Ontogeny and ultradian rhythms of adrenocorticotropic and cortisol in the late-gestation fetal horse

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Abstract

Fetal maturation and the timing of parturition in both sheep and primates are thought to be controlled by the hypothalamic-pituitary-adrenal axis but little is known about the endocrinology of the equine fetus. We investigated the ontogeny of plasma concentrations of adrenocorticotropic hormone (ACTH), cortisol and corticosteroid binding capacity in the late-gestation fetal horse. We also wished to determine whether there is ultradian rhythmic release of ACTH and cortisol in fetal horses and we compared fetuses to maternal and non-pregnant adult horses. Six fetuses, 278–304 days gestation (term \( \approx 335 \) days), were catheterized and sampled daily until delivery. Mean (± s.e.m.) ACTH concentrations increased significantly from 159 ± 21 pg/ml to 246 ± 42 pg/ml over the last 2 days before parturition. Fetal cortisol increased significantly from 3-1 ± 1.0 to 13.4 ± 3.7 ng/ml (mean ± s.e.m.) over the last 9 days before delivery. The slope of regressions for ACTH and cortisol concentrations with respect to time were positive in all subjects and statistically significant in 3 of 6 for ACTH and 5 of 6 for cortisol. Fetal corticosteroid binding capacity declined from 49.5 ± 20.5 to 16.1 ± 2.2 ng/ml (mean ± s.e.m.) over the last 10 days before parturition. However, the greatest changes in ACTH, cortisol and corticosteroid binding capacity occurred very late in gestation, during the last 48 to 72 h before parturition. Significant peaks and nadirs in plasma ACTH concentration were detected in all 20 experiments and in plasma cortisol concentration in 17 of 20 experiments using Cluster analysis. We found statistically significant periods of oscillation between 11 and 64 min in plasma ACTH (19 of 20 experiments) and cortisol (15 of 20 experiments) using power spectral density analysis. Statistically significant periods between 11 and 17 min were detected in 11 of 20 experiments for ACTH and in 8 of 20 for cortisol. We conclude that: 1) at the end of gestation, equine fetal plasma ACTH and cortisol concentrations increase while corticosteroid binding capacity decreases suggesting that there is a disproportionately large increase in unbound cortisol at this time; 2) the secretion of ACTH and cortisol is rhythmic in both fetal and adult horses; 3) most animals exhibit a period of oscillation between 11 and 17 min; and 4) there is no apparent developmental change from late gestation to adulthood in the ultradian oscillator influencing ACTH and cortisol secretion in this species.


Introduction

A large body of evidence supports the hypothesis that the hypothalamic-pituitary-adrenal axis plays an important role in controlling fetal maturation and the timing of parturition in both ruminants and primates (Challis & Brooks 1989, Jaffe 1986). In the sheep, the species that has been the most intensively studied, an increase in fetal cortisol is responsible for promoting fetal maturation, the induction of placental 17-hydroxylase activity, enhanced estrogen production and the initiation of labor (Challis & Olson 1988). In the primate, there is no dramatic increase in fetal cortisol or placental 17-hydroxylase activity as there is in the sheep but there is an increase in fetal adrenocorticotropic hormone (ACTH) and dehydroepiandrosterone (DHEA) (Challis & Olson, 1988). Fetal DHEA is aromatized to estrogens which act at the myometrium to initiate labor. Though the specific mechanisms that control fetal maturation and the timing of parturition in these species are different, the fetal hypothalamic-pituitary-adrenal axis plays a necessary role.

While there is less known about the control of fetal maturation and the timing of parturition in the horse, the available evidence suggests that the mechanisms employed in this species are different from those in both the sheep and the primate. For example, it is known that equine
maternal plasma concentrations of a family of steroids, the so-called 'progestagens' that are structurally similar to progesterone (Holton et al. 1991), increase to high levels in the first, decrease in the second and increase again in the last third of gestation before falling again within days or hours of delivery (Holton et al. 1975). Equine maternal total estrogens rise and peak at around the middle of gestation and then decline at the end of gestation though they remain elevated through delivery compared with the levels in non-pregnant mares (Nett et al. 1973). These changes are in contrast to the consistent increases in estrogen and progesterone observed throughout pregnancy to within days of parturition in the sheep and primate. However, there is little information about the change in the concentrations of these hormones in equine fetal plasma or in the target tissues such as the myometrum and no data is available concerning the biological activity of the progestagens in horses nor how the levels of these hormones are controlled. The available evidence suggests that the horse employs different mechanisms to control the timing of parturition, compared with the sheep and primate.

The purpose of this study was three-fold. First, to develop a chronically instrumented fetal horse preparation in our laboratory. This has previously only been accomplished by Silver and co-workers at Cambridge University (Comline et al. 1975). Second, to begin to study the mechanisms that control fetal maturation and the timing of parturition in the horse by investigating whether or not there is an ontogenetic increase in hypothalamic-pituitary-adrenal axis activity at the end of gestation. Other workers have reported on the ontogenetic pattern of ACTH and cortisol in the fetal horse (Silver & Fowden 1994), but the present study is the first to document the ontogenetic change in corticosteroid binding capacity during the late gestational period. Third, we wished to characterize the ultradian pattern of release of ACTH and cortisol in the fetus and the adult horse. Changes in the pattern of release of ACTH and cortisol may play an important role in controlling visceral maturation and the timing of parturition. We compared fetal ultradian rhythms to those in the corresponding pregnant mares and to those in non-pregnant mares to determine if there is a developmental change in frequency of ACTH and cortisol pulses.

Materials and Methods

Eight adult non-pregnant mares and six pregnant mares were used in this study. Mares were bred at the University of Florida, with breeding dates confirmed using standard methods. Pregnant mares were anesthetized for surgery with xylazine, glycerol guiacolate and sodium thioumal and maintained with halothane and oxygen. Mares were positioned in right lateral recumbency with the pelvic limbs elevated so as to roll the abdomen ten degrees from lateral towards dorsal. A midline incision was made and the uterus was exposed. The uterus and fetal membranes were incised and arterial and venous catheters were introduced into fetal umbilical arteries and veins (n=2) or median arteries and veins (n=4). The fetal membranes and the uterus were closed and the catheters were directed either to the flank to exit the skin or through the dorso-cranial wall of the vagina. The abdomen was closed and a cloth pouch to contain the catheters was sutured to the skin in the flank region before the mares were recovered.

Preparation of mares for experiments

Before study, the mares were placed in stocks and chronically implanted catheters were opened and flushed. A percutaneous jugular venous catheter was maintained in pregnant mares throughout the study. The non-pregnant mares were not chronically catheterized. On the day of an experiment, the non-pregnant mares were placed in stocks and lidocaine (2%) was injected s.c. over the area of the external jugular vein to produce local analgesia. An i.v. catheter was placed in the left jugular vein to allow access to venous blood. All mares were permitted to stand undisturbed for at least 1 h following catheterization before the beginning of the sampling period.

Sampling protocols for ontogenetic and ultradian studies

Fetuses were sampled daily between 0900 and 1030 h until delivery. The sampling protocol for all ultradian studies consisted of a 2-h period during which 4 ml samples of blood were drawn at 5 min intervals. This represents a total collection volume of 100 ml blood from each animal studied. This volume of blood is small relative to the estimated blood volume in the fetus (body weight = 25–35 kg), pregnant mares (200–375 kg), and non-pregnant mares (300–400 kg). Ultradian experiments were performed in 5 of 6 fetuses and their mothers while in one fetus, two experiments were performed (Subject 6 in Tables). All ultradian studies were begun between 0930 and 1030 h. Blood samples were collected into polypropylene tubes containing 200 μl of 0·3 m Na4EDTA (Sigma, St Louis, MO, USA) and placed on ice immediately after collection. Following the study, the samples were centrifuged at 3000 r.p.m. at 4 °C for 15 min. Plasma was stored in several aliquots at −20 °C until assay. Blood for blood gas analysis was collected anaerobically in heparinized syringes that were capped and stored on ice until assay. Blood gas analysis was performed using a Radiometer BMS 3Mk2 blood microsystem and PHM 73 pH and blood gas analyzer (Radiometer, Copenhagen, Denmark).

Assays

Plasma ACTH concentrations were measured by radioimmunoassay (RIA) using anti-ACTH antiserum
generated in this laboratory, human hACTH-(1–39) (Peninsula Labs, Belmont, CA, USA) for standard, and
\(^{125}\text{I}\)-labelled ACTH made by iodination of the hACTH-
(1–39) standard using the chloramine-T method. Equine
ACTH is homologous to hACTH-(1–39) (Ng et al. 1981).
Before assay, ACTH was extracted from plasma on glass.
This assay has been completely described elsewhere (Bell
& Wood 1991). Plasma cortisol concentrations were
measured as previously described by RIA using a poly-
clonal rabbit antisemur obtained from Radioassys Systems
Laboratory (Carson, CA, USA) and [1,2,6,7-\(^{3}\text{H}(\text{N})\] hydrocortisone (New England Nuclear, Boston, MA,
USA) (Wood & Rudolph 1983). Before assay, plasma was
deproteinized with 20 vol ethanol. Both hormone assays
were measured in triplicate at each time point for purposes
of statistical analysis.

Corticosteroid binding capacity was measured using
methods previously described (Ballard et al. 1982). Briefly,
15 ng cortisol, 65 \(\mu\)l plasma and 5 \(\mu\)l [1,2,6,7-\(^{3}\text{H}(\text{N})\]
cortisol (New England Nuclear) were incubated at 37 \(^\circ\)C
for 30 min and then at 4 \(^\circ\)C for 30 min. Unbound cortisol
was absorbed by adding 15 \(\mu\)l charcoal (85 mg/ml charcoal
in 0-05 M PBS) followed by a 20 min incubation at room
temperature and centrifugation at 3000 r.p.m. at 4 \(^\circ\)C for
5 min. Supernatant (25 \(\mu\)l) was then removed and the
activity was counted. Corticosteroid binding capacity was
calculated by multiplying the total cortisol concentration
(the sum of the plasma cortisol plus the 15 ng added
cortisol) by the fraction of trace bound (sample counts/min
divided by total counts/min). All samples were assayed in
triplicate.

Analyses

First order regressions were calculated for daily ACTH and
cortisol values in each subject using SigmaPlot 5-0 (Jandel
Scientific, San Rafael, CA, USA). ACTH and corti-
costeroid binding capacity were subjected to Kruskal-
Wallis one-way analysis of variance (ANOVA) on ranks
and Dunn’s method for pairwise multiple comparison
testing. Cortisol values were subjected to one-way
ANOVA and Student-Newman-Keuls test was used for
pairwise multiple comparison testing. For all infer-
ential statistics, the null hypothesis was rejected when
\(P<0.05\).

The hormone values from each time series were sub-
ject to statistical analysis for the detection of significant
peaks and nadirs using Cluster analysis (Veldhuis &
Johnson 1986). In this analysis, the replicate measuremen-
t of hormone concentration in each sample were used to
determine measurement error. Minimum and maximum
coefficients of variation, 1\% and 25\% respectively, and a
Cluster size of 1 were used as criteria for detection of peaks
and nadirs. The \(t\)-statistic used in the analysis (1-75) was
for a 5\% false positive rate and for a \(1 \times 1\) (one point
determines a peak and one point determines a valley)
configuration measured in triplicate. The minimum
change necessary for peak or valley detection was
20 pg/ml for ACTH and 1 ng/ml for cortisol. These
values were chosen as they are the minimum levels of
detectability of the assays.

In addition to Cluster analysis, all the endocrine data
were subjected to frequency analysis using fast Fourier
transform (FFT) (Jasper & Engeland 1991). This is a
method for detecting the most prominent frequencies of
oscillation in a time series. Before calculating the FFT in
each time series, data were subjected to trend removal,
time domain windowing and direct-current bias removal
(Jasper & Engeland 1991). Data were tapered to zero at
the beginning and end of each series using a cosine taper
window. This analysis yields a series of FFT coefficients
which represent apparent frequencies in the original time
series. Power spectra (the squared magnitudes of the FFT
coefficients) were normalized as the fraction of total power
within an experiment to allow for comparison between
animals. To determine which individual frequencies were
statistically significant, power spectra were subjected to
Permutation Rank Test (Odell et al. 1975). Both the
frequency analysis and the statistical analysis of the nor-
malized spectra were performed using a computer program
developed by Dr M Jasper (Jasper & Engeland 1991).
In each group, normalized spectra were analyzed by one-
way ANOVA corrected for repeated measures and a \(\text{a posteriori}\)
comparison of means by Duncan’s multiple range test. The
analyses by ANOVA and Duncan’s test were used to
detect rhythms common to most animals in that group.
Values are expressed as means ± S.E.M.

This study was approved by the Institutional Animal
Care and Use Committee of the University of Florida.

Results

Chronic instrumentation of equine fetuses

In all, 9 fetuses were catheterized for this study. In three
cases, fetal blood gases deteriorated, ACTH and cortisol
values did not decrease after surgery and the fetuses were
spontaneously delivered 3 to 4 days following surgery.
Therefore, values from these animals were not presented
though the reason for the high values and the prompt
delivery after surgery was not determined.

Of the 6 fetuses from which data is presented, the
average time from surgery to delivery was 11-2 days and
ranged from 6 to 20 days (Table 1). The mean gestational
age at the time of surgery was 293 days with a range of
278-304 days. The average gestational age at delivery was
305 days while the average length of gestation in the
equine is 335 days. We estimate, therefore, that the
surgical instrumentation of the fetuses advanced the day of
parturition by 30 days, or 9% of normal gestation. Surgery
on fetal sheep in this laboratory advances parturition
approximately 6% (6-8 days) of normal (Cudd & Wood,
TABLE 1. Data from six fetuses. Age (day of gestation) at surgery, delivery, and experiments for detection of ultradian rhythms, outcome, sampling site, mean ± S.E.M. and range for pH and blood gas values. Blood gases were measured daily.

<table>
<thead>
<tr>
<th>Fetus number</th>
<th>Age at surgery</th>
<th>Age at delivery</th>
<th>Age at experiments</th>
<th>Outcome</th>
<th>Sample site</th>
<th>pH mean ± S.E.M. (range)</th>
<th>PCO₂ mean ± S.E.M. (range)</th>
<th>PO₂ mean ± S.E.M. (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>303</td>
<td>309</td>
<td>305</td>
<td>Alive</td>
<td>Umb. V.</td>
<td>7.394 ± 0.034 (7.262–7.442)</td>
<td>41.4 ± 4.8 (34.6–60.3)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>295</td>
<td>305</td>
<td>304</td>
<td>Stillborn</td>
<td>Aorta</td>
<td>7.403 ± 0.018 (7.290–7.463)</td>
<td>43.4 ± 4.2 (38.3–58.7)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>278</td>
<td>284</td>
<td>none performed</td>
<td>Stillborn</td>
<td>Aorta</td>
<td>7.306 ± 0.034 (7.268–7.373)</td>
<td>39.9 ± 0.1 (39.8–40.0)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>291</td>
<td>304</td>
<td>303</td>
<td>Stillborn</td>
<td>Aorta</td>
<td>7.334 ± 0.007 (7.356–7.314)</td>
<td>48.0 ± 2.2 (44.7–63.0)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>304</td>
<td>316</td>
<td>310</td>
<td>Alive</td>
<td>Umb. V.</td>
<td>7.400 ± 0.017 (7.262–7.469)</td>
<td>39.2 ± 0.8 (35.4–45.0)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>289</td>
<td>309</td>
<td>293, 300</td>
<td>Died during dystocia</td>
<td>Aorta</td>
<td>7.413 ± 0.007 (7.365–7.468)</td>
<td>45.6 ± 1.5 (35.5–58.6)</td>
<td>27.3 ± 0.9 (22.0–36.0)</td>
</tr>
</tbody>
</table>
unpublished observations). The reduced length of gestation in horses following surgery and instrumentation compared with that in normal, non-operated spontaneous births, raises the issue of whether these pregnancies may have been terminated by a mechanism not consistent with normal birth. However, fetal blood gases were between 7.262 and 7.469 for pH, 34.6 and 63.0 mm Hg for PCO\(_2\) and 17.3 and 43.4 mm Hg for PO\(_2\) during the entire study period. These values are similar to those reported for healthy, chronically catheterized fetal horses (Taylor et al. 1992). Despite this, only 2 of 6 fetuses were delivered alive. One fetus died prior to delivery due to dystocia (the fetus was alive during attempts to manually correct the presentation) but the causes of death of the other 3 could not be determined. In our experience, this preparation is more difficult and less often successful in fetal horses compared with fetal sheep. Nevertheless, our success rate is similar to that reported in the rhesus monkey and other non-human primates where published success rates vary from 21% (Parshall & Silverstein 1969) to 78-6% (Harrison et al. 1982) live births following fetal surgery but without chronic instrumentation, an added negative factor that was present in our preparations.

Arterial and venous catheters were placed in all fetuses. In many cases, especially early in our experience with this preparation, we were unable to draw samples from all the catheters. The use of different sites for blood sampling reflects this difficulty. We do not believe that variations in the sampling site significantly altered our results. In 2 fetuses only venous samples could be obtained while in another 2 fetuses only arterial samples could be obtained. However, in 2 fetuses, we were able to maintain both artery and vein catheters and were able to compare the arterial and venous concentration of ACTH and cortisol. The arterial values for ACTH were higher in 6 samples by an average of 23 pg/ml, lower in 7 cases by an average of 122 pg/ml and the same in 2 cases. The mean arterio-venous difference for all samples was \(-62 \pm 28\) pg/ml. For cortisol, in no cases did we detect a higher venous value compared with the arterial sample and the mean arterio-venous difference was \(-0.7 \pm 0.2\) ng/ml. These data suggest that the equine placenta does not secrete ACTH or cortisol.

### Ontogeny of fetal ACTH, cortisol and corticosteroid binding capacity

Fetal plasma ACTH and cortisol concentrations were measured daily until parturition. While, in most subjects, cortisol rose progressively over the last 3 days before parturition with little day-to-day fluctuation, ACTH exhibited a higher degree of day-to-day variability. A first order regression was calculated from the ACTH and cortisol values for each subject. For ACTH, the slopes of all 6 regressions were positive and the \(r\) values were statistically significant for 3 of 6 animals (Table 2). For cortisol, the slopes were positive for all 6 subjects with a statistically significant \(r\) value for 5 of 6 subjects.

Overall, fetal plasma ACTH increased significantly only on the last day before parturition (from \(159 \pm 21\) pg/ml to \(281 \pm 32\) pg/ml). The cortisol increased significantly over the last three days before parturition (from \(3.0 \pm 0.7\) ng/ml to \(5.8 \pm 0.9\) ng/ml). Corticosteroid binding capacity increased significantly 10-14 days before birth (from \(61 \pm 20\) ng/ml to \(80 \pm 20\) ng/ml). The mean values for each day are shown in the following figures: ACTH, cortisol and corticosteroid binding capacity.

#### Table 2. First order regression coefficients for ACTH and cortisol by subject (* indicates significance at \(P<0.05\))

<table>
<thead>
<tr>
<th>Fetus number</th>
<th>Slope</th>
<th>(r)</th>
<th>Slope</th>
<th>(r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td></td>
<td></td>
<td>Cortisol</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50.4</td>
<td>0.975*</td>
<td>1.9</td>
<td>0.886*</td>
</tr>
<tr>
<td>2</td>
<td>11.25</td>
<td>0.381</td>
<td>0.6</td>
<td>0.720*</td>
</tr>
<tr>
<td>3</td>
<td>5.6</td>
<td>0.752*</td>
<td>0.3</td>
<td>0.416</td>
</tr>
<tr>
<td>4</td>
<td>12.3</td>
<td>0.783*</td>
<td>4.3</td>
<td>0.872*</td>
</tr>
<tr>
<td>5</td>
<td>24.1</td>
<td>0.48</td>
<td>2</td>
<td>0.823*</td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>0.068</td>
<td>1</td>
<td>0.815*</td>
</tr>
</tbody>
</table>

#### Figure 1. Mean ± s.e.m. values for fetal ACTH, cortisol and corticosteroid binding capacity (cort. binding cap.) on days prior to parturition. ACTH and cortisol increased while corticosteroid binding capacity decreased significantly at the end of gestation. * indicates statistical significance at \(P<0.05\).
TABLE 3. Mean ACTH and cortisol values for fetuses (Fet), pregnant mares (PM) and non-pregnant mares (NPM). The bottom row shows the mean values measured in all experiments by subject group. Subject: numeral for fetuses and mothers and character for non-pregnant mares (6a identifies the second experiment performed in subject 6).

<table>
<thead>
<tr>
<th>Subject</th>
<th>ACTH (pg/ml)</th>
<th>Cortisol (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fet</td>
<td>PM</td>
</tr>
<tr>
<td>1/a</td>
<td>176</td>
<td>90</td>
</tr>
<tr>
<td>2/b</td>
<td>117</td>
<td>43</td>
</tr>
<tr>
<td>4/c</td>
<td>61</td>
<td>37</td>
</tr>
<tr>
<td>5/d</td>
<td>389</td>
<td>68</td>
</tr>
<tr>
<td>6/e</td>
<td>126</td>
<td>130</td>
</tr>
<tr>
<td>6a/f</td>
<td>118</td>
<td>102</td>
</tr>
<tr>
<td>g</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>165</td>
<td>78</td>
</tr>
</tbody>
</table>

The mean ACTH and cortisol values for each ultradian experiment are presented in Table 3. The mean ACTH values for all time series were 165, 78 and 57 pg/ml for fetuses, pregnant mares and non-pregnant mares respectively. The mean values of cortisol were 6, 17 and 36 ng/ml for fetuses, pregnant mares and non-pregnant mares. Examples of time series data for the fetuses, pregnant mares and non-pregnant mares are presented in Figs 2 and 3. The time series data for each experiment were subjected to Cluster analysis. Using this technique, we detected significant peaks and valleys in plasma ACTH in all animals studied, and significant peaks and valleys in plasma cortisol in all fetuses, in all but one pregnant mare, and in all but two non-pregnant mares (Table 4). In fetuses, we detected between 1 and 5 peaks and valleys for both ACTH and cortisol. The mean number of peaks and valleys for fetuses was 2.5 for ACTH and 3.0 and 3.5 respectively for cortisol. In pregnant mares, we detected between 2 and 4 significant peaks and valleys in ACTH and between 0 and 3 peaks and valleys for cortisol. The mean number of peaks and valleys in pregnant mares was

FIGURE 2. Time series values of ACTH for (a) fetus 6, (b) pregnant mare 5 and (c) non-pregnant mare a. * indicate significant peaks or valleys detected by Cluster analysis.

FIGURE 3. Time series values of cortisol for (a) fetus 6, (b) pregnant mare 5 and (c) non-pregnant mare a. * indicate significant peaks or valleys detected by Cluster analysis.

Ultradian rhythmicity of plasma ACTH and cortisol concentrations

The mean ACTH and cortisol values for each ultradian experiment are presented in Table 3. The mean ACTH values for all time series were 165, 78 and 57 pg/ml for fetuses, pregnant mares and non-pregnant mares respectively. The mean values of cortisol were 6, 17 and 36 ng/ml for fetuses, pregnant mares and non-pregnant mares. Examples of time series data for the fetuses, pregnant mares and non-pregnant mares are presented in Figs 2 and 3. The time series data for each experiment were subjected to Cluster analysis. Using this technique, we detected significant peaks and valleys in plasma ACTH in all animals studied, and significant peaks and valleys in plasma cortisol in all fetuses, in all but one pregnant mare, and in all but two non-pregnant mares (Table 4). In fetuses, we detected between 1 and 5 peaks and valleys for both ACTH and cortisol. The mean number of peaks and valleys for fetuses was 2.5 for ACTH and 3.0 and 3.5 respectively for cortisol. In pregnant mares, we detected between 2 and 4 significant peaks and valleys in ACTH and between 0 and 3 peaks and valleys for cortisol. The mean number of peaks and valleys in pregnant mares was

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2-8 for ACTH and 1-7 and 1-8 for cortisol respectively. In non-pregnant mares, we detected between 2 and 6 peaks and valleys for ACTH and between 0 and 4 for cortisol. The mean number of peaks and valleys were 3-5 and 3-8 for ACTH and 1-3 and 1-6 respectively for cortisol.

Frequency analysis permitted us potentially to identify periods of oscillation between 10 and 60 min, based on a sampling frequency of 5 min and a time series lasting 2 h. FFT of the time series data generated 33 frequency bins representing periods of 10 min to infinity. The frequency bin representing 64 min, the frequency closest to 60 min or one half the length of the time series, was the maximum resolvable frequency.

Examples of individual frequency analyses for fetuses, pregnant mares and non-pregnant mares are presented in Figs 4 and 5. Frequency analysis detected significant periods of oscillation for ACTH in all experiments except in one non-pregnant adult (Table 5). In all the other experiments, either 1 or 2 significant period(s) of oscillation for ACTH were detected. Four of 6 fetuses, 1 of 6 pregnant mares, and 6 of 8 non-pregnant mares exhibited significant periods between 11 and 17 min (mean 14 min, median 13 min). Four of 6 fetuses, 4 of 6 pregnant mares, and 2 of 8 non-pregnant mares exhibited periods between 27 and 40 min (mean 34 min, median 34 min). These longer periods may be multiples of shorter periods. Alternatively, the longer periods may indicate that a more complex rhythm is present with more than one period detectable.

Pulse frequency in the cortisol data was similar to that in the ACTH data with the exception that the distribution of frequencies was skewed towards longer periods. Three of 6 fetuses, 3 of 6 pregnant mares, and 2 of 8 non-pregnant mares had significant periods between 11 and 17 min. One fetus, 1 pregnant mare, and 2 non-pregnant mares had significant periods between 27 and 40 min. On the other hand, 4 fetuses, 4 pregnant mares, and 2 non-pregnant mares had significant periods longer than 40 min. In 5 experiments (1 fetus, 1 pregnant mare, and 3 non-pregnant mares), there was no significant rhythm in plasma cortisol concentration detectable with this method.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Fet ACTH P</th>
<th>Fet ACTH V</th>
<th>PM ACTH P</th>
<th>PM ACTH V</th>
<th>NPM ACTH P</th>
<th>NPM ACTH V</th>
<th>Fet cortisol P</th>
<th>Fet cortisol V</th>
<th>PM cortisol P</th>
<th>PM cortisol V</th>
<th>NPM cortisol P</th>
<th>NPM cortisol V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/a</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
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Figure 4. ACTH frequency analyses for (a) fetus 6, (b) pregnant mare 5 and (c) non-pregnant mare a. The values are presented as the fraction of total power calculated by fast Fourier transform for frequencies between 0 (infinite period of oscillation) and 0.5 (10 min frequency period) cycles/min. The frequency periods that were determined to be statistically significant by Permutation Rank Test are identified by the period duration in minutes.
To detect the most robust rhythms in the times series, we subjected the FFT results by group to one-way ANOVA. When considering all fetal experiments, we found significant periods in ACTH at 11, 12, 13, 17, 25 and 40 min and significant periods in cortisol at 13, 15 and 64 min (Fig. 6). In the pregnant mares, the significant periods in ACTH were at 16, 32 and 53 min and in cortisol at 17, 29 and 64 min. In the non-pregnant mares, we found significant periods in ACTH at 12, 17, 21, 29 and 64 min and in cortisol at 12, 16, 27, 36 and 53 min. When all frequency analysis results were combined, we found significant periods at 12, 13, 17, 25, 32, and 53 min for ACTH and at 12, 17, 21, 29, and 53 min for cortisol.

**Table 5.** Significant pulse frequencies in minutes detected by fast Fourier transform and Permutation Rank Test for ACTH and cortisol for fetuses (Fet), pregnant mares (PM), and non-pregnant mares (NPM). Blank fields indicate no experiments while — indicates that significant rhythms were not detected. Subject: numeral for fetuses and mothers and character for non-pregnant mares (6a identifies the second experiment performed in subject 6)

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**Discussion**

**Ontogeny**

The purpose of this study was to characterize the ontogenetic changes in ACTH, cortisol, corticosteroid binding capacity and the ultradian pattern of ACTH and cortisol release in the equine fetus. We found that fetal plasma ACTH increases very late, within the last 48 h before parturition and that ACTH in individual fetuses exhibited a high degree of day-to-day variability near the end of gestation. Fetal plasma cortisol also increases at the end of gestation and, like ACTH, the increase begins very late, during the last 48–72 h or 1–2% of gestation. Others have found that while there was a small apparent increase, there was no significant rise in equine fetal ACTH at the end of gestation (Silver & Fowden 1994). However, like us, they found that fetal ACTH variability and cortisol concentrations increased significantly at the end of gestation. The apparent increase in equine fetal cortisol, days before the increase in ACTH observed in our studies and those of others (Silver & Fowden 1994), is similar to that observed in fetal sheep (Rose et al. 1978) and can be explained by the increasing sensitivity of the fetal adrenal cortex in both the equine (Silver & Fowden 1994) and the sheep (Wood 1989) fetus at the end of gestation. An increase in fetal adrenal sensitivity to ACTH during this part of development can result in a rise in cortisol in response to small or statistically difficult to detect increases in ACTH.

We found that corticosteroid binding capacity decreases during the final days of gestation in the fetal horse. Because total cortisol increases during this period, unbound plasma cortisol must rise out of proportion to the total cortisol concentration at the end of gestation. Corticosteroid binding capacity has previously been measured in neonatal and
adult horses (Irvine & Alexander 1987). They found corticosteroid binding capacity to be low in the neonatal period (about 10 ng/ml, a level that is comparable to our late fetal values of 16 ng/ml), and higher in adults (about 41 ng/ml or similar to our early fetal values of 49.5 ng/ml). Fetal corticosteroid binding capacity also decreases at the end of gestation in rats (Martin et al. 1977), mice (Savu et al. 1977) and baboons (Oakey 1975). In fetal sheep, the corticosteroid binding capacity begins to rise after 120 days gestation and continues to increase until parturition (Ballard et al. 1982). It has been hypothesized that the fetal pituitary-adrenal axis controls the expression of fetal corticosteroid binding globulin in fetal sheep (Ballard et al. 1982, Jacobs et al. 1991). However, in the adult rat, increases in plasma cortisol result in a decrease in corticosteroid binding globulin (Smith & Hammond 1992). The findings that fetal cortisol increases while corticosteroid binding capacity decreases at the end of gestation in non-ruminants suggests that control of corticosteroid binding globulin synthesis is different in these species.

In comparison to the horse, the ontogenetic rise in cortisol in the fetal sheep begins sooner, as early as 17 days or 12% before parturition (Magyar et al. 1980). Because of the different ontogenetic pattern, we postulate that the role of cortisol in the development of the fetus and the timing of parturition in the horse is likely to be different to that in the sheep. Results of experiments from other laboratories support this hypothesis. The administration of dexamethasone to the gravid mare does not induce parturition (Drost 1972) or does so only after repeated high doses over a much longer time compared with the sheep (Aim et al. 1974). It has recently been reported that cytochrome P450c17 cannot be detected even in term equine placentae (Mason et al. 1993). Therefore, cortisol does not induce 17-hydroxylase and 17,20 lyase activities in the horse placenta near term as occurs in the sheep. An important function of late rising cortisol in the equine fetus may be to orchestrate developmental changes necessary for survival. For example, gluconeogenic enzymes in fetal horses increase very late in gestation or over the same time period that cortisol values increase suggesting that this process may be cortisol dependent (Fowden et al. 1992). Silver and co-workers have reported that plasma cortisol is low in foals born prematurely (Silver et al. 1984) and that these foals exhibit poor cardiovascular stability, abnormal mentation and other manifestations of incomplete development and hypoadrenocorticism (Rossdale et al. 1984). In the present study, the cortisol values on the last day before parturition ranged from 3 to 20 ng/ml. Interestingly, the cortisol values for individual fetuses measured the last day before parturition and ranked from highest to lowest corresponded to fetal gestational ages ranked from oldest to

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Mean fractions of total power for ACTH and cortisol in all fetuses, pregnant mares and non-pregnant adults. For details see legends to Figs 4 and 5. Statistically significant frequencies are identified by the period duration given in minutes.
youngest in all cases. Our findings, and those of others, suggest that final visceral development is later and more rapid in the horse than in the sheep, that the late increase in equine fetal cortisol is required for final development and for perinatal survival and that the late onset of the preparturient rise in fetal plasma cortisol places the equine fetus at a disadvantage for preterm survival.

Ultradian rhythms

We have also characterized the ultradian release of ACTH and cortisol in the equine fetus and pregnant mare and have compared this to the non-pregnant mare. We used two methods for demonstrating pulsatility. Cluster analysis is a statistical method which conservatively detects pulsatility in individual time series records, taking into account the inherent variability of the assay methodology. Cluster analysis detected statistically significant pulses in ACTH and cortisol in most experiments, regardless of whether the subjects were adult or fetal horses. The second method of analysis, power spectral density analysis, was chosen as a sensitive method for identifying the frequencies of oscillation within a time series. This method of analysis allows for the detection of more than one rhythm in the pattern of the release of a measured hormone (Benton & Yates 1990). Power spectral density analysis is, therefore, a sensitive method for separating component oscillations. To distinguish 'signal' from 'noise', we used the Permutation Rank Test (Odell et al. 1975), a nonparametric statistical method designed to identify those component rhythms which are significantly stronger than random noise. Using these methods for frequency analysis, we found a remarkable consistency of ultradian rhythms in all the animals studied, with most demonstrating significant rhythms between 11 and 17 min. Power spectral density analysis allows detection of periods as short as twice the sampling frequency (Odell et al. 1975), 10 min in the present study. It is conceivable that we might have detected even faster rhythms had we sampled more frequently. The significant rhythms with longer periods could be separate rhythms or, possibly, multiples or harmonics of the basic faster rhythms. The design of these experiments does not allow us to distinguish between these possibilities.

We were successful in identifying more rapid rhythms in plasma ACTH concentration than in plasma cortisol concentration. This is most likely the result of the more rapid half-disappearance time of ACTH (1–2 min) compared with cortisol (approximately 30 min) from plasma. Because of the long half-disappearance time and binding of cortisol in plasma, a time equivalent to 4 or 5 times the half-disappearance time may be required for changes in cortisol secretion rate to produce steady-state changes in plasma cortisol concentration (Veldhuis & Johnson 1988).

In adult horses, pulsatile release of ACTH every 16 to 24 min into cavernous sinus blood has been reported (Redekopp et al. 1986). The present report is the first, however, to demonstrate ultradian rhythms in ACTH and cortisol concentrations in pregnant and fetal horses. The rhythms in the present study were demonstrable in most of the animals although they were not present in all. We cannot readily explain this observation. However, we believe that the sampling of blood from an animal with a large blood volume eliminates the possibility that the sampling regimen itself stimulates ACTH secretion.

The ultradian rhythms of ACTH and cortisol found in horses are comparable to those found in other species. For example, Wood et al. (1982) reported rhythms in plasma ACTH concentration in conscious dogs of approximately 10 min/cycle. Carnes and co-workers (Carnes et al. 1990) reported ACTH ultradian rhythms in rats with periods of approximately 20 and 100 min. A larger body of work has been focused on rhythmicity in plasma cortisol concentration. Investigators have reported rhythms between 3 and 160 min, depending upon species and experimental conditions. For example, Benton & Yates (1990) reported rhythms of 3, 6, and 90 min in dogs. Rhythms with periods between 75 and 128 min in primates and between 53 and 120 min in rats have been reported by Tapp et al. (1984). Yellon and co-workers have found a cortisol ultradian rhythm in fetal sheep with periods of 54 and 23 min depending on gestational age (Apostolakis et al. 1992). Jasper and Engeland used a novel microdialysis sampling method for measuring corticosterone in adrenal cortical extracellular fluid (Jasper & Engeland 1991). They found an interpulse interval of 51–54 min using Pulsar (Merriam & Wachter 1982) to detect individual 'peaks' and 'valleys' and an interpulse interval of 60–80 min using power spectral density analysis. This method allowed for the detection of rapid changes in corticosterone secretion rate, without the influence of plasma binding proteins and plasma clearance rate of corticosterone.

Basal hormonal values are commonly derived from a single plasma sample. However, it is evident from these studies that obtaining a single sample from fetal and adult horses to assess basal ACTH or cortisol plasma concentrations would potentially introduce error compared with averaging the values from serial samples. ACTH and cortisol peaks and valleys were often over 50% different from the mean values for the entire sampling series. The period of oscillation for most animals was 11–17 min. Therefore, in order to obtain a value for ACTH and cortisol that accurately reflects the mean basal value for these hormones it would be advisable to obtain multiple samples over a period of time longer than the expected period of oscillation, every 5 min over a period of 20 min for example, and to average the values obtained.

To our knowledge, this study is 1 of only 3 reports of ACTH ultradian rhythmicity in fetal animals. Challis and co-workers reported ACTH rhythms in fetal sheep of between 30 and 60 min (Challis et al. 1981) while others have reported rhythms of 40 min (Apostolakis et al. 1992).
ultradian pattern of release of ACTH and cortisol when comparing the late gestation fetus to adults. However, the observation that ACTH release was synchronized in one fetus and mother raises the possibility that the actions of non-labor contractions might change the rhythm of ACTH release in the fetus in late gestation and perhaps accelerate fetal adrenal maturation.

Summary and conclusion

We have developed a chronically instrumented fetal horse preparation, a difficult feat that has only been previously accomplished by Silver and colleagues in Cambridge, UK (Comline et al. 1975). We have found that equine fetal ACTH concentrations are quite variable during the last week of gestation before finally increasing the day before parturition. We found that there is an increase in fetal plasma concentrations of cortisol and a decrease in cortisol binding capacity in the last 48–72 h before parturition. We conclude that there is an increase in fetal hypothalamic-pituitary-adrenal activity near term and that the increase in unbound plasma cortisol concentration is proportionately greater than that of the total plasma cortisol concentration due to a decrease in plasma corticosteroid binding capacity at the end of gestation. The results of this study demonstrate ultradian rhythms in plasma ACTH and cortisol concentrations in late-gestation fetal horses, in pregnant mares and in non-pregnant mares. The period of the ACTH rhythm varies among animals, but appears to be rapid (11–17 min) in most. The cortisol rhythms appear to be the result of the ACTH rhythms and the longer half-disappearance rate for cortisol. We conclude that the ultradian rhythm in plasma ACTH concentration is robust and demonstrable as early as the late fetal period.

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