Title

Progesterone and 5α-dihydroprogesterone (DHP) in Cyclic Mares, and in Ovariectomized Mares and Geldings After Progesterone Administration

Authors

Conley, AJ
Scholtz, EL
Legacki, EL
et al.

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RESEARCH

5α-dihydroprogesterone concentrations and synthesis in non-pregnant mares

A J Conley[1], E L Scholtz[1], E L Legacki[1], C J Corbin[1], H K Knych[2], G D Dujovne[1], B A Ball[3], B C Moeller[2] and S D Stanley[2]

[1]Department of Population Health & Reproduction, School of Veterinary Medicine, University of California, Davis, California, USA
[2]Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California, USA
[3]Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington Kentucky, USA

Correspondence should be addressed to A J Conley: ajconley@ucdavis.edu

Abstract

In vivo and *in vitro* evidence indicates that the bioactive, 5α-reduced progesterone metabolite, 5α-dihydroprogesterone (DHP) is synthesized in the placenta, supporting equine pregnancy, but its appearance in early pregnancy argues for other sites of synthesis also. It remains unknown if DHP circulates at relevant concentrations in cyclic mares and, if so, does synthesis involve the non-pregnant uterus? Jugular blood was drawn daily from cyclic mares ($n = 5$). Additionally, ovariectomized mares (OVX) and geldings were administered progesterone (300 mg) intramuscularly. Blood was drawn before and after treatment. Incubations of whole equine blood and hepatic microsomes with progesterone were also investigated for evidence of DHP synthesis. Sample analysis for progesterone, DHP and other steroids employed validated liquid chromatography–tandem mass spectrometry methods. Progesterone and DHP appeared a day (d) after ovulation in cyclic mares, was increased significantly by d3, peaking from d5 to 10 and decreased from d13 to 17. DHP was 55.5 ± 3.2% of progesterone concentrations throughout the cycle and was highly correlated with it. DHP was detected immediately after progesterone administration to OVX mares and geldings, maintaining a relatively constant ratio with progesterone (47.2 ± 2.9 and 51.2 ± 2.7%, respectively). DHP was barely detectable in whole blood and hepatic microsome incubations. We conclude that DHP is a physiologically relevant progestogen in cyclic, non-pregnant mares, likely stimulating the uterus, and that it is synthesized peripherally from luteal progesterone but not in the liver or blood. The presence of DHP in pregnant perissodactyla as well as proboscidean species suggests horses may be a valuable model for reproductive endocrinology in other exotic taxa.

Key Words

- female reproduction
- steroidogenesis
- novel progestin
- equine
- progesterone

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Introduction

The results of recent *in vivo* and *in vitro* studies, utilizing liquid chromatography–tandem mass spectrometry (LC-MS/MS) for steroid determination, confirm that 5α-dihydroprogesterone (DHP) is the major progestogen supporting equine pregnancy (Scholtz *et al.* 2014), but nothing is known about DHP in non-pregnant mares. DHP is bioactive, stimulating endometrial growth and expression of progesterone-induced genes in ovariectomized (OVX) mares, and it maintained pregnancy for almost 2 weeks in mares after luteolysis

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was induced 14 days post ovulation (Scholtz *et al.* 2014). DHP concentrations in early pregnant mares were lower than, but closely paralleled, progesterone concentrations until 80–90 days of gestation (Holtan *et al.* 1991, Scholtz *et al.* 2014, Legacki *et al.* 2016*b*) after which their secretory patterns began to diverge. Progesterone begins a progressive decline around this time, whereas DHP concentrations begin to increase. This coincides with the period in equine gestation when pregnancy can survive without ovarian progestogenic support (Holtan *et al.* 1979). The chorioallantoic placenta, together with the endometrium of pregnant mares, has been shown to have the capacity to synthesize DHP from progesterone both *in situ* (Moss *et al.* 1979) and *in vitro* (Hamon *et al.* 1991). Equine placentas express robust 5α-reductase enzyme transcript and activity (Legacki *et al.* 2017), the loss of which in post-parturient placentas (Legacki *et al.* 2018) coincides with the pre-parturient decline in all 5α-reduced pregnanes before foaling (Legacki *et al.* 2016*a*). However, DHP was not detected by immunoassay in any samples from nine cyclic females in the only study that appears to have investigated this physiologically important progestogen in non-pregnant mares (Hamon *et al.* 1991). This is surprising because, as noted earlier, DHP was detected soon after ovulation in mares that became pregnant (Atkins *et al.* 1976, Holtan *et al.* 1991, Scholtz *et al.* 2014, Legacki *et al.* 2016*b*). Therefore, one of the goals of the current investigations was to examine concentrations of DHP and progesterone (as well as other possible metabolites) in cyclic mares for the first time using a recently developed LC-MS/MS-based method (Legacki *et al.* 2016*b*).

There can be little doubt that the placenta is the major site of 5α-reduction of progesterone to DHP and other 5α-reduced metabolites in mid-to-late equine pregnancy (Legacki *et al.* 2017, 2018) when progesterone becomes undetectable (Holtan *et al.* 1991, Scholtz *et al.* 2014, Legacki *et al.* 2016*b*). However, the source of DHP in early pregnant mares, before placental development is advanced, remains unknown. Even outside the Perissodactyla, the mare is not alone in exhibiting a predominance of 5α-reduced pregnanes together with surprisingly lowprogesterone concentrations during pregnancy. Both the African elephant (Hanks & Short 1972, Hodges *et al.* 1997), and its close relative among the Proboscidea the rock hyrax (Heap *et al.* 1975, Kirkman *et al.* 2001), experience unusually low progesterone concentrations and high concentrations of DHP during pregnancy. In the case of the African elephant, luteal tissue itself is thought to be the secretory source of DHP (Hodges *et al.* 1994, 1997) but in the rock hyrax metabolism of progesterone to DHP is thought to take place in blood (Heap *et al.* 1975, Makawiti *et al.* 1991, Kirkman *et al.* 2001). Other tissues may also be capable of metabolizing progesterone to DHP. The liver for instance is well known to exhibit high levels of 5α-reductase expression (Normington & Russell 1992). These 'extra-placental' sites of progesterone metabolism would be especially relevant if DHP circulates in cyclic mares at concentrations that are comparable to those seen in early equine pregnancy. In fact, our prior studies on equine gestation (Scholtz *et al.* 2014) monitored several cycles prior to establishing pregnancies and provided convincing evidence that DHP circulates in cyclic mares at concentrations that are similar to those seen in early pregnancies. Given the potential sites of DHP synthesis, tissues of immediate interest in non-pregnant mares would include the ovary (corpus luteum), the uterus (endometrium), whole blood and liver.

Based on the previously published reports, additional experiments were conducted to ascertain the likely involvement of the ovaries and the non-pregnant uterus as potential sites of conversion of progesterone to DHP. To this end, OVX mares and geldings (lacking both gonads and uterus) were administered progesterone. Bloods samples were taken before administration and at frequent intervals thereafter and analyzed for the presence of DHP using LC-MS/MS as previously described (Scholtz *et al.* 2014). In addition, *in vitro* incubations with progesterone were conducted with both equine hepatic microsomes and whole blood to evaluate these tissues as potential sites of DHP synthesis potentially contributing to circulating concentrations.

Materials and methods

All animal procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee. Mixed breed, light horse mares ($n=5$) were made to bleed daily by jugular venipuncture through an estrous cycle. Serum was separated and stored frozen at −20°C. Progesterone (Sigma-Aldrich) was dissolved in 20% benzyl alcohol (Fisher Scientific, Pittsburgh, PA, USA) and 80% filter-sterilized cottonseed oil (Sigma-Aldrich) to a concentration of 50 mg/mL for injection. One injection of progesterone (300 mg) was administered intramuscularly to ovariectomized (OVX) mares ($n=3$) and geldings ($n=3$). Peripheral blood samples were collected immediately prior to injection (time 0) and then 1, 2, 3, 4, 5, 6, 12, 24, 48 and 72 h after injection by jugular

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venipuncture into heparinized, evacuated glass tubes (Beckton Dickinson, Franklin Lakes, NJ, USA). Samples were placed on ice until processed by centrifugation at 2000***g*** for 20 min at 4°C and stored (−20°C). Serum and plasma samples were analyzed subsequently by LC-MS/MS using published methods (Scholtz *et al.* 2014, Legacki *et al.* 2016*b*). Serum from cyclic mares was analyzed using the more comprehensive method that can detect a variety of pregnanes and pregnenes including progesterone, DHP, 17αOH-progesterone and a number of 5α-reduced progesterone metabolites among other steroids (Legacki *et al.* 2016*b*). Plasma from OVX mares and geldings was analyzed for progesterone and DHP only (Scholtz *et al.* 2014).

Heparinized jugular blood (4 mL) from geldings (n=3) was incubated for a total of 6 h at 37°C with slow rocking after addition of progesterone (final concentration ~6 ng/mL) with and without finasteride (30 μM) to inhibit any apparent 5α-reductase activity (Corbin *et al.* 2016). Aliquots (0.5 mL) were taken at time 0, 0.5, 1.0 and 3.0 h and extracted with ten volumes (5 mL) of methylene chloride. At 6 h the remaining 2 mL were extracted with 20 mL of methylene chloride. The organic phase was evaporated completely and samples were reconstituted with 200 μL of 50:50 water and methanol and shaken for 1 min in preparation for analysis by LC-MS/MS. Additional experiments were conducted with equine liver microsomes (20 μg; described below) incubated with 3 μM progesterone with and without finasteride (30 μM) and ketoconazole (30 μM), to inhibit cytochrome P450-mediated metabolism (Murray & Zaluzny 1988). All incubations were performed in 50 mM KPO_4, 1 mM EDTA pH 7.4 buffer with a generating system consisting of 17 mM glucose-6-phosphate, 1 mM NADPH, 2 mM NADP and 1 unit of glucose-6-phosphate dehydrogenase (Sigma) for a total of 2 h at 37°C after the evaporation of the inhibitor, if used, in the tube. Aliqouts (100 μL) were taken at 30 and 60 min and the reaction was stopped by extraction with ten volumes (1 mL) methylene chloride (Fisher Scientific, Fair Lawn, NJ). At 2 h the final 300 μL were extracted in 3 mL methylene chloride. The organic phase was evaporated completely and samples were analyzed subsequently by LC-MS/MS (Legacki *et al.* 2016*b*).

Equine livers 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) 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.

Statistical analyses were performed using commercially available software (SAS ver. 9.4, SAS Institute Inc., Cary, NC, USA). For comparison of DHP and P4 in cyclic mares, continuous data were square root transformed and analyzed using repeated-measures ANOVA in proc GLM. Differences in steroid concentrations between days of the cycle were subjected to paired t-tests. Pearson correlation coefficients were calculated using proc corr. Proc GLM was also used to analyze the repeated measures, linear and quadratic effects of time on progesterone and DHP concentrations in OVX mares and geldings, and of time on progesterone concentrations in incubations of whole blood. Progesterone and DHP data from geldings were log transformed before analysis.

Results

Progesterone and its 5α-reduced metabolite, DHP and 17αOH-progesterone were the only steroids detected consistently, appearing during the luteal phase. Progesterone (P<0.05), DHP (P<0.01) and 17αOH-progesterone (P<0.05) all increased significantly from the day of ovulation to day 3 post ovulation, and progesterone (P<0.01), DHP (P<0.05) and 17αOH-progesterone (P<0.01) all decreased significantly from day 13 to day 17 of the cycle. There was a significant linear (P<0.001) and quadratic (P<0.001) effect of time on steroid concentrations, reflecting this mid-luteal phase increase in concentrations, but no time by steroid interaction (P>0.25). Progesterone and DHP concentrations were highly correlated (r=+0.89, P<0.001), as both were with 17αOH-progesterone (r=+0.84 and r=+0.85, respectively, P<0.001). Peak progesterone (8–10 ng/mL) and DHP (4–5 ng/mL) concentrations were reached by cycle days 5–10 and decreased concomitantly between cycle days 14–16 (Fig. 1). On average, DHP was 55.5±3.2% of progesterone concentrations consistently throughout the cycle. Though highly correlated with both progesterone and DHP, 17αOH-progesterone remained <1 ng/mL on average even at peak mid-cycle concentrations.

Intramuscular injection of progesterone into OVX mares was followed by its appearance in systemic blood to over 2 ng/mL at 1 h and over 3 ng/mL by 2 h (Fig. 2), though this was not statistically significant (P<0.08).

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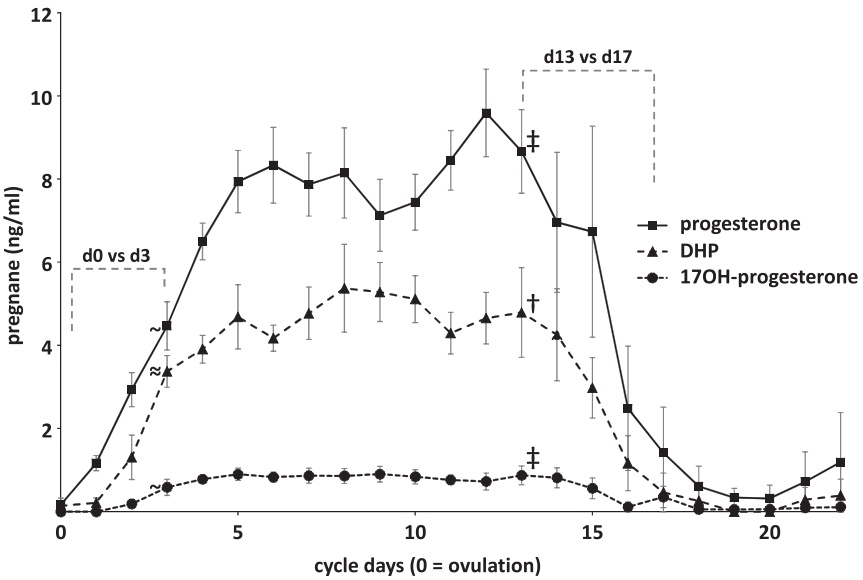

Figure 1
Progesterone (squares, solid line), 5α-dihydroprogesterone (DHP, triangles, dashed line) and 17αOH-progesterone (dots, dotted line) concentrations (ng/mL) measured by liquid chromatography–tandem mass spectrometry in daily jugular blood samples taken from cyclic mares ($n=5$). Time of ovulation was defined as cycle day (d) 0. The means ± S.E.M.s are depicted. ~$P<0.05$, ≈$P<0.01$, represents significant differences in steroid concentrations between cycle d0 and 3. †$P<0.05$, ‡$P<0.01$, represents significant differences in steroid concentrations between cycle d13 and d17. DHP, 5α-dihydroprogesterone.

Progesterone concentrations declined linearly with time ($P<0.01$) from a numerical peak of 3.53±0.51 ng/mL at 6 h to 0.77±0.10 ($P<0.05$) at 72 h after injection. Similarly, DHP concentrations increased to almost 1 ng/mL at 1 h, peaking (numerically) by 12 h at 1.46±0.22 ng/mL), and decreasing linearly thereafter ($P<0.05$), maintaining a relatively constant ratio with progesterone for 48 h. Numerical peak concentrations of progesterone at 6 h decreased significantly to 72 h post-injection ($P<0.05$). Similarly, DHP decreased from a numerical concentration peak at 5–72 h post injection ($P<0.01$). On average, DHP

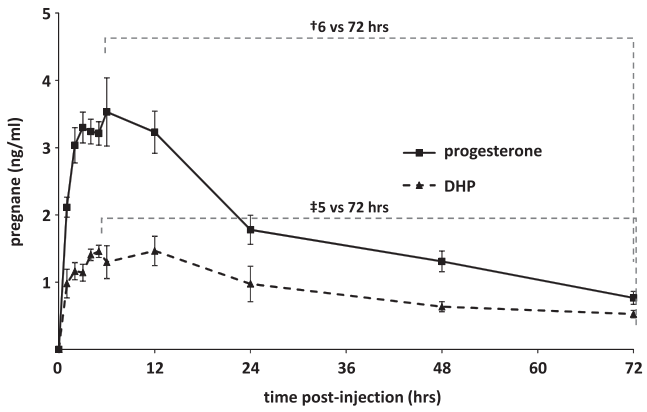

Figure 2
Progesterone (solid line) and 5α-dihydroprogesterone (DHP, dashed) measured by liquid chromatography–tandem mass spectrometry in ovariectomized mares ($n=3$) administered 300 mg of progesterone by intramuscular injection (time = 0). Blood samples were taken hourly for 6 h and at 12, 24, 48 and 72 h thereafter. The means ± S.E.M.s are depicted. †Progesterone concentrations decreased from a numerical peak at 6–72 h-post-injection ($P<0.05$). ‡DHP concentrations decreased ($P<0.01$) from a numerical peak at 5–72 h-post-injection. DHP, 5α-dihydroprogesterone.

was 47.2±2.9% of progesterone concentrations in these mares. Similar patterns were observed in geldings except that the data from these three animals had to be log transformed for analysis and were plotted individually on a log scale (Fig. 3). Probably due to intravenous absorption, progesterone concentrations in one gelding had already reached 72 ng/mL at 1 h (Fig. 3A) at which point DHP was 32 ng/mL (Fig. 3B), over ten-fold higher than concentration of both progesterone and DHP in the other two geldings. In these two geldings, the temporal appearance and concentrations of progesterone and DHP (Fig. 3A) were similar to that seen in OVX mares after progesterone administration (Fig. 2). After log transformation, there was a significant linear decline in progesterone ($P<0.01$) and DHP ($P<0.01$) with time in the geldings. Overall, the ratio of DHP:progesterone as a percentage in all three geldings (52.2±2.7%) was not significantly different from that seen in OVX mares.

Progesterone concentrations in incubations of whole blood from geldings decreased linearly ($P<0.02$) with time by more than 30% over 3 h of incubation from almost 6 to less than 4 ng/mL (Fig. 4). The inclusion of the 5α-reductase enzyme antagonist, finasteride, did not significantly change the rate of progesterone metabolism in these incubations ($P>0.9$). DHP was not detectable at any point up to 3 h, but was detectable in the residual blood remaining after 6 h of incubation. The volume of blood remaining at the 6-h time point represented a four- to five-fold larger sample volume for extraction than that was analyzed at the earlier time points. The DHP detected at 6 h was a tiny fraction of the remaining progesterone itself at that point (<6%). There were also

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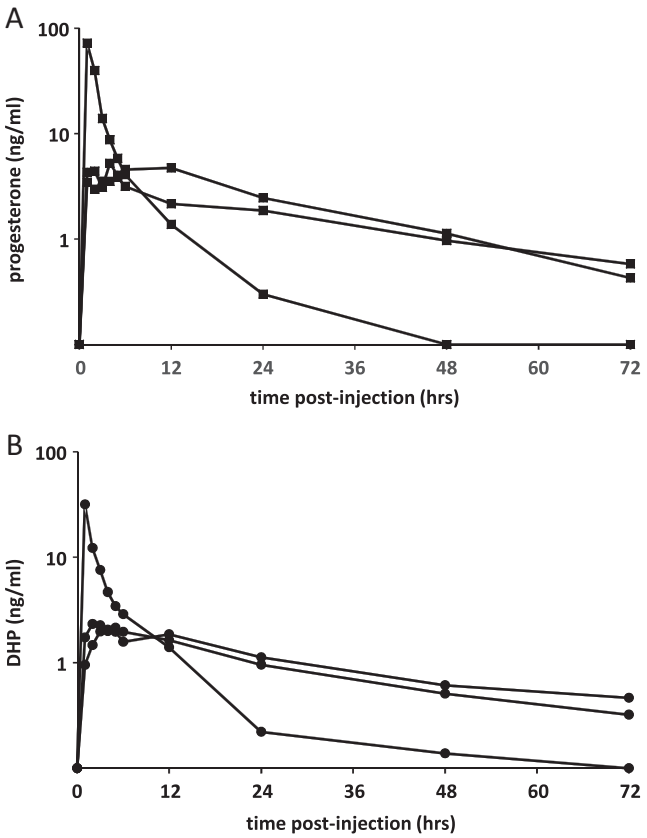

Figure 3
Progesterone (A) and 5α-dihydroprogesterone (DHP, B) measured by liquid chromatography–tandem mass spectrometry in geldings ($n=3$) administered 300 mg of progesterone by intramuscular injection (time = 0). Jugular blood samples were taken hourly for 6 h and at 12, 24, 48 and 72 h thereafter. Profiles represent those of each individual gelding. DHP, 5α-dihydroprogesterone.

detectable, though still relatively minor, concentrations of 20αOH-progesterone, representing <15% of the metabolized substrate after 3 h (data not shown). In contrast to blood, progesterone metabolism by hepatic microsomes was rapid and complete within an hour of incubation though the major, presumed hydroxylated products were not identifiable. Progesterone metabolism by hepatic microsomes was inhibited by finasteride and by ketoconazole, but DHP was undetectable nonetheless even under these conditions (data not shown).

Discussion

These are the first data defining DHP and progesterone concentrations through the cycle of non-pregnant, cyclic mares although, among others in the Perissodactyl order, data on DHP during the cycle have been reported for three zebra species (Klima *et al.* 1999). It has proven to be

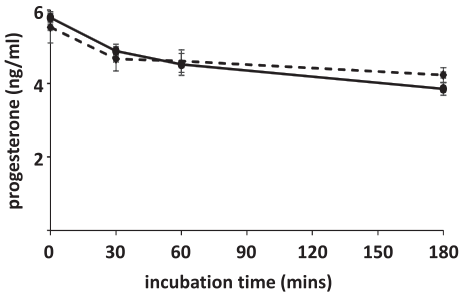

Figure 4
Progesterone metabolism in incubations of whole equine blood over 3 h. Progesterone was added to whole blood (time = 0) from geldings ($n=3$), incubated at 37°C and sampled at 0, 30, 60 and 180 min. The disappearance of progesterone was monitored by liquid chromatography–tandem mass spectrometry. Only minor (<10%) of detectable metabolites were quantified (data not shown). The means ± S.E.M.s are depicted. The disappearance of progesterone was linear with time ($P<0.02$), but there was no effect of finasteride and no time × finasteride interaction.

extremely difficult to develop an immunoassay capable of discriminating DHP and other (mostly pregnancy-associated) 5α-reduced metabolites from progesterone sufficiently, without careful, preparatory chromatography prior to immunoassay (Klima *et al.* 1999). Most immunoassays for progesterone cross-react considerably with DHP (Wynn *et al.* 2018). The only report using an immunoassay developed specifically to measure DHP failed to find any in cyclic, non-pregnant mares (Hamon *et al.* 1991). The results reported here demonstrate that DHP is easily detectable in the luteal phase of cyclic mares by LC-MS/MS and at slightly more than half the concentration of progesterone. The overall steroid analytical profile was notably simpler than that seen in pregnant mares (Legacki *et al.* 2016*b*), DHP being the only 5α-reduced pregnane detected. In previous studies, we demonstrated that concentrations quite similar to those seen here during the luteal phase, achieved in OVX mares by administration of DHP, strongly stimulated an endometrial response (Scholtz *et al.* 2014). DHP administered to maintain (only near) physiological concentrations (as confirmed here) was adequate to maintain early pregnancy after prostaglandin-induced luteolysis (Scholtz *et al.* 2014). Moreover, *in vitro* bioactivity studies in cells expressing the equine progesterone receptor along with a steroid-responsive luciferase reporter construct indicated that progesterone and DHP are equipotent progesterone receptor agonists in horses (Scholtz *et al.* 2014). Thus, collectively, DHP likely exerts progestogenic influence on the uterus during the luteal phase. Though highly correlated, DHP concentrations were consistently lower than progesterone (for example, cycle day 8, 5.3 ± 1.1 vs

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8.1 ± 1.1 ng/mL, respectively). Therefore, progesterone probably exerts greater stimulation on the uterus of cyclic mares than does DHP during normal luteal phases by virtue of consistently higher circulating concentrations.

The results of the present study are also of relevance to the question of the tissue source and involvement of the reproductive tract in formation of DHP in non-pregnant mares. Data from *in vitro* and *in vivo* experiments provide convincing evidence that DHP is synthesized by 5α-reductase activity from progesterone (Raeside *et al*. 2015, Corbin *et al*. 2016) in the placenta (Legacki *et al*. 2016*a*, 2018) and/or the associated endometrium during pregnancy (Moss *et al*. 1979, Hamon *et al*. 1991). Based on detectable DHP in blood samples from early pregnant mares, others speculated that non-placental sites of synthesis might also exist (Atkins *et al*. 1976) but provided no insight into the possible source. The evidence presented here indicates that DHP is synthesized from progesterone, rapidly and effectively, outside the reproductive tract in non-pregnant mares and even geldings. These observations are consistent with the notion that neither ovaries nor a uterus are necessary to convert progesterone to DHP efficiently and at ratios that approximate those reported here in intact cyclic mares. In contrast to horses, the corpus luteum of elephants synthesizes and secretes DHP (Hodges *et al*. 1994, 1997). Preliminary studies indicate that, unlike elephants, equine corpora lutea have little capacity for 5α-reduction of progesterone *in vitro* (data not shown) and metabolize progesterone to DHP in the absence of ovaries altogether. Other major organs or body systems were investigated as possible sites where progesterone could be metabolized to DHP. Hepatic microsomes were shown to metabolize progesterone rapidly to multiple metabolites, principally by cytochrome P450-mediated (ketoconazole-sensitive) pathways to presumably hydroxylated products, not 5α-reduced pregnanes like DHP. Therefore, while liver metabolism is extensive, DHP remains a minor metabolite even when P450 enzyme activity is inhibited. Ultimately, the question of whether or not there is a single organ primarily responsible for the metabolism of progesterone to DHP in cyclic mares remains to be determined. Regardless, though placental expression of transcripts encoding 5α-reductases is substantial during pregnancy (Legacki *et al*. 2017, 2018), these data make it clear that progesterone can be metabolized to DHP efficiently in the absence of a placenta and even a reproductive tract.

The possibility that progesterone could perhaps be metabolized to DHP in other tissues was also explored. Incubations of progesterone with whole blood were explored based on the results of similar studies reportedly demonstrating DHP synthesis from progesterone in blood from the rock hyrax (Heap *et al*. 1975, Makawiti *et al*. 1991, Kirkman *et al*. 2001). This would not be predicted to occur in erythrocytes that lack an endoplasmic reticulum and nucleus, where 5α-reductase activity is localized and membrane bound (Scheer & Robaire 1983). The extent of DHP synthesis in blood was not easy to determine from those studies (Heap *et al*. 1975, Makawiti *et al*. 1991, Kirkman *et al*. 2001). Identification of products was achieved after isolation from low-resolution thin layer or high-performance liquid chromatography with recrystallization. The investigation of metabolism was limited to the loss of progesterone only (Heap *et al*. 1975), to the synthesis of DHP and the 5β-isomer of DHP (5βDHP; (Makawiti *et al*. 1991)), or these (DHP and 5βDHP) plus allopregnanolone and 17αOH-progesterone (Kirkman *et al*. 2001). In the latter study (Kirkman *et al*. 2001), 17αOH-progesterone was referred to mistakenly as 17αOH-DHP. Only the earlier paper on progesterone metabolism (Heap *et al*. 1975) included negative control treatments to show that enzymatic turnover could be diminished by inhibiting oxidative phosphorylation. Only the earlier two studies ran time-course experiments (Heap *et al*. 1975, Makawiti *et al*. 1991). Though previous studies in ovine and bovine fetal blood identified 20αOH-progesterone as a major progesterone metabolite (Nancarrow 1983), none of the studies on the rock hyrax included this as a possible metabolite of interest in their analysis. Our data indicate this is present, and accumulates with time, but remains a minor metabolite of equine blood. Lastly, studies employing hepatic microsomes failed to find evidence of DHP synthesis by this tissue, which instead rapidly synthesized what in all likelihood were a multitude of hydroxylated metabolites. Ketoconazole was very effective at inhibiting the otherwise rapid disappearance of progesterone in these experiments, consistent with metabolism by hepatic P450 enzymes but DHP was never detected. Based on these observations, it seems highly unlikely that DHP synthesis in the systemic circulation of the horse arises by either metabolism in blood itself or by synthesis in the liver. If indeed there is a principle organ involved, further studies will be required to resolve the question of the primary site of DHP synthesis in non-pregnant and early pregnant mares (Atkins *et al*. 1976, Holtan *et al*. 1991, Scholtz *et al*. 2014, Legacki *et al*. 2016*b*).

In addition to defining DHP concentrations in cyclic, non-pregnant mares, the application of this analytical method, with the ability to simultaneously detect and quantify steroids from multiple classes

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(Legacki *et al.* 2016*b*), confirmed the presence of 17αOH-progesterone in the luteal phase as previously observed from the results of immunoassay (Meinecke 1987). This is consistent with the expression of the requisite steroidogenic enzymes in equine luteal tissue, specifically CYP17A1 (Albrecht *et al.* 2001). In comparison with pregnancy, however, few other steroids were detected during the cycle in the mares in the current study, none consistently or quantifiably, and the steroid profile generated is far simpler than suggested by others (Meinecke 1987). No doubt, the cyclic fluctuations in androgens and estrogens reported previously are below the level of detection and quantitation of the steroid analytical method employed here. For instance, both androstenedione (~0.4 ng/mL) and 17αOH-progesterone (~4 ng/mL) were detected, peaking in concentration when luteal tissue was under maximal stimulation by equine chorionic gonadotropin in early pregnant mares (Legacki *et al.* 2016*b*). The levels of the androgens and estrogens detected during the luteal phase in cyclic mares (Meinecke 1987) are likely just below the limits of detection of the method we utilized. Still, the secretion of 17αOH-progesterone (and DHP) during the luteal phase in mares is notable in not being seen in other livestock, but is instead similar to observations made during the luteal phase of the menstrual cycle in women. DHP (Milewich *et al.* 1995) and 17αOH-progesterone (Thorneycroft *et al.* 1971) concentrations both increase concomitant with progesterone secretion, and the human corpus luteum similarly expresses CYP17A1 (Doody *et al.* 1990). As previously noted relative to equine pregnancy (Conley 2016), and now with observations during the estrous cycle, it seems the reproductive endocrinology of mares, as in other facets of reproductive function (Ginther 2012), shares some remarkable similarities to that of women.

Conclusion

From these data, we conclude that DHP in cyclic mares is synthesized from luteal progesterone rapidly and in significant amounts outside of the reproductive tract. Though 5α-reduction of progesterone systemically is apparently efficient, DHP concentrations remain lower than those of progesterone in cyclic mares and during the early stages of equine pregnancy. Thus, DHP synthesis is initially dependent on luteal progesterone in cyclic and early pregnant mares, which is consistent with the high correlation between the two, and the fact that 5α-reduction occurs peripherally. This suggests that DHP is unlikely to provide any more useful information clinically than progesterone itself in cyclic mares. In contrast, concentrations of DHP relative to progesterone have potentially significant clinical value in pregnancy. Given the prominence of DHP in pregnant elephants and the rock hyrax (Proboscodea), and in zebras (Perissodacyla), the extent to which horses provide a model for reproductive endocrinology of cyclicity and gestation warrants investigation in a broader range of species.

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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