegPCR (version 2013.0; (Ruijter *et al.* 2009)). All reactions were pipetted using the epMotion Automated Pipetting Systems (Eppendorf; Hauppauge, NY, USA). The ΔC_t for each gene of interest was calculated by subtracting the C_t of the housekeeping genes (geometric mean of β -actin (*ACTB*) and β -glucuronidase (*GUSB*)) from the C_t of the gene of interest. Gene expression data are presented as relative quantification values. Changes in relative abundance of specific transcripts were examined by calculating the ΔC_t (Livak & Schmittgen 2001).

Statistical analysis

Analysis of variance was conducted using the Proc Mixed procedure in SAS (SAS Statistical Software, SAS Institute Inc., Cary, NC, USA). The correlations among the steroids in tissues were determined with the Proc Corr procedure. Data that did not meet the standards for normality were log transformed before analysis. The data were graphed using the means and standard errors calculated for each steroid. Differences in abundance of mRNA of steroidogenic enzymes based on ΔC_t values were examined using a two-tailed *t*-test in SAS. Differences among means and correlation coefficients were considered significant when $P < 0.05$.

Results

Estimates of 5 α -reductase activity were based on the rate of accumulation of measured 5 α -reduced metabolites of progesterone, and there was robust activity in microsomes from placentas collected at GD300. 3 β ,20 α DHP was found in the greatest amounts, synthesized at a rate of 12.0 ± 1.9 nmol/mg/h. Similarly, allopregnanolone was synthesized at rate of 3.5 ± 0.8 nmol/mg/h and DHP at 1.8 ± 0.3 nmol/mg/h in addition to trace amounts of 20 α DHP (Fig. 1). Finasteride, an inhibitor of 5 α -reductase activity, decreased the synthesis of all 5 α -reduced metabolites ($P < 0.05$) by 70% on average. In stark contrast to the active 5 α -reductase metabolism evident in microsomes from placentas collected at GD300, no 5 α -reduced progesterone metabolites were detected in incubations with the microsomal preparations from placentas collected at term (Fig. 1; $P < 0.001$).

The same microsomal preparations from placentas collected at GD300 and at term demonstrated rapid synthesis of androstenedione from DHEA, a measure of 3 β HSD activity. Androstenedione was synthesized at a rate of 165.0 ± 9.8 and 160.0 ± 11.7 nmol/mg/h in microsomes from placentas collected at GD300 and term, respectively. Trilostane, a specific inhibitor of 3 β HSD enzyme activity, inhibited the synthesis

Figure 1 5 α -Reductase enzyme activity (nmol/mg/h) in microsomal preparations (12 μ g protein) from equine placentas collected at 300 days of gestation (GD300) and at foaling (term) measured by metabolism of progesterone to 5 α -reduced products including dihydroprogesterone (DHP), 3 β ,20 α -dihydroxy-DHP (3 β ,20 α DHP), allopregnanolone (3 α DHP) and 20 α -hydroxy-DHP (20 α DHP), the sum of which is also shown (total). The 5 α -reduced metabolism of progesterone was conducted in the absence (control, black) or presence (finasteride, shaded) of a specific enzyme inhibitor to verify active synthesis. *Indicates finasteride-inhibited product formation ($P < 0.05$). **Note, no 5 α -reduced progesterone metabolites were detectable in incubations with microsomes from term placentas ($P < 0.001$).

Figure 2 3 β -Hydroxysteroid dehydrogenase/ Δ^5 -4 isomerase (3 β HSD) enzyme activity (nmol/mg/h) in microsomal preparations (12 μ g protein) from equine placentas collected at 300 days of gestation (GD300) and at foaling (term) measured by metabolism of dehydroepiandrosterone (DHEA) to androstenedione. The metabolism of DHEA to androstenedione by 3 β HSD was conducted in the absence (control, black) or presence (trilostane, shaded) of a specific enzyme inhibitor to verify active synthesis. Note: there were no significant differences between microsomes from GD300 and term placentas in apparent 3 β HSD activity. *Indicates trilostane inhibited androstenedione formation ($P < 0.05$).

Figure 3 Aromatase activity (nmol/mg/h) in microsomal preparations (12 µg protein) from equine placentas collected at 300 days of gestation (GD300) and at foaling (term) measured by the release of tritiated water following the aromatization of [1β-3H]-androstenedione. The metabolism of [1β-3H]-androstenedione was conducted in the absence (control, black) or presence (letrozole, shaded) of a specific enzyme inhibitor to verify active synthesis. Note: there were no significant differences between microsomes from GD300 and term placentas in apparent aromatase activity. **Indicates letrozole inhibited aromatase activity ($P < 0.01$).

of androstenedione ($P < 0.05$) by 30 and 37% in the GD300 and term microsomes respectively (Fig. 2). There was no significant difference in 3βHSD enzyme activity between the GD300 and term placental microsomes.

Aromatase activity, determined by the release of $^3\text{H}_2\text{O}$ from tritiated androstenedione, was also detectable in placental microsomes from GD300 and term placentas and was inhibited >99% by letrozole (Fig. 3; $P < 0.01$). The variability of the measured activity among the term placentas from the UK mares was extreme (0.01–2.25 nmol/mg/h). Consequently, additional samples were collected from mares foaling at UCD, which were assayed for comparison with similar results. Aromatase activity in the term placental samples from UCD exhibited a similarly wide range, from 0.01 to 3.01 nmol/mg/h. The data were combined and the levels of aromatase activity in microsomes from placentas collected at GD300 and at term (1.49 ± 0.32 and 1.01 ± 0.18 nmol/mg/h, respectively) were not significantly different from one another.

Incubations conducted in placental microsomes with progesterone as substrate in the presence of finasteride were examined for the synthesis of 17αOH-progesterone and androstenedione, as evidence of 17α-hydroxylase/17,20-lyase (P450c17) activity. There was detectable 17αOH-progesterone in incubations of microsomes from placentas collected at GD300, which was completely abolished by addition of ketoconazole, an inhibitor of cytochrome P450 activity. Based on 17αOH-progesterone accumulation in incubations with these specimens, the rate of P450c17 enzyme activity was detectable (0.08 ± 0.03 nmol/mg/h; Table 1). No androstenedione or testosterone was detected, even in the absence of ketoconazole. In contrast to placentas from GD300, there was no detectable metabolism of progesterone to 17αOH-progesterone or androstenedione in any of the microsomal preparations from term placentas.

Testicular microsomes were examined for P450c17 enzyme activity (Table 1) using the same incubation conditions as for placental microsomes. Progesterone was metabolized to 17αOH-progesterone, androstenedione and testosterone at rates of 30.5 ± 16.6 , 20.7 ± 8.7 and 3.4 ± 1.1 nmol/mg/h respectively. Product synthesis was inhibited in the presence of ketoconazole by 80% overall ($P < 0.01$). Additional incubations were also conducted with DHEA as substrate to measure 3βHSD enzyme activity and with [1β-3H]-androstenedione to measure aromatase activity. Apparent 3βHSD activity was 45.0 ± 22.7 nmol/mg/h, which was reduced to 9.3 ± 4.4 nmol/mg/h in the presence of trilostane, also over 80% inhibition of enzyme activity ($P < 0.01$). Aromatase activity in testicular microsomes averaged 0.44 ± 0.41 nmol/mg/h and was almost completely inhibited by letrozole (>99%).

The quality of RNA isolated from placentas collected from mares at GD300 and post-parturiently was high overall (9.4 ± 0.2 and 9.4 ± 0.3 respectively, 10 point scale), and essentially identical. The abundance of transcript encoding P450arom (CYP19A1) and 3β-HSD (HSD3B1) in the pre- and post-parturient placentas were not different (Table 2). However, the abundance of transcript encoding 5α-reductase (SRD5A1) was significantly lower (higher ΔCt) in the post-parturient placentas than that of the placentas collected at GD300 (Table 2; $P < 0.05$).

Table 1 Progesterone metabolism to 17OH-progesterone, androstenedione and testosterone (P450c17 activity, nmol/mg/h) in microsomes from placentas (day 300 of gestation, $n = 5$) and testes ($n = 4$), in the presence or not of ketoconazole, an enzyme inhibitor.

Tissue	17OH-progesterone	Androstenedione	Testosterone	Total
Placenta	0.08 ± 0.03^a	0^a	0^a	0.08 ± 0.03^a
Placenta + keto	0^b	0^a	0^a	0^b
Testis	40.3 ± 22.0^c	23.7 ± 10.0^b	3.9 ± 1.1^b	67.9 ± 32.4^c
Testis + keto	8.3 ± 5.4^d	2.8 ± 0.9^c	1.2 ± 0.1^c	12.3 ± 3.4^d

Finasteride (100 µM) was included in incubations with placental microsomes to minimize 5α-reductase activity that was minimal in preparations from testes.

^{a,b,c,d}Within columns (steroid products), means with different superscripts differ, $P < 0.01$.

Table 2 Relative mRNA transcript abundance for *CYP19A1* (encodes aromatase cytochromes P450), *HSD3B1* (encodes 3 β -hydroxysteroid dehydrogenase/ Δ 5-4 isomerase), and *SRD5A1* (encodes 5 α -reductase type 1) in equine chorioallantois at 10 months gestational age ($n=4$) and postpartum ($n=4$).

	GD300	Term
<i>CYP19A1</i>	-8.05 ± 0.40	-5.82 ± 1.77
<i>HSD3B1</i>	-0.46 ± 0.80	-2.31 ± 0.90
<i>SRD5A1</i>	3.39 ± 0.59^a	8.27 ± 0.69

Data expressed as mean ($\pm$ S.E.M.) Δ Ct as determined by quantitative polymerase chain reaction (qPCR) using the geometric mean of β -actin (*ACTB*) and β -glucuronidase (*GUSB*) as reference transcripts. Lower values of Δ Ct represent higher mRNA expression.

^aValues differ between GD300 and post-parturient (term) placentas ($P < 0.05$).

Discussion

The equine placenta is thought to be reliant on the fetus for the provision of steroid substrates that it cannot synthesize in sufficient quantities itself for the lack of expression of the requisite enzymes (Conley 2016, Legacki *et al.* 2017). The best known and documented example is the provision of DHEA by the fetal gonads, which is used by the placenta for the synthesis of estrogens (Pashen & Allen 1979, Raeside *et al.* 1979). The equine placenta is thought to be incapable of synthesizing androgens *de novo*, though data supporting this view are scant (Ainsworth & Ryan 1966, Mason *et al.* 1993). We have previously reported that the transcript abundance of *CYP17A1*, the gene encoding P450c17 the enzyme required for androgen synthesis, was extremely low in placenta compared with fetal gonads and adrenal glands (Legacki *et al.* 2017). The results of the current studies are consistent with these data, quantitatively demonstrating that the level of androgen synthesis from progesterone by placental microsomes is trivial compared with the capacity of testicular microsomes. However, the placenta can utilize fetal DHEA by virtue of expressing 3 β HSD (Pashen *et al.* 1982, Mason *et al.* 1993, Chavatte *et al.* 1995) and aromatase (Ainsworth & Ryan 1966, Mason *et al.* 1993, Marshall *et al.* 1996, 1999) activity, which the current results also confirm is robust pre- and post-partum. In addition, the placenta is thought to utilize pregnenolone derived from the fetus for 5 α -reduced pregnane synthesis based on high concentrations delivered via the umbilical artery and markedly lower concentrations in the umbilical vein (Ousey *et al.* 2003). This is consistent with very low *CYP11A1* transcript abundance in placenta compared with the fetal adrenals and gonads (Legacki *et al.* 2017). The results of these recent studies suggest further that the fetal gonads are likely a significant source of the high concentrations of pregnenolone seen in the fetal circulation (Legacki *et al.* 2017). Collectively then, the supply of pregnenolone and DHEA by the equine fetus compensates for the relative lack of transcripts encoded by *CYP11A1* and *CYP17A1* in the placenta and supports

both 5 α -reduced pregnane and estrogen synthesis during equine pregnancy. This is an unusual and particularly complex example of a feto-placental unit that is required to support steroid synthesis during gestation.

In addition to clarifying the enzymatic basis for placental pregnane and estrogen synthesis during pregnancy, the results of the current study also provide insight into mechanisms involved in the observed decline in 5 α -reduced pregnane concentrations that precede foaling in mares (Legacki *et al.* 2016a). The ability of the equine placenta to 5 α -reduce progesterone has been demonstrated *in vitro* (Hamon *et al.* 1991) and *in situ* (Moss *et al.* 1979, Marshall *et al.* 1999), but none to our knowledge have examined the enzyme activity at parturition. The presented data indicate that the capacity for 5 α -reduction disappears at the time of parturition though the capacity for metabolism of Δ 5 steroids (pregnenolone and DHEA) by 3 β HSD does not change at term from levels seen at GD300. Furthermore, the utilization of Δ 4 androgens (androstenedione) for estrogen synthesis by aromatase in the placenta also remained unchanged. The maintenance of 3 β HSD and aromatase enzyme activities argues strongly that the loss of 5 α -reductase in term placentas is not due to a general decline of placental enzymatic function at parturition. Similarly, it is unlikely to simply reflect tissue devitalization or protein deterioration after placental delivery given the high quality of RNA extracted and the correspondence between 5 α -reductase activity and *SRD5A1* transcript abundance, which was also lower in post-partum than GD300 placentas. Rather, these data suggest collectively that there is a selective reduction of 5 α -reductase in the placenta and is consistent with the dramatic decline in 5 α -reduced pregnanes in mares prior to foaling (Legacki *et al.* 2016a). Earlier studies have clearly documented the induction of expression of P450c17 and P450arom in the ovine placenta that fuels increased estrogen synthesis before parturition (Anderson *et al.* 1975, Flint *et al.* 1975, France *et al.* 1988, Mason *et al.* 1989). Cows may also exhibit an induction of *CYP17A1* preceding parturition (Shenavai *et al.* 2012). The opposite appears to be the case in the mare. In fact, the authors are unaware of any evidence for selective suppression of placental steroidogenic enzyme expression as a prelude to parturition in any species.

One of the concerns of the present experiment remains the potential for agonal degradation of placental tissues and associated steroidogenic enzyme activities during the interval from whenever endometrial separation occurred and the placenta was delivered to the time of collection. The major factor would be the onset of tissue anoxia that follows the lack of perfusion and the time before autolysis or necrosis begins. The placental tissues collected in Davis were within a few minutes of passage as these mares and their foals were under close and constant observation, but may have been hours in the case of

the tissues collected in Lexington. Placental enzyme activities measured after microsomal isolation were robust in both cases nonetheless. Since 5 α -reductase is in the microsomal fraction (Corbin *et al.* 2016), like 3 β HSD and aromatase (Conley *et al.* 2011), loss of activity due to necrosis or autolysis would be expected to affect this and all in that cellular compartment, not any one activity selectively, as noted earlier. Moreover, placental tissue is unusually resistant to anoxia (Schneider 2009). The high RIN values from isolated placental transcripts in this study are consistent with maintenance of cellular integrity until the time of tissue collection and processing. The RIN values observed compared very favorably to those of RIN values for human placental samples collected and stored under ideal conditions (Martin *et al.* 2017). Metabolic changes can be detected within a few minutes following separation of the human placenta from their uterine blood supply (Cirelli *et al.* 2000, Serkova *et al.* 2003). These metabolic responses can include a halving or more of adenosine triphosphate concentrations rapidly (Serkova *et al.* 2003). Yet, re-perfusion after as much as 3 h of anoxia is followed by normalization of those cellular parameters (Schneider 2009) suggesting cellular viability is maintained. Consistent with these published observations, viable trophoblast for cell culture and perfusion can be isolated successfully from bovine placentas hours after collection (Conley & Ford 1987), further verifying that there is an extended period of tissue viability after loss of placental perfusion. However, to what degree enzyme activities in post-parturient placentas reflect those in placentas in the days immediately preceding parturition in mares will require additional, very carefully timed studies.

Lastly, it is noteworthy that the inhibition of 3 β HSD by trilostane was markedly more effective in testicular than in placental microsomes even though levels of activity were robust in both, within an order of magnitude of each other. To date, there is no evidence for the existence of multiple isoforms of 3 β HSD in the horse as there is in human and other species. However, there are differences in the sensitivity of human isoforms encoded by *HSD3B1* and *HSD3B2* to trilostane as well as differences in the mode of inhibition (Thomas *et al.* 2004). Similar observations of differential sensitivity of enzyme activity led to the discovery of isoforms of aromatase (Corbin *et al.* 1995). It is of interest to note that investigators have previously reported substantial differences between the equine placenta and equine fetal adrenal glands in the rates of utilization of pregnenolone and DHEA as substrates for 3 β HSD activity. Fetal adrenal tissues converted far less pregnenolone to progesterone than they did DHEA to androstenedione, whereas placental 3 β HSD utilized both substrates to a similar degree (Pashen *et al.* 1982). Kinetic studies on placental and

testicular microsomes would be necessary to properly investigate the evidence for the possible existence of multiple equine *HSD3B* isoforms and would require molecular studies to verify it, as for the porcine aromatases (Corbin *et al.* 1999, 2001). Such studies are well beyond the scope of those conducted but remain an intriguing possibility suggested by the apparent differences in sensitivity of placental compared with testicular 3 β HSD to inhibition by trilostane. If true, this would represent yet another similarity between mares and women in steroidogenesis during pregnancy (Conley 2016).

In conclusion, the data presented quantify activities of several enzymes in the equine placenta before and at the time of parturition for the first time. The equine placenta has a pronounced capacity for steroid metabolism by expression of 5 α -reductase, 3 β HSD and aromatase activities but has little capacity for androgen synthesis lacking significant P450c17 activity. Parturition is associated with a selective loss of 5 α -reductase activity and transcript abundance without any such changes in either 3 β HSD or aromatase. The loss of placental 5 α -reductase activity and expression is consistent with the dramatic decline in 5 α -reduced pregnane metabolites that occurs in the days before foaling and likely contributes substantially to 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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