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# Salivary cortisol in stallions: the relationship with plasma levels, daytime profile and changes in response to semen collection

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**Summary**

The aim of this study was to investigate the usefulness of salivary cortisol to monitor the hypothalamic-pituitary-adrenocortical axis activity in horses. Plasma and saliva samples were collected in 4 stallions at two hours intervals in one day. Saliva samples were collected in five stallions hourly during four days. Salivary cortisol was also monitored in 15 min intervals one hour before and during two hours following semen collection. Saliva collection is easily performed, non-invasive and non-stressful. Plasma and salivary cortisol were positively correlated ($r_s$=0.83). Salivary cortisol showed a diurnal pattern with maximum levels in the early morning and lowest in the afternoon (2.76 nmol vs. 1.73 nmol/l). Semen collection led to a significant increase in salivary cortisol (p = 0.019). The measurement of salivary hormones may be useful to assess the welfare of horses and offers an alternative way for a variety of clinical and endocrinological investigations.

**Keywords:** horse, cortisol, saliva, daytime profile, welfare

**Speichel-Kortisol bei Hengsten: Die Beziehung zu Plasmawerten, Tagesprofilen und Veränderungen als Reaktion auf die Samenabnahme**

Das Ziel dieser Studie war, bei Pferden die Brauchbarkeit des Speichel-Kortisols für die Überwachung der Aktivität der Hypothalamus-Hypophysen-Nebennierenrinden-Achse zu untersuchen.

Plasma- und Speichelproben wurden bei 4 Hengsten in einem zweistündigem Intervall an einem Tag genommen. Speichelproben wurden bei 5 Hengsten stündlich während 4 Tagen genommen. Speichel-Kortisol wurde auch in 15minütigen Intervallen eine Stunde vor und bis 2 Stunden nach der Samenabnahme überwacht.

Das Sammeln von Speichel ist leicht auszuführen, nicht invasiv und nicht belastend. Die Speichelproben wurden mit einem Wattetupfer (Hartmann, München, Deutschland) gesammelt, der für 20 Sekunden von dem Versuchspferd gekaut wurde.

Plasma- und Speichel-Kortisol korrelierten positiv miteinander ($r_s$= 0,83).

Das Speichel-Kortisol zeigte einen Tagesrythmus mit maximalen Spiegeln am frühen Morgen und niedrigsten Werten am Nachmittag (2,76 nmol/l vs. 1,73 nmol/l). Die Samenabnahme führte zu einem signifikantem Anstieg des Speichel-Kortisols (p=0,019).

Die Messung von Speichelhormonen ist möglicherweise sinnvoll, um das Wohlergehen der Pferde abzuschätzen und bietet einen alternativen Weg für eine Vielfalt von klinisch und endokrinologischen Untersuchungen.

**Schlüsselwörter:** Pferd, Kortisol, Speichel, Tagesprofil, Wohlergehen

## Introduction

Stress is associated with an activation of the hypothalamic-pituitary-adrenocortical (HPA) system. Corticotropin-releasing factor is secreted from the hypothalamus, causing a release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH is responsible for the release of corticosteroid hormones such as corticosterone and cortisol from the adrenal cortex into the general circulation.

Monitoring the activity of the HPA axis has provided information about the welfare of horses during competition (*Covalesky* et al., 1992), transport (*Clark* et al., 1993) and weaning (*Lindner* et al., 1993). Cortisol accounts for almost 90% of the circulating corticosteroids in horses (*James* et al., 1970). Plasma free cortisol represents the biologically active concentration of that glucocorticoid but its measurement demands laborious procedure. Equine plasma cortisol exhibits circadian as well as ultradian patterns, hence point samples are of little or no use to assess the activity of the HPA axis (*Evans* et al., 1977; *Irvine* and *Alexander,* 1994). The measurement of cortisol in saliva offers several advantages over blood sampling: saliva is easily collected using non-invasive methods, non-stressful to collect, easy to perform, plentiful in supply, glucocorticoids are free from the binding protein and the concentration of cortisol in saliva is independent of flow rate (e.g.. *Riad-Fahmy* et al., 1983). In recent years several groups reported on the correlation between plasma and salivary cortisol (*Fell* et al., 1985; *Parrot* et al., 1989; *Vincent* and *Michell,* 1992), the circadian pattern of salivary cortisol and its usefulness as an indicator for responses to acute stress such as transport (*Zanella* and *Unshelm,* 1994) in different species but not in horses. The aim of the present study was to investigate the usefulness of salivary cortisol to monitor the HPA axis in stallions. Therefore plasma and salivary cortisol levels were compared during daytime and salivary cortisol was monitored in samples taken pre and post semen collection. Semen collection was chosen as a model for an acute activation of the HPA axis as ejaculation leads to a marked and long lasting increase in plasma cortisol levels (*Rabb* et al., 1989).

## Animals, materials and methods

Five stallions, 4 to 17 years of age, housed in the Gynäkologische und Ambulatorische Tierklinik of Munich university were used during this experiment. The animals were kept in conventional horse pens. They were fed a commercial horse diet three times a day at 7:00 h, 12:00 h and 17:00 h and had ad libitum access to hay and water. The stallions were used for teaching procedures and were familiar with blood sampling. All animals were experienced semen donors.

*Sample collection and cortisol measurement*

Blood was collected by jugular puncture and EDTA (1 mg / ml blood) was added, followed by centrifugation at 3,000 g for 15 min at 4°C. Saliva samples were collected using cotton buds (Hartmann, München, Germany) which were chewed by the experimental animal for about 20 seconds. The cotton buds were centrifuged for 10 minutes at 3,000 g and 4°C. Both plasma and saliva samples were divided in aliquots, placed in Eppendorf test tubes and stored frozen at -20°C until assays were performed. Plasma and salivary cortisol were measured in duplicates using a radioimmunoassay technique developed by J. Goode, Dept. of Behavioural Physiology, Institute of Animal Physiology and Genetic Research, Babraham, Cambridge, UK. Minimum assay detection was 4 pg per tube, intra-assay coefficient of variation was 4.3%. To avoid influence of intra-assay variation all samples were processed in one assay. Standards, samples (100 µl saliva and 20 µl plasma) and quality controls were dried under nitrogen in micronic tubes after ethanol extraction and 100ml of PBS buffer (ph 7.4) was added to each tube. 100 ml of antiserum against cortisol raised in rabbits (Klinger, St. Albans, Herts, UK) supplied as a freeze dried material was dispensed into 100 µl aliquots, after being reconstituted with 1 ml of PBS buffer. The antiserum aliquots were mixed with 35 ml of PBS buffer to give a working solution which was used in the assay (100 µl per tube, except the tubes used for non-specific binding). After 4 hours of incubation, 10,000 cpm of Cortisol-3-(O-carboxymethyl)oximino-(2-[$^{125}$I]iodohistamine) (Amersham-Buchler, Braunschweig, Germany ) diluted in PBS buffer was added to each tube. The antibody bound fraction was separated using 100 µl of anti-rabbit IgG raised in donkey, coupled with cellulose (SacCell, IDS, Boldon, UK) after 24 hours of incubation. After centrifugation followed by aspiration of the supernatant the radioactivity of the pellet was measured using a gamma-counter (1417 Wizard, Wallach, Turku, Finland) and cortisol concentrations were calculated with the software package MultiCalc 2.0 (Wallach, Turku, Finland). To test recoveries after ethanol extraction a known amount of hydrocortisone (Sigma-Aldrich Chemie, Deisenhofen, Germany) was added to horse double stipped plasma (sieved characoal, Sigma-Aldrich Chemie, Deisenhofen, Germany) and showed to be higher than 85%.

*Relationship between plasma and salivary cortisol*

Blood and saliva samples were collected every two hours starting at 8.00 h until 16.00 h in one day in four stallions. Several routine management procedures such as oestrus detection and exercise were undertaken during the sampling day.

*Salivary cortisol profile in relation to daytime*

Daily profiles in salivary cortisol levels were monitored in the stallions for four days (two consecutive days followed by a week interval followed by two more consecutive days). Samples were collected at hourly intervals starting at 7.00 h until 18.00h.

*Salivary cortisol levels in response to semen collection*

After 5 basal samples were taken every 15 minutes from 13:00 h to 14:00 h the stallions were exposed to an oestrus mare. After mounting a dummy mare followed by semen collection via an artificial vagina the stallions were taken to their home pen and one saliva sample was collected immediately (5 minutes ± 2 post semen collection). During the following 170 minutes 11 additional saliva samples were collected in 15 minutes intervals. The animals were tested in separated days.

*Statistical analysis*

For the analysis the statistical package Stat View TM-3 (Abacus Concepts, 1988) was used. To minimise the effects of ultradian fluctuations, described for plasma cortisol in previous studies (Evans et al., 1977; Irvine and Alexander, 1994), mean hourly values obtained for individual horses during the four experimental days were grouped in two hours intervals. Salivary cortisol monitored in association with semen collection was divided in 1) basal levels, 2) 5 minutes to 50 minutes post semen collection, 3) 65 minutes to 110 minutes post semen collection and 4) 125 minutes to 170 minutes post semen collection.

## Results

All results are presented as mean ± standard deviation (SD).

*Saliva collection*

Immediately after putting the cotton buds into the horses' mouth, the animals started to chew on it. As chewing led to an increased secretion of saliva, with the technique used in this experiment it was possible to collect 1–1.5 ml of saliva. A centrifuged volume of 100 µl was sufficient to measure salivary cortisol.

*Blood and salivary cortisol*

There was a positive correlation between salivary and plasma cortisol levels (Spearman rank correlation, $r_s = 0.83$, samples = 20, $p = 0.0003$, two-tailed). The amount of cortisol present in saliva ranged from 3 % to 5 % of total plasma cortisol levels. Figure 1 shows the cortisol profiles in plasma and saliva (mean of 4 stallions).

*Salivary cortisol profile in relation to daytime*

Friedman two way analysis of variance showed a significant difference between the samples taken in the intervals described when all animals were included in the analysis (DF 5, samples 6, cases 5, $chi_r^2 = 18.95$, $p = 0.002$). The highest levels were found in the early morning (2.76 nmol/l ± 1.05), the lowest in the late afternoon (1.73 nmol/l ± 0.65). Figure 2 shows the daytime profile in salivary cortisol (7.00h–18.00h) based on the mean of the five stallions being involved in this experiment.

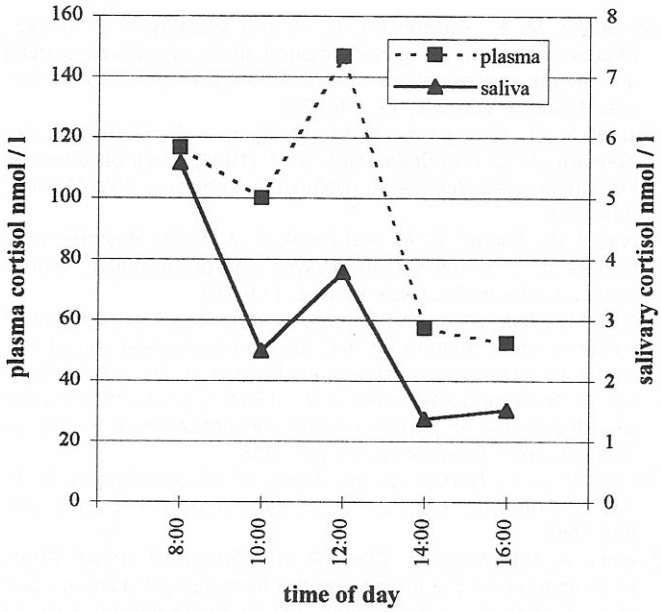


Fig. 1: Relationship between plasma and salivary cortisol. The results are presented as mean of four stallions in nmol/l. The animals were sampled in the same day. ($r_s$ = 0.83)

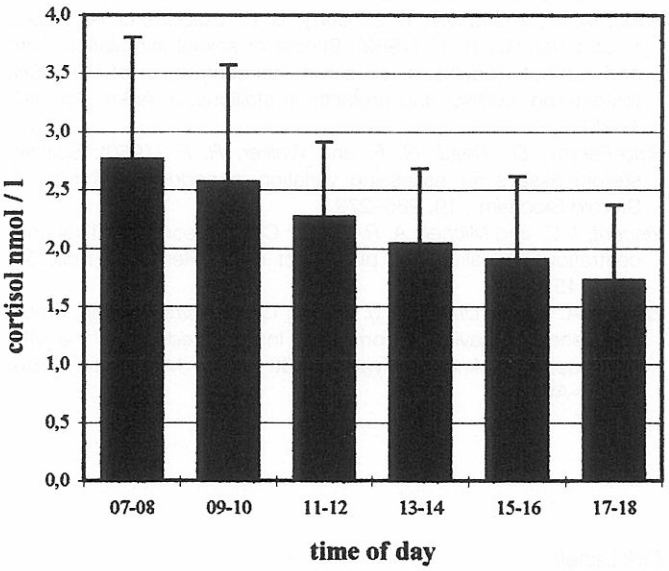


Fig. 2: Salivary cortisol profile during daytime. Cortisol levels measured in salivary samples of five stallions collected on four days hourly from 7.00 h to 18.00 h and grouped in two hour intervals. Results are presented as mean ± SD in nmol/l.

*Semen collection and salivary cortisol*

Semen collection led to a significant increase in salivary cortisol (Friedman two way analysis of variance, DF 3, samples 4, cases 5, $chi_r^2$ = 11.15, p = 0.019). Wilcoxon matched pair t-test showed significant higher ($p < 0.05$) cortisol levels in the stallions' saliva between 35 and 65 min post semen collection. The cortisol level reached 190 % of the mean basal value at 50 minutes after ejaculation. 80 minutes post semen collection cortisol levels were not different ($p > 0.05$) to pre-collection values. Figure 3 shows the levels of salivary cortisol before and after semen collection (mean ± SD of five stallions).

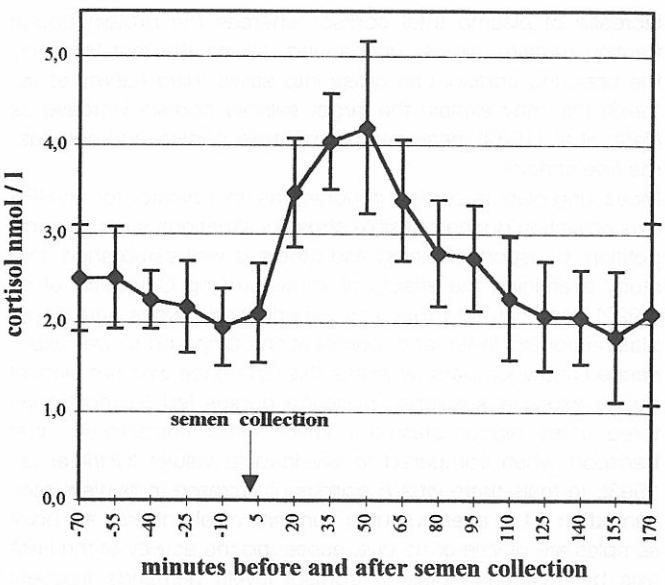


Fig. 3: Salivary cortisol profile before and after semen collection. Results are presented as mean ± SD of five stallions. The arrow marks the point of semen collection.

## Discussion and conclusion

The results of the present study support the work done in other species and indicates that salivary cortisol levels reflect also in horses the concentration of this glucocorticoid in plasma (*Fell* et al., 1985; *Parrot* et al., 1989; *Vincent* and *Michell,* 1992). Cortisol levels in saliva were higher in the early morning samples and showed its lowest levels in the late afternoon. This profile is similar to that reported for plasma cortisol levels in the horse (*Irvine* and *Alexander,* 1994). Although it was possible to show that there is a clear diurnal pattern in salivary cortisol, marked were the inter- and intraindividual differences in daytime profile of cortisol levels observed during the present work. Such observations agree with the variability in plasma cortisol profiles reported by *Evans* et al. (1977) and support the finding that minimal environmental disturbance may cause a disruption of the circadian pattern in plasma cortisol secretion (*Irvine* and *Alexander,* 1994). In contrast to the salivary cortisol profile described above, the profile of cortisol in plasma and saliva as presented in Figure 1 shows a marked peak at 12.00 h. This difference may be due to the small number of samples (4 stallions, sampled in only one day) and to the 2 hours collecting interval. As there is data, indicating an episodic cortisol secretion in horses with a mean peak frequency ranging from about one to two hours (*Evans* et al., 1977; *Irvine* and *Alexander,* 1994) that is superimposed upon the circadian rhythm, the effect of such ultradian fluctuations will be stronger when collecting samples in 2 hours instead of 1 hour intervals. Furthermore, as there was some evidence for a 2 hours rhythm in salivary cortisol in the present study, the hourly collected samples were presented as means of 2 hours to minimise these effects.

Exposure to an oestrus mare and ejaculating into an artificial vagina led to a significant increase in salivary cortisol which is in agreement with *Rabb* et al. (1989) who reported a long lasting increase in plasma cortisol levels following ejaculation. However, this study showed maximum levels around 165 % of basal values 20 minutes post ejaculation. During an acute activation of the HPA axis plasma free cortisol is responsible for the

increase of plasma total cortisol whereas the protein bound fraction remains almost unchanged. Giving the fact that only the unbound cortisol can cross into saliva (*Riad-Fahmy* et al., 1983) this may explain the larger salivary cortisol increase as *Rabb* et al. (1989) measured plasma total cortisol and not plasma free cortisol.

Measuring plasma cortisol in horses as an indicator for an HPA axis activation during possible stressful situations such as competition, transport, weaning and others is well established. In a study examining the effects of show-jumping *Covalesky* et al. (1992) demonstrated that less experienced horses had higher plasma cortisol levels after competition compared to well experienced show-jumpers, whereas this difference was not evident during exercise sessions. Trailering horses led to more than three times higher plasma cortisol levels immediately after transport when compared to pre-loading values (*Clark* et al., 1993). In foals there was a significant increase in cortisol concentration 24 h after weaning (*Lindner* et al., 1993). As point samples are of little or no use, assessing the activity of the HPA axis by measuring plasma cortisol levels demands frequent blood sampling preferably using a jugular catheter. The present study examined the measurement of salivary cortisol in stallions showing fluctuations of cortisol concentrations in saliva as observed in diurnal changes as well as in response to an acute activation of the HPA axis represented by sexual stimulation and semen collection. Saliva collection in horses is very easy to perform and as a non-invasive and non-stressful method it offers several advantages over blood sampling. It allows frequent sampling without the risks of jugular catheterisation and is not restricted by legislation as is the use of jugular catheters for experimental investigations in several countries. Furthermore, as a lack of cortisol circadian pattern has been identified in horses suffering from Cushing disease by *Dybdal* et al. (1994) collecting saliva may offer a diagnostic alternative to frequent blood sampling in order to monitor cortisol profiles in those horses.

Giving the fact that the reliability of using saliva instead of blood as a body fluid to determine the free fraction of low molecular weight hormones, such as steroids, has been well established (*Riad-Fahmy* et al., 1983), saliva may become the body fluid of choice for monitoring welfare in horses but also for a variety of endocrinological and clinical investigations.

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