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Short Communication

# Monitoring the circadian rhythm of serum and salivary cortisol concentrations in the horse

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ABSTRACT

Daily fluctuations of cortisol concentration in the blood or saliva have been repeatedly reported. However, several contradictions in the existing literature appear on this subject. The present study was performed to definitively establish options for testing adrenocortical function. To the best of our knowledge, this is the first study to evaluate parallel circadian rhythms in salivary and serum cortisol concentrations during a 24-h period. Twenty horses were examined under the same conditions. Blood and saliva samples were taken every 2 h for 24 h to determine the daily changes in cortisol concentrations of saliva and serum at rest and to determine the relationship between salivary and serum cortisol levels. Cosinor analysis of group mean data confirmed a significant circadian component for both serum and salivary cortisol concentrations ($P < 0.001$ in both cases). The serum cortisol circadian rhythm had an acrophase at 10:50 AM (95% CI, 10:00 AM–11:40 AM), a MESOR of 22.67 ng/mL, and an amplitude of 11.93 ng/mL. The salivary cortisol circadian rhythm had an acrophase at 10:00 AM (95% CI, 9:00 AM–11:00 AM), a MESOR of 0.52 ng/mL, and an amplitude of 0.12 ng/mL. We found a significant but weak association between salivary and serum cortisol concentrations; the Pearson correlation coefficient was 0.32 ($P < 0.001$). The use of salivary cortisol level as an indicator of hypothalamic-pituitary-adrenal axis activity may be warranted. However, the salivary cortisol levels are more likely to be correlated with free plasma cortisol than with the total plasma cortisol concentration.



## 1. Introduction

In horses, just as in other animals and human beings, the intensity of many biological processes varies during the day. Certain hormones of blood and saliva may have a diurnal pattern. Circadian rhythms for cortisol in plasma and saliva have been reported [1–5].The cardinal differences among the studies are in the sampling duration (1–6 h) and number of horses used (4–18 horses). Some equine studies have found relatively large differences between morning and evening plasma cortisol concentrations [1,4,5], whereas others could not confirm a difference [6] or could only occasionally find circadian changes [7,8]. Cortisol daily peak levels usually occur in the morning, whereas the lower values occur in the evening [1–3]. However, some experiments show that the circadian rhythm could be disrupted if the daily routine of the horses was changed [1,9–11]. Collecting samples from horses (as with other animals) usually means increased stress for the animal. When examining a stress hormone, it is sensible to use the least stressful sampling procedure. In some horses, collection of a saliva sample provokes less stress than does collection of blood. An even more important advantage of using saliva samples is that the cortisol in the saliva passively diffuses into the salivary glands and therefore is not bound to proteins. Salivary cortisol constantly provides information about the free cortisol concentration [2,12].

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According to several reports, measuring the salivary cortisol concentration is a useful method for assessing the activity of the hypothalamic-pituitary-adrenal (HPA) axis [2,13,14]. Several researchers have described a close relationship between the salivary and blood cortisol levels in different species, including the horse [2,3,12,15]. Other studies could not find a positive correlation between equine serum and salivary cortisol concentrations [16,17]. Further, Lebelt et al [2] reported a daily fluctuation pattern in salivary cortisol concentrations, whereas other researchers could not confirm this phenomenon [3].

Measurement of the salivary cortisol levels is well suited for the human praxis for measuring stress [18] and as a screening test for Cushing's disease [19]. Stress has been assessed in horses by measuring the salivary cortisol concentrations [10,12]; however, reliably using salivary cortisol content, as in human practice, should be further studied in horses [3,4].

The aim of the present study was to clarify the discrepancies in the literature about serum cortisol circadian rhythm and to determine whether salivary cortisol has a circadian rhythm.

## 2. Materials and methods

### 2.1. Horses

Twenty clinically healthy horses (3 thoroughbred, 4 Shagya–Arabian, and 13 Hungarian sport horses) were used in the experiment. They were housed in the Clinic for Large Animals, Üllő, Dóra major, Hungary. Although no horses were working, they were in good physical condition throughout the study period. The age of the animals ranged from 3 to 13 y. Eight of these horses were mares, 7 were geldings, and 5 were stallions. All animals were kept in conventional horse pens and fed a commercial horse diet (ie, oats and wheat bran) 3 times daily at 7:00 AM, 12:00 PM, and 6:00 PM. They had ad libitum access to hay and water. The experiment was performed at the end of August. During this period, there was daylight for approximately 14 h and darkness for 10 h.

### 2.2. Sample collection

Starting at 2:00 PM, blood and saliva samples were obtained from each of the 20 horses every 2 h for 24 h. Blood samples were obtained by venipuncture of the vena jugularis with the use of S-Monovette 7.5-mL Z tubes (Sarstedt, Nümbrecht, Germany), followed by centrifugation at 2,000× *g* for 10 min.

Saliva samples were collected on cotton swabs held with a surgical arterial clamp. After the experimental animal had chewed the swabs for approximately 20 s, the swabs were mechanically squeezed out. The saliva sample was then centrifuged for 5 min at 2,000× *g*. The serum and saliva samples were placed in Eppendorf test tubes and stored frozen at −20°C until the assay [20].

Special care was used to minimize stress during the sampling. Throughout the study, the same person handled all animals. The experimental horses were accustomed to clinical routines and frequent blood sampling. To cause the least pain to the animals, thin 22-G sterile needles (BD Microlance TM3, Becton Dickinson S.A., Madrid, Spain) were used for venipuncture. Horses did not need any special restraint during blood and saliva samplings. They stayed calm and learned quickly to accept the swab. At night, the samples were collected under a headlamp. We tried to minimize unnecessary noise [2].

### 2.3. Cortisol measurement

Cortisol analyses were performed in duplicates by using direct radioimmunoassay with a HPLC preparation of cortisol-3-corticosterone methyloxidase, coupled with 2-[$^{125}$I] iodohistamine as tracer for specific antibodies raised against cortisol-3-CMO-BSA [21]. Peeters et al [12] have described the details of the validation of this technique. In brief, lower detection limits of the assays were 0.2 nM and 8 nM in saliva and serum, respectively. The CVs for between-run assays were <11.3% for both saliva and serum. Intra-assay CVs were <5.6%.

### 2.4. Statistical analysis

To determine whether there was either diurnal variation or circadian rhythm in the measured serum and salivary cortisol concentrations, we used the Cosinor program of Refinetti et al [22], based on the least squares cosine fit method of Halberg et al [23], for the group means and separately computed cosine fits to the hormone values for each horse over the trial period (n = 12 data points/series). Circadian variables such as acrophase (the clock time of the maximum value of the curve), MESOR (true mean of the oscillating variable over its entire period), and amplitude (half of the difference between the maximal and minimal value in the curve) were extracted from the cosinor analysis. To assess the association between salivary and serum cortisol concentrations, the Pearson correlation test was used in the R 2.12.2. statistics software. The significance level was set at $P < 0.05$.

## 3. Results

Cosinor analysis of group mean data confirmed a significant circadian component for both serum and salivary cortisol concentrations ($P < 0.001$ in both cases). The 24-h serum and salivary cortisol concentration profiles are presented graphically as the mean ± SEM in Figures 1 and 2, respectively. The serum cortisol circadian rhythm had an acrophase at 10:50 AM (95% CI, 10:00 AM–11:40 AM), a MESOR of 22.67 ng/mL, and an amplitude of 11.93 ng/mL. The salivary cortisol circadian rhythm had an acrophase at 10:00 AM (95% CI, 9:00 AM–11:00 AM), a MESOR of 0.52 ng/mL, and an amplitude of 0.12 ng/mL.

We found a significant but weak association between salivary and serum cortisol concentrations; the Pearson correlation coefficient was 0.32 ($P < 0.001$).

## 4. Discussion

Our present study confirmed the existence of a cortisol circadian rhythm in horses. In agreement with previous