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Serum and tissue pregnanes and pregnenes after dexamethasone treatment of cows in late gestation

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Abstract

Dexamethasone (DEX) initiates parturition by inducing progesterone withdrawal and affecting placental steroidogenesis, but the effects of DEX in fetal and maternal tissue steroid synthetic capacity remains poorly investigated. Blood was collected from cows at 270 days of gestation before DEX or saline (SAL) treatment, and blood and tissues were collected at slaughter 38 h later. Steroid concentrations were determined by liquid chromatography tandem mass spectrometry to detect multiple steroids including 5α-reduced pregnane metabolites of progesterone. The activities of 3β-hydroxysteroid dehydrogenase (3βHSD) in cotyledonary and luteal microsomes and mitochondria and cotyledonary microsomal 5α-reductase were assessed. Quantitative PCR was used to further assess transcripts encoding enzymes and factors supporting steroidogenesis in cotyledonary and luteal tissues. Serum progesterone, pregnenolone, 5α-dihydroprogesterone (DHP) and allopregnanolone (3αDHP) concentrations (all <5 ng/mL before treatment) decreased in cows after DEX. However, the 20α-hydroxylated metabolite of DHP, 20αDHP, was higher before treatment (≈100 ng/mL) than at slaughter but not affected by DEX. Serum, cotyledonary and luteal progesterone was lower in DEX- than SAL-treated cows. Progesterone was >100-fold higher in luteal than cotyledonary tissues, and serum and luteal concentrations were highly correlated in DEX-treated cows. 3βHSD activity was >5-fold higher in luteal than cotyledonary tissue, microsomes had more 3βHSD than mitochondria in luteal tissue but equal in cotyledonary sub-cellular fractions. DEX did not affect either luteal or cotyledonary 3βHSD activity but luteal steroidogenic enzyme transcripts were lower in DEX-treated cows. DEX induced functional luteal regression and progesterone withdrawal before any changes in placental pregnene/pregnane synthesis and/or metabolism were detectable.

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Introduction

The establishment and maintenance of pregnancy, and the initiation of parturition, have long been recognized to rely on hormones including progestins, estrogens and prostaglandins, the secretion of which are suppressed or promoted dependent on stage of gestation (Thorburn *et al.* 1977). In cattle, we and others have focused on progesterone and estrogens (estrone, estradiol and estrone sulphate) as the major steroids involved, placental synthesis and target tissues (Conley & Ford 1987, Hoffmann & Schuler 2002, Schuler *et al.* 2008, Nguyen *et al.* 2012, Conley & Reynolds 2014, Schuler *et al.* 2018), along with placental enzyme expression (Conley *et al.* 1992, Schuler *et al.* 2006). However, recent studies in our laboratory investigating steroid secretion during equine pregnancy have identified 5α-reduced pregnanes, 5α-dihydroprogesterone (DHP) specifically, as an important progestin (Scholtz *et al.* 2014, Legacki *et al.* 2016, 2017, 2018, Reynolds *et al.* 2018) activating the progesterone receptor in a species-specific manner (Scholtz *et al.* 2014). Other direct metabolites of DHP exhibit other biopotent effects. As potent neuro-active steroids, 5α-reduced pregnanes, such as allopregnanolone (5α-pregnan-3α-ol-20-one or 3αDHP), have been hypothesized to suppress activation of the fetal pituitary–adrenal axis until parturition (Conley & Neto 2008, Brunton *et al.* 2014, Conley & Reynolds 2014). Measuring 5α-reduced pregnanes by immunoassay is difficult and requires mass spectrometry with appropriate method development (Legacki *et al.* 2016), an approach that has been little utilized in ruminant species to date. To the authors' knowledge, only one study has applied mass spectrometry to monitor changes in steroid hormone concentrations during gestation and parturition in cattle (Martins-Júnior *et al.* 2014), but 5α-reduced steroids were not included in that analysis.

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Thus, the studies examining changes in hormone concentrations in late pregnancy and at parturition in cattle have to date investigated only a limited number of steroids. None, to our knowledge, have examined 5α-reduced pregnane concentrations during gestation in cattle.

The role of fetal adrenal activation and cortisol secretion in fetal preparation for (Silver 1990), and the initiation of (Thorburn & Challis 1979), parturition are well known and have been investigated extensively in sheep in particular (Challis *et al.* 2000). Notwithstanding the significant contribution of the corpus luteum to pregnancy maintenance even in the final weeks of bovine gestation, a similar sequence of events is hypothesized to initiate parturition in cattle (Comline *et al.* 1974). The administration of synthetic corticoids, like dexamethasone (DEX), induces parturition in late gestation and is thought to trigger events in a way that resembles the spontaneous, natural birth process initiated by fetal adrenal activation (Adams & Wagner 1970). Recent, comprehensive studies (Shenavai *et al.* 2012) have compared many facets of placental gene expression and steroid secretion in cows induced to calve using DEX administration as well as a progesterone receptor antagonist, prostaglandin F2α, and natural spontaneous birth. Others studying DEX-induced parturition in cattle (Adams & Wagner 1970, Comline *et al.* 1974, Hirayama *et al.* 2012) have focused on the effects on the placenta (Hoffmann & Schuler 2002) with less attention given to luteal function. The current studies were conducted to re-evaluate DEX-induced parturition in cows in late gestation (day 270) by examining (1) the array of pregnenes (Δ5, pregnenolone; Δ4, progesterone and metabolites) and pregnanes (DHP and metabolites) in serum and in tissues, (2) the expression of enzymes (both transcript abundance and catalytic activities) involved in placental progesterone synthesis, as well as (3) luteal tissue progesterone concentrations and 3βHSD enzyme activity. It was hypothesized that DEX treatment would alter placental pregnene and pregnane synthesis and thereby facilitate progesterone withdrawal in the late gestation cow.

Materials and methods

Animals, treatments and sample collection

Experiments were approved by the Institutional Animal Use and Care Advisory Committee at the University of California, Davis and North Dakota State University (NDSU), and were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Cows were synchronized as previously described (Larson *et al.* 2006) and pregnancies established (day 0) by artificial insemination with frozen semen from a single sire. At gestational day 270, cows were randomly assigned to one of two treatments. Cows were either injected with saline alone (SAL, 4 mL; $n=8$) or DEX (dexamethasone sodium phosphate) suspended in saline at 10 mg/mL (4 mL; $n=9$). Blood samples were taken by jugular venipuncture just before treatment and at slaughter and allowed to clot. Serum was harvested and stored at −20°C. Thirty-eight hours after treatment, cows were slaughtered at the NDSU Meats Laboratory, a federally inspected facility, using approved methodology (AVMA 2013). At slaughter, the entire reproductive tract was obtained, and placenta (caruncle (maternal placental tissue) and cotyledon (fetal placental tissue)) and corpus luteum were dissected. Samples of caruncle, cotyledon and corpus luteum were snap-frozen in liquid nitrogen-cooled isopentane as described previously (Reynolds *et al.* 2015, 2018) and were stored at −80°C until they were used for analysis of tissue steroid concentrations and steroidogenic activities.

Microsomal and mitochondrial enrichment

Placental 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 µg/µL aprotinin) and then briefly sonicated. Homogenates were centrifuged at 15,000 ***g*** for 10 min to recover mitochondria and the supernatant was transferred to a new tube and centrifuged again at 100,000 ***g*** for 1 h to recover microsomes, as previously described and validated (Moran *et al.* 2002, Corbin *et al.* 2016, Legacki *et al.* 2017, 2018, Reynolds *et al.* 2018). The resulting pellets (15,000 ***g*** – mitochondrial; 100,000 ***g*** – microsomal) were recovered and resuspended. The concentrations of crude protein in each were determined using the Pierce BCA Protein Reagent (Thermo Scientific). Microsomal preparations were stored in aliquots at −80°C.

Standards and solutions

Standards were purchased from Steraloids (Newport, RI): 5α-dihydroprogesterone (5α-pregnan-3,20-dione, DHP), allopregnanolone (5α-pregnan-3α-ol-20-one or 3αDHP), 5α-pregnan-3β, 20α-diol (3β,20αDHP), 5α- pregnan-20α-ol-3-one (20αDHP), pregnenolone, progesterone, 20αOH-progesterone, androstenedione (A_4), testosterone dehydroepiandrosterone (DHEA), d^7-androstenedione (A_4-d^7), d^3-testosterone (T-d^3) and d^9-progesterone (P_4-d^9). 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

Both 3βHSD and 5α-reductase enzyme activities were examined in sub-cellular fractions of placental tissues. The activity of 3βHSD was determined as described earlier (Conley *et al.* 2012), with minor modifications. Cotyledonary 3βHSD activity was 4-fold higher than that in caruncular samples (data not shown) and thus subsequent studies focused on microsomal and mitochondrial sub-cellular fractions from cotyledonary tissue samples. Luteal tissues were treated similarly, but

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reactions utilized less protein incubated for shorter periods because of the high reaction rates relative to placental samples. Accordingly, reactions were conducted with 10–20 µg of microsomal or mitochondrial protein in phosphate buffer (100 mM K_3PO_4, 1 mM EDTA pH 7.4) with pregnenolone (10 µM final) as substrate at 37°C for 0.25–2 h and 10 mM βNAD. The incubation time was chosen, based on preliminary time course experiments with sample pools, to ensure that substrate was not limiting. Parallel reactions were conducted with addition of trilostane (100 µM), a specific inhibitor of 3βHSD, to verify that the steroids detected after incubation resulted from active synthesis. In addition, 5α-reductase activity was assessed in microsomal incubations conducted with 3 µM progesterone with and without finasteride (100 µM; specific 5α-reductase inhibitor) to confirm active synthesis, essentially as described previously for equine epididymis (Corbin *et al.* 2016). These reactions were supported by co-incubation with NADPH and a generating system (1 mM $NADP^+$, 10 mM glucose-6-phosphate and 1.25U glucose-6-phosphate dehydrogenase).

Steroid product analysis

The LC-MS/MS method used has been validated as described previously for serum/plasma (Legacki *et al.* 2016) and tissue steroids (Legacki *et al.* 2017). This method detects progesterone, pregnenolone, DHEA, A_4, DHP and several of its metabolites including 3αDHP (allopregnanolone), 20αDHP, 3β,20αDHP. Briefly, d^7-A_4 and d^9-P_4 internal standards were added to all serum samples which were extracted subsequently with methyl-tert butyl ether (1:5). The products of reactions for 5α-reductase and 3βHSD were extracted and prepared similarly. Frozen tissues were ground mechanically (Precellys, Rockville, MD, USA) using stainless steel beads (3.2 mm), extracted with a 50:50 mix of methanol:water and shaken at 6500 rpm for 60 s. Samples were then stored for 20 min at −20°C and shaken again using the same parameters. The supernatant was removed, put into glass screw top tubes 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 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 × 75 glass tubes and dried in a Zymark Turbovap Concentrator (Hopkinton, MA, USA) at 45°C under N_2. Samples were reconstituted with 200 µL of 50:50 mix of water and methanol. The reverse-phase gradient separation was performed on an Agilent UHPLC C18 analytical column (2.1 × 50 mm, 1.8 µm ps) with two mobile phases delivered at 0.4 mL/min, an injection volume of 20 µL and a column temperature of 40°C. Mobile phase A and B were water with 0.2% formic acid and methanol, respectively. An elution gradient was held at 40% B for the first 0.2 min, 40–60% B from 0.2 to 1 min, 60–80% B from 1 to 10 min, 80–90% B from 10.0 to 10.1 min, 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 (Bruker Daltronics Inc., Billerica, MA, USA). Detection and quantitation of all analytes were accomplished using multiple reaction monitoring with a minimum of two transitions per analyte. Inter- and intra-accuracy and precision were assessed at four QC concentrations for all analytes (six replicates). All analytes were 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) pregnenolone, 20αDHP and 3β,20αDHP had ≤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.

Transcript abundance

Expression levels of transcripts encoding steroid synthesizing and metabolizing enzymes were determined by quantitative reverse-transcriptase/polymerase chain reaction (qPCR) analysis using SYBR green chemistry and the ΔΔCT method (Livak & Schmittgen 2001) using primers as previously described (Reynolds *et al.* 2015, 2018). All samples were compared to a reference pool comprising RNA isolated from bovine caruncles (maternal portions of the placenta) and fetal adrenal glands from the SAL-treated cows from this study. In addition, expression of β-actin was used as the endogenous reference.

Statistical analysis

The differences in concentration among each of the measurable steroids in serum, tissue and products of steroidogenic enzyme assays were subjected to ANOVA using the Proc Mixed function in SAS (SAS Statistical Software, SAS Institute Inc.). Analysis of serum steroid concentrations before and after treatment incorporated a repeated-measures design. Main effects of treatment, and for serum data treatment by time interactions, were determined and Pearson's correlation co-efficients were determined. Differences among means were examined by orthogonal contrasts. The data were graphed using the means and standard errors. Expression of select transcripts was analyzed as the ΔCT by subtracting the CT of the ACTB from the CT of the transcript of interest (Livak & Schmittgen 2001). Expression data are presented as median and range for ΔCT.

Results

Serum steroid concentrations

Progesterone was present at concentrations ranging from 3 to 5 ng/mL before treatment (Fig. 1). Progesterone concentrations did not change with time in the SAL injected cows but were lower in cows after DEX treatment with significant main effects of treatment and time ($P < 0.05$) and a significant treatment by time interaction ($P < 0.01$). Luteal weight also tended to be lower in DEX- than in SAL-treated cows (5.24 ± 0.30 vs 5.82 ± 0.25 g, $P < 0.08$) (Supplementary Figure 1 (see section on Supplementary data given at the end of

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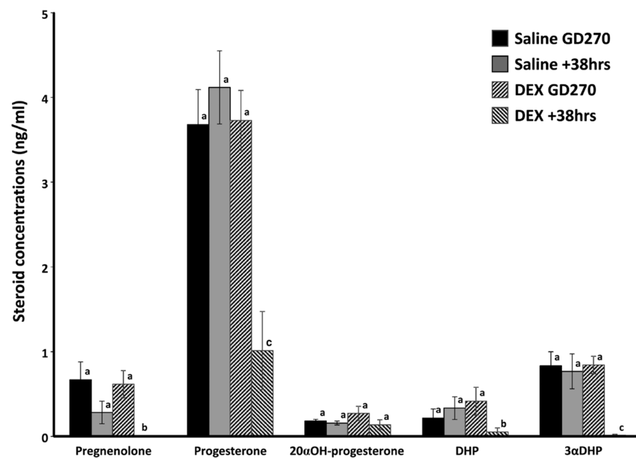

Figure 1 Serum steroid concentrations (ng/mL) in saline- (SAL) and dexamethasone-treated (DEX) cows before and 38 h after treatment on day 270 of gestation. Steroids concentrations were determined by LC-MS/MS, as described in Materials & methods. For each steroid depicted, differences between means are indicated, $^{a,b}P<0.05$, $^{a,c}P<0.01$.

the article)). Several other 5α-reduced pregnanes and pregnenes were detected included allopregnanolone (3αDHP), pregnenolone ($P<0.05$), DHP and 20αOH-progesterone, all of which were <1 ng/mL on average (Fig. 1). Dexamethasone treatment led to a significant decrease in serum concentrations of pregnenolone, DHP ($P<0.05$) and allopregnanolone at the time of slaughter ($P<0.01$; Fig. 1). Notably also, 20αOH-DHP was by far the most abundant steroid measured, present in some cows at >100 ng/ml (Fig. 2), more than 20-fold higher than progesterone and over 100-fold higher than the other measured 5α-reduced pregnanes before treatment.

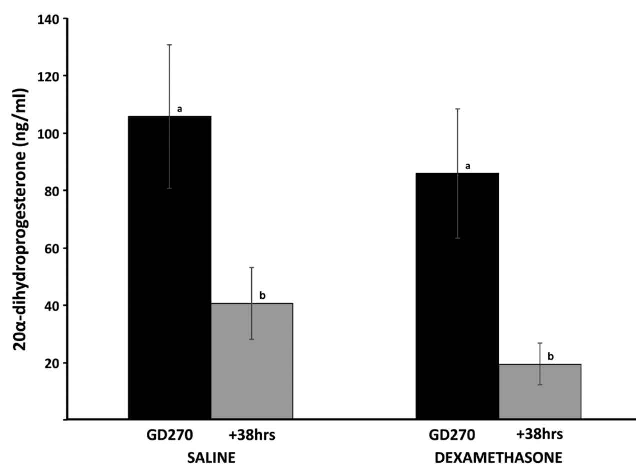

Figure 2 Serum concentrations (ng/mL) of 20α-hydroxy-5α-dihydroprogesterone (20αOH-DHP) in saline- (SAL) and dexamethasone-treated (DEX) cows before and 38 h after treatment on day 270 of gestation. Steroids concentrations were determined by LC-MS/MS, as described in Materials & Methods. [a],[b]Means with different superscripts differ, $P<0.05$.

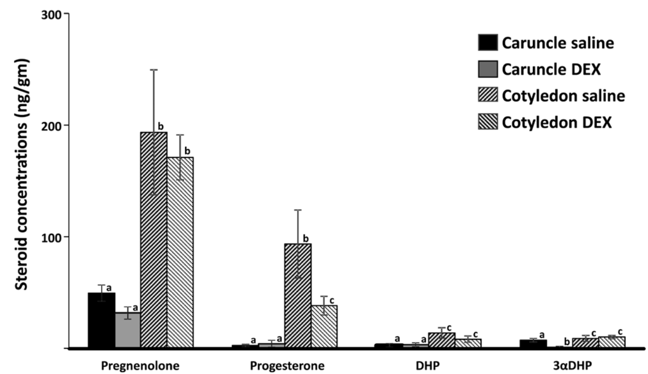

Figure 3 Steroid concentrations (ng/g) in cotyledonary and caruncular tissues from saline- (SAL) and dexamethasone-treated (DEX) cows collected at slaughter 38 h after treatment on day 270 of gestation. Steroids concentrations were determined by LC-MS/MS, as described in Materials & Methods. For each steroid depicted, differences between means are indicated, $^{a,b}P<0.01$, $^{a,c}P<0.05$, $^{b,c}P<0.05$.

There was a decrease in 20αDHP with time ($P<0.05$), but no main effects of DEX and no interaction.

Tissue steroid concentrations

The cotyledonary (fetal) component of the placentome had significantly higher tissue concentrations of pregnenes (pregnenolone and progesterone; $P<0.01$) and pregnanes (DHP and allopregnanolone; $P<0.05$) than the caruncular (maternal) component of the placentomes (Fig. 3). Pregnenolone was the highest of the measured steroids in the cotyledon (182.3 ± 51.5 ng/g tissue) and progesterone (90.4 ± 30.0 ng/g tissue) the next highest. Pregnenolone was also the steroid in highest concentrations in the caruncle (47.6 ± 7.3 ng/g tissue). Treatment with DEX reduced progesterone in cotyledons (93.44 ± 30.53 vs 38.16 ± 8.44 ng/g tissue, $P<0.05$) and allopregnanolone in caruncles (7.29 ± 1.72 vs 0.88 ± 0.88 ng/g, $P<0.05$), but did not affect cotyledonary pregnenolone or DHP (Fig. 3). There was no correlation between cotyledonary and serum concentrations of progesterone.

Steroid concentrations in luteal tissue (Fig. 4) were, on average, far higher than those in placental tissues. Luteal tissue progesterone concentrations (33.4 ± 1.9 µg/g) were 10-fold higher ($P<0.001$) than any other steroid detected in the SAL-treated cows (pregnenolone, 3.2 ± 0.2 µg/g; DHP, 1.9 ± 0.2 µg/g; all others were <1 µg/mL; 20αOH-DHP was undetectable). Luteal progesterone concentrations in SAL-treated cows were significantly higher than those in luteal tissue from DEX-treated cows (33.4 ± 1.9 vs 19.1 ± 2.3 µg/g, respectively; Fig. 4; $P<0.05$). In DEX-treated cows, concentrations of progesterone in luteal tissue were positively correlated with those in serum over all ($r=+0.69$, $P<0.05$) even including what appeared to be a single outlying data point. If this single data point was removed from the

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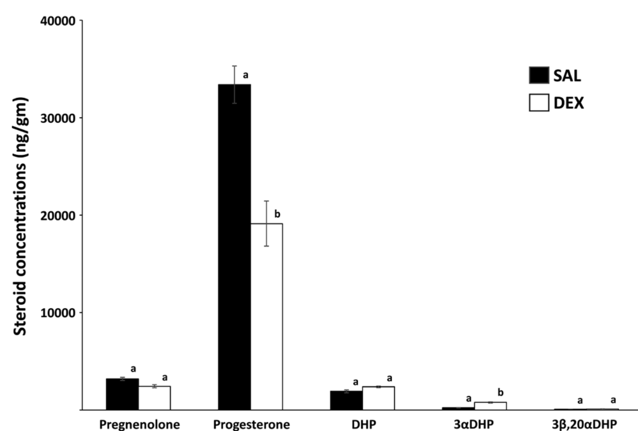

Figure 4 Steroid concentrations (ng/g) in corpus luteum tissues from saline- (SAL) and dexamethasone-treated (DEX) cows collected at slaughter 38 h after treatment on day 270 of gestation. Steroids concentrations were determined by LC-MS/MS, as described in Materials & methods. For each steroid depicted, differences between means are indicated, $^{a,b}P<0.05$.

analysis, the correlation co-efficient increased markedly ($r=+0.98$, $P<0.0001$). In SAL-treated cows, there was no significant correlation between serum progesterone and luteal tissue progesterone concentrations, but luteal progesterone was negatively correlated with serum pregnenolone ($r=-0.81$, $P<0.05$).

Enzyme activities

The activity of 3βHSD in cotyledonary microsomes was on average four-fold higher than caruncular samples and trilostane effectively inhibited >90% of product accumulation (data not shown). Cotyledonary microsomal and mitochondrial fractions were examined further (Table 1). The differences in rate of conversion of pregnenolone to progesterone between cotyledonary microsomal and mitochondrial proteins from SAL- and DEX-treated cows were not large relative to the mean activities themselves and not greatly affected by DEX (range of mean rates, 6.02–7.26 nmol/mg/h; Table 1). There was a significant main effect of sub-cellular fraction on 3βHSD activity ($P<0.01$) and a significant interaction ($P<0.05$) between treatment

Table 1 Activity (nmol/mg/h) of 3β-hydroxysteroid dehydrogenase in microsomal and mitochondrial protein isolated from cotyledonary and corpus luteum tissue from saline- (SAL; $n=8$) and dexamethasone- (DEX; $n=9$) treated cows in late gestation (270 days), 38 h after treatment.

	Cotyledon		Corpus luteum	
	Microsomal	Mitochondrial	Microsomal	Mitochondrial
SAL	6.45 ± 0.07[a]	6.69 ± 0.06[a]	42.79 ± 0.67[a]	26.72 ± 0.87[b]
DEX	6.02 ± 0.05[a]	7.26 ± 0.07[b]	43.05 ± 0.66[a]	32.15 ± 0.32[b]

Shown are the means ± standards errors of the means.
[a,b]Within cotyledon and corpus luteum, means with different superscripts differ, $P<0.01$.

and sub-cellular fraction ($P<0.05$). Cotyledonary mitochondrial 3βHSD was significantly higher in DEX than SAL-treated cows ($P<0.01$). On average, however, there was 2.35-fold more total microsomal than mitochondrial protein isolated from cotyledonary tissues, which was similar in tissues from SAL-treated and DEX-treated cows (microsomal/mitochondrial protein, 2.44- vs 2.27-fold, respectively). Overall there was far more 3βHSD activity in luteal than cotyledonary microsomes and mitochondria (Table 1). Within luteal tissue there was a main effect of sub-cellular fraction, with significantly more activity in microsomes than mitochondria ($P<0.001$). However, there was no main effect of DEX treatment and no interaction, only a tendency for higher luteal 3βHSD activity in mitochondria from DEX- than from SAL-treated cows ($P<0.07$).

Estimates of 5α-reductase activity in microsomal protein were based on the rate of accumulation of DHP. Caruncular tissues synthesized DHP at a rate of 2.02 ± 0.24 nmol/mg/h and 2.15 ± 0.29 nmol/mg/h in SAL- and DEX-treated animals, respectively, which was significantly greater than in cotyledonary tissues ($P<0.05$). Cotyledonary tissues synthesized DHP at a rate of 1.16 ± 0.20 and 1.47 ± 0.27 nmol/mg/h in SAL- and DEX-treated animals, respectively. Dexamethasone treatment did not affect the activity of 5α-reductase in either caruncular or cotyledonary tissue. Finasteride inhibited DHP synthesis, verifying active synthesis by 5α-reductase (data not shown).

Transcript analysis

The abundance of transcripts encoding enzymes and co-factors involved in steroid synthesis and metabolism in the placenta demonstrated the predominant steroidogenic role of the cotyledon over the caruncle. The abundance of StAR ($P<0.001$), CYP11A1 ($P<0.0001$) and its redox partner FDX ($P<0.05$, though not FDXR, $P>0.3$; Fig. 5) and of HSD3B1 ($P<0.001$; Fig. 6) were all significantly higher in cotyledonary than in caruncular tissues. In contrast, the abundance of SRD5A1 was greater ($P<0.001$) in caruncular than cotyledonary tissues. Of the placental steroidogenic enzyme transcripts investigated, the only detected effect of DEX was observed for SRD5A2 where DEX-treated cows had greater abundance ($P<0.001$) in caruncular tissue than caruncular tissue of SAL-treated cows or cotyledonary tissue of either treatment (Fig. 6). Transcripts encoding CYP11A1, FDX, FDXR, StAR and HSD3B1 (Fig. 7) were more abundant in luteal than placental tissues from SAL- and DEX-treated cows ($P<0.01$). In addition, transcript abundance was significantly lower in luteal tissue from DEX- than from SAL-treated cows for CYP11A1 ($P<0.005$), as well as FDX, FDXR, StAR and HSD3B1 ($P<0.05$).

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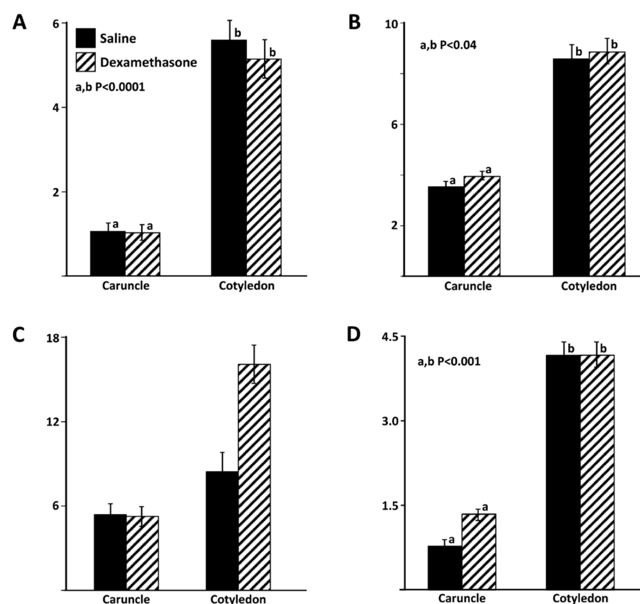

Figure 5 Quantitative reverse transcriptase polymerase chain reaction (qPCR) analysis of transcript abundance in tissues collected at slaughter from cows treated 38 h earlier with dexamethasone (DEX) or saline (SAL). (A) CYP11A1 (encoding cytochrome P450 cholesterol side chain cleavage). (B) Ferrodoxin (FDX). (C) Ferrodoxin reductase (FDXR). (D) Steroid acute regulatory protein (StAR). Shown are the means and standard errors. [a,b]Means with different superscripts differ, $P<0.05$.

Discussion

The results of this study provide new insight into the synthesis of pregnenes (progesterone, pregnenolone) and pregnanes (5α-reduced progesterone metabolites) in late pregnant cattle, both placental and luteal contributions to systemic levels, as well as the events triggered by DEX that induce bovine parturition. The 5α-reduced pregnane, 20α-DHP, was identified for the first time as an unusually abundant progesterone metabolite in these studies. To the best of our knowledge, this is the highest 5α-reduced pregnane ever identified in a ruminant.

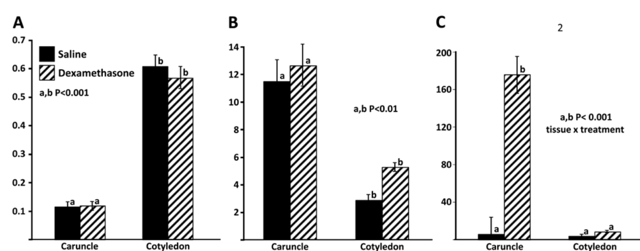

Figure 6 Quantitative reverse transcriptase polymerase chain reaction (qPCR) analysis of transcript abundance in tissues collected at slaughter from cows treated 38 h earlier with dexamethasone (DEX) or saline (SAL). (A) HSD3B1 (encoding 3β-hydroxysteroid dehydrogenase/Δ5-4 isomerase). (B) SRD5A1 (5α-reductase type 1). (C) SRD5A2 (5α-reductase type 2). Shown are the means and standard errors. [a,b]Means with different superscripts differ, $P<0.05$.

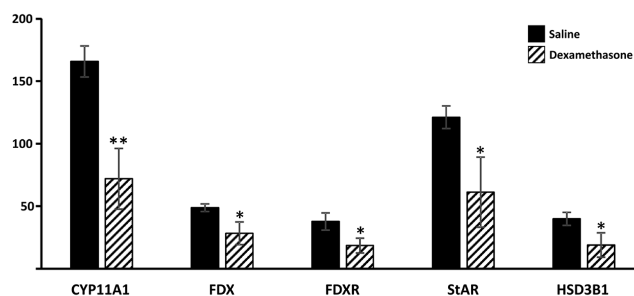

Figure 7 Quantitative reverse transcriptase polymerase chain reaction (qPCR) analysis of transcript abundance in luteal tissue collected at slaughter from cows treated 38 h earlier with dexamethasone (DEX) or saline (SAL). CYP11A1 (encoding cytochrome P450 cholesterol side chain cleavage), ferrodoxin (FDX), ferrodoxin reductase (FDXR), steroid acute regulatory protein (StAR) and HSD3B1. Shown are the means and standard errors. Means differ significantly as shown, $^{*}P<0.05$, $^{**}P<0.005$.

This suggests that 5α-reduction is a major route of progesterone metabolism in cattle, contrasting with the pregnant ewe wherein 5α-reduced pregnanes were typically less than 1 ng/mL in systemic blood (Reynolds *et al.* 2018). However, progesterone metabolism was not influenced by DEX, which successfully induced progesterone withdrawal, apparently by precipitating functional luteal regression based on the significantly lower luteal progesterone concentrations as well as transcripts encoding CYP11A1 and other enzymes and co-factors supporting progesterone synthesis in the corpora lutea of DEX-treated cows. The tendency for luteal weights to be less in DEX- compared with SAL-treated cows further suggests that structural regression had likely also begun, even though a significant decrease luteal weight might not be expected to be evident for 48 h (Juengel *et al.* 1993). These observations are consistent with results from an extensive series of experiments first reported in what the authors described as pilot studies on DEX induction of bovine parturition (Hoffmann *et al.* 1979). Thus, from the current data, it seems that DEX has no major effect on placental progesterone synthesis or metabolism (within 38 h) after administration (parturition reportedly occurs within 72 h after DEX; Adams & Wagner 1970, Shenavai *et al.* 2012) and that luteal regression precedes any subsequent, pre-parturient changes in placental steroidogenesis.

The relative contribution of the placenta to circulating progesterone concentrations in cows in late gestation was also clarified in the current studies by observations in the DEX-treated cows. Cotyledonary tissue concentrations of progesterone were not correlated with systemic progesterone concentrations, but luteal tissue concentrations were. The higher concentrations of pregnenolone and progesterone in cotyledonary than caruncular tissues was consistent with higher cotyledonary than caruncular 3βHSD enzyme activity and the higher transcript abundance of StAR, CYP11A1,

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FDX and HSD3B1 in the fetal component of the placenta. These data are consistent with a significant capacity for pregnene synthesis by the bovine placenta during gestation (Shemesh 1990, Conley *et al.* 1992), which may become relevant in the absence of a functional corpus luteum (Conley & Ford 1987). The extraordinarily high progesterone concentrations of luteal compared to cotyledonary tissue (>30,000 vs 100 ng/g) suggest that, despite the approximately 500-fold greater mass of cotyledons than of corpora lutea (Reynolds *et al.* 1990, Vonnahme *et al.* 2007), there is a more significant contribution of ovarian secretion to systemic progesterone concentrations in late gestation. The strong positive correlation between luteal tissue and systemic progesterone concentrations in DEX-treated cows is equally consistent and supportive evidence that luteal secretion is the major determinant of progesterone concentrations late in bovine gestation, as has been suggested by others (Schuler *et al.* 2008). Again, all the above data support the view that functional luteal regression more likely initiates progesterone withdrawal and parturition following DEX treatment in cattle.

Pregnenolone is the proximate precursor for the synthesis of progesterone, as well as all other pregnanes, and tissue concentrations are therefore relevant in terms of understanding substrate availability for steroidogenesis. Serum pregnenolone concentrations are reported here for the first time in pregnant cows, along with placental and in luteal tissue concentrations and 3βHSD enzyme activities. Cotyledonary pregnenolone concentrations were twice those of progesterone on average, despite what appears to be robust 3βHSD enzyme activity that were very similar in magnitude to previously published estimates in microsomes (Tsumagari *et al.* 1994). In fact, 3βHSD enzyme activity was as high in bovine cotyledon as it was in ovine cotyledon (Reynolds *et al.* 2018), which is adequate to sustain progesterone synthesis at levels compatible with maintenance of pregnancy in sheep. End product inhibition of placental 3βHSD activity by progesterone and 20αOH-progesterone is one potential reason why placental progesterone synthesis might not be maximized as long as luteal progesterone secretion dominates. Higher concentrations of pregnenolone than progesterone suggest again that substrate supply was not limiting in placental progesterone synthesis but 3βHSD may have been. *In vitro* cultured and perfused placentomal tissues recovered from cows after prostaglandin-induced luteolysis at 200 days of gestation showed increased progesterone synthesis from pregnenolone over tissues from SAL-treated cows (Conley & Ford 1987). Thus, excess pregnenolone coupled with the capacity of cotyledonary tissues to increase 3βHSD activity may provide the bovine placenta with the ability to compensate for the loss of luteal progesterone until late in gestation. Though 3βHSD activities were shown to be higher in luteal tissue by 5-fold over cotyledonary tissue, the difference in progesterone concentrations of several orders of magnitude suggest that cotyledonary 3βHSD activity may be limited not by the amount of enzyme but by other elements, perhaps reducing equivalents or the redox environment. This is also consistent with the lack of any demonstrated effect of DEX on the abundance of HSD3B1 or any of the transcripts encoding enzymes and associated proteins in placental tissues. Removal of the ovaries results in cows calving early in the absence of ovaries (Mcdonald *et al.* 1953, Estergreen *et al.* 1967) suggesting that, whatever contribution it might make, the capacity for placental 'compensation' wanes near term.

Some have concluded, primarily from *in vitro* studies, that bovine CL lose progesterone synthetic capacity as gestation proceeds (Mills & Morrissette 1970) and that the placenta plays an increasingly significant role supplementing luteal progesterone production as gestation advances (Shemesh 1990). Several researchers reported slight decreases in progesterone concentrations in luteal tissue in the second half of gestation (Melampy *et al.* 1959, Stormshak & Erb 1961, Bowerman & Melampy 1962, Erb *et al.* 1968) though correlations with ovarian venous concentrations were poor (Erb *et al.* 1968). Others have reported no difference in ovarian progesterone secretion between early and late gestation from *in vitro* perfusion studies (Mills & Morrissette 1970). A recent study, conducted to investigate concentrations of progesterone, pregnenolone and some 5α-reduced pregnanes in luteal tissue from cyclic cows using gas chromatography mass spectrometry, examined the effects of prostaglandin F2α-induced luteolysis (Waite *et al.* 2005). The response of the corpus luteum to prostaglandin F2α was rapid in these cyclic cows and was evident within 4 h of treatment. Despite a more protracted period between treatment and tissue collection, the results reported here for corpora lutea of late gestation bore a remarkable similarity in terms of the range of concentrations of progesterone and pregnenolone found in cyclic corpora lutea. There was slightly more progesterone and slightly less pregnenolone in the corpora lutea of pregnancy such that the progesterone:pregnenolone ratio was close to 10 compared with about 4 as reported for luteal tissue from cyclic cows (Waite *et al.* 2005). There were also 5α-reduced pregnanes at much lower concentrations in luteal tissues from late gestation cows including DHP (1–2 μg/g), which was not detected in corpora lutea of cyclic cows. Prostaglandin treatment of cyclic cows resulted in a decrease in luteal concentrations of progesterone, 20βOH-progesterone and pregnenolone and a significant increase in 3βOH DHP 24 h after treatment. In the current study, by comparison, while the decrease in luteal tissue pregnenolone was not significant, progesterone was significantly lower and the 3αOH DHP was significantly increased 38 h after DEX treatment. The tendency for lower luteal weight, coupled with the significant decreases in CYP11A1, FDX, FDXR, StAR and HSD3B1 transcript abundance, is consistent

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with functional regression, even though pregnenolone was not significantly affected by DEX treatment, and there was no demonstrated effect on 3βHSD activity. This might also reflect the delay between transcription, translation and protein processing needed to support steroidogenesis. Systemic concentrations of 20αOH DHP were already declining and were unaffected by DEX, so increased progesterone metabolism seems to be an equally unlikely explanation for the decrease in luteal progesterone. Changes in redox state of cells might have an influence on the availability of reducing equivalents needed to support 3βHSD *in vivo* (Sherbet *et al.* 2007) and, since these are supplied *in vitro*, an *in vivo* effect on the redox state of luteal cells could be easily missed. Additional studies will be required to address these otherwise incongruous results. Regardless, pregnenolone concentrations were still 10-fold higher in luteal than cotyledonary tissues even though progesterone concentrations were four orders of magnitude higher in luteal than cotyledonary tissues. This speaks to the remarkable steroid synthetic capacity of the corpus luteum of late gestation in cattle even if function is in decline (Mills & Morrissette 1970, Shemesh 1990).

The measurement and effective partitioning of 3βHSD into the microsomal (endoplasmic reticulum) and mitochondrial compartments (Thomas *et al.* 1988, 1989) has rarely been investigated in a physiological context, certainly not in bovine tissues. Enzyme activity and transcript data were consistent with one another, indicating that cotyledonary expression of 3βHSD was higher than caruncular expression. Within the cotyledonary tissues then, the data presented here suggest that both microsomal and mitochondrial intra-cellular compartments contribute significantly to progesterone synthesis by the bovine placenta. It is noteworthy also that the demonstration of 3βHSD activity in the present experiments is consistent with the enzyme being located on the cytoplasmic side of both microsomes and mitochondria. Activities were robust and apparently fueled effectively by NAD^+ supplied in the buffer, which would be unable to cross intact membrane in charged form. The higher specific activity in the mitochondrial fraction was offset by the larger size (based on recovered protein) of the microsomal compartment in the cotyledonary tissues. No differences were noted in 3βHSD activity between microsomes and mitochondria in placentas from SAL- and DEX-treated cows, so whether or not there is regulation of compartmental distribution of the enzyme is unclear. However, there were higher levels of 3βHSD activity in luteal microsomes than mitochondria, both of which were substantially higher than in cotyledonary tissue. There were also higher levels of activity in luteal mitochondria from DEX-treated than SAL-treated cows. This might reflect a membrane-stabilizing effect of the glucocorticoid (Madsen-Bouterse *et al.* 2006). Whether or not there is a mechanism controlling the partitioning of 3βHSD into microsomal or mitochondrial compartments of steroidogeneic tissues requires additional investigation and remains an intriguing possibility.

The activity of 5α-reductase in placental microsomes was slightly lower than that of 3βHSD, but of a similar order of magnitude. Based on these data, and correlations between luteal and systemic progesterone concentrations, it is reasonable to conclude that cotyledonary 3βHSD does not contribute significantly to circulating progesterone levels when the corpus luteum is functional. Since there was no detectable 20α-DHP in cotyledonary tissue, it seems equally unlikely that placental 5α-reductase contributes to circulating 20α-DHP. DEX had no effect on 20α-DHP either, but increased SRD5A2 expression in caruncular tissue which would certainly indicate that caruncular SRD5A2 expression has little influence on 20αDHP concentrations in systemic blood. In any case, 5α-reduced pregnane concentrations were universally low in placental tissues. Given the modest levels of 5α-reduced pregnanes in luteal tissue compared with progesterone itself, it is equally unlikely that this is the source of these metabolites either, even though this is the case in the elephant (Hodges *et al.* 1997). The rate of peripheral 5α-reduction of progesterone is certainly impressive in ovariectomized mares and geldings (Conley *et al.* 2018) and is likely of significance in cattle and women (Milewich *et al.* 1977, 1995). In aggregate, the results of this study provide little evidence to support the proposition that placental pregnene synthesis or metabolism has a major influence on maternal progesterone or 5α-reduced pregnane concentrations in cows with a functional corpus luteum at term. Similarly, it seems unlikely that reduced circulating pregnanes/enes at parturition involve changes in placental synthesis or metabolism.

In conclusion, the data presented here suggest it is more likely that that DEX initiates progesterone withdrawal and parturition in cattle in late gestation by inducing luteal regression than by effects on placental progestin synthesis or metabolism. This includes pregnenolone, the concentrations of which were unaffected by DEX in either placental or luteal tissues. High circulating concentrations of 20αDHP suggest that 5α-reduction is a major route of progesterone metabolism in the cow, and low placental and luteal concentrations suggest that metabolism likely occurs peripherally. DEX appears not to have a dramatic or rapid effect on 3βHSD enzyme activity in the placenta or in luteal tissue overall, even though transcript abundance for enzymes and co-factors supporting progesterone synthesis were decreased in luteal tissues of DEX-treated cows. Further studies are required to determine the mechanisms behind DEX-induced functional regression of the CL in cattle in late gestation.

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Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-18-0558.

Declaration of interest

Lawrence P Reynolds is on the editorial board of *Reproduction*. Lawrence P Reynolds was not involved in the review or editorial process for this paper, on which he is listed as an author. The other 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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