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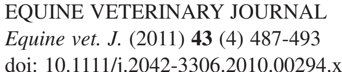

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Comparison between blood serum and salivary cortisol concentrations in horses using an adrenocorticotropic hormone challenge

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Keywords: horse; cortisol; ACTH challenge; saliva; stress

Summary

Reasons for performing study: **In horses, serum cortisol concentration is considered to provide an indirect measurement of stress. However, it includes both free and bound fractions. The sampling method is also invasive and often stressful. This is not the case for salivary cortisol, which is collected using a more welfare-friendly method and represents a part of the free cortisol fraction, which is the biologically active form.**

Objectives: **To compare salivary and serum cortisol assays in horses, in a wide range of concentrations, using an adrenocorticotropic hormone (ACTH) stimulation test, in order to validate salivary cortisol for stress assessment in horse.**

Methods: **In 5 horses, blood samples were drawn using an i.v. catheter. Saliva samples were taken using swabs. Cortisol was assayed by radioimmunoassay. All data were treated with a regression method, which pools and analyses data from multiple subjects for linear analysis.**

Results: **Mean ± s.d. cortisol concentrations measured at rest were 188.81 ± 51.46 nmol/l in serum and 1.19 ± 0.54 nmol/l in saliva. They started increasing immediately after ACTH injection and peaks were reached after 96 ± 16.7 min in serum (356.98 ± 55.29 nmol/l) and after 124 ± 8.9 min in saliva (21.79 ± 7.74 nmol/l, $P<0.05$). Discharge percentages were also different (225% in serum and 2150% in saliva, $P<0.05$). Correlation between serum and salivary cortisol concentrations showed an adjusted $r^2 = 0.80$ ($P<0.001$). The strong link between serum and salivary cortisol concentrations was also estimated by a regression analysis.**

Conclusions: **The reliability of both RIAs and regression found between serum and salivary cortisol concentrations permits the validation of saliva-sampling as a noninvasive technique for cortisol level assessment in horses.**

Abbreviations

ACTH:	Adrenocorticotropic hormone
CBG:	Cortisol binding globulin
HPA axis:	Hypothalamic-pituitary-adrenal axis
MDL:	Minimum detection limits

Introduction

Cortisol levels are used in man and in domestic and wild animal research. In main studies, serum cortisol concentration is considered to be an indirect measurement of stress (Mears and Brown 1997; Hennessy *et al*. 2001; Rushen *et al*. 2001; Möstl and Palme 2002; Krawczel *et al*. 2007). In horses, serum cortisol concentration is often used to assess stress induced by transport (Clark *et al*. 1993; Cavallone *et al*. 2002; Fazio *et al*. 2008), competition (Covalesky *et al*. 1992) and training (Alexander *et al*. 1991; Cayado *et al*. 2006), and stress associated with stereotypies (McGreevy and Nicol 1998). The increase in serum cortisol during acute stress is largely made up of free cortisol. When assessing stress, it is more useful and relevant to measure free cortisol than total cortisol in serum (Alexander and Irvine 1998; Hellhammer *et al*. 2009). Faecal cortisol metabolites are also used to assess long-term stress, but do not allow detection of minor and transient increases in plasma cortisol (Schmidt *et al*. 2009).

Salivary cortisol results from passive diffusion into the salivary glands and constantly provides information about free cortisol concentration. This concentration is, therefore, now used by more researchers as an index of serum free cortisol (Francis *et al*. 1987; Vincent and Michell 1992; Castro *et al*. 2000; Roy *et al*. 2001; Mormède *et al*. 2007; Wolf *et al*. 2008). Salivary cortisol concentration represents a part of free cortisol concentration. In horses, stress has already been assessed by salivary cortisol concentration during isolation (Harewood and McGowan 2005), weaning (Moons *et al*. 2005), transport (Schmidt *et al*. 2010a,b) or during new manipulations (Jongman *et al*. 2005). However, these researchers were not concerned with the relationship between serum and salivary cortisol concentrations in horses; they based their hypothesis on man or other species studies. The present study is a confirmation of these previous hypotheses.

In the saliva-sampling method, there is no risk of complications, unlike jugular catheterisation or venipuncture. Saliva-sampling is effectively a noninvasive method, painless and stress-free for horses that are used to be manipulated by man. This method is also easier than blood-sampling (Fell *et al*. 1985; Vincent and Michell 1992; Bushong *et al*. 2000; Negrao *et al*. 2004; Hellhammer *et al*. 2009). Nowadays, cortisol concentration can be assayed by using immunoassays. A direct radioimmunological method called

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radioimmunoassay (RIA) was used, based on a competition between unlabelled cortisol and cortisol radiolabelled with ^{125}iodide, used in terms of sensitivity against the specific sites of an antibody previously described (Sulon *et al*. 1978). This technique has been widely validated in man (van Eck *et al*. 1996; Nicolson *et al*. 1997; Calixto *et al*. 2002) and equine studies (Hughes *et al*. 2006).

A positive correlation between equine serum and salivary cortisol concentrations has been established (Lebelt *et al*. 1996; Pell and McGreevy 1999; van der Kolk *et al*. 2001) or not (Elsaesser *et al*. 2001). Nevertheless, none of these studies provided a regression model to explain the relationship between serum and salivary cortisol concentrations. To establish this relationship, samples of saliva and blood are taken in parallel and in the present study adrenocorticotropic hormone (ACTH) administration, inducing a high cortisol secretion was used to extend the range of sample concentrations (Calixto *et al*. 2002; Bousquet-Mélou *et al*. 2006).

The first aim of the present study was to develop a sensitive and specific RIA to measure cortisol concentration precisely in serum and saliva. The secondary objectives were to study the kinetics of cortisol in both blood and saliva after an adrenal stimulation and to assess if their relationship could permit the use of saliva sampling instead of blood-sampling to assess hypothalamic-pituitary-adrenal (HPA) axis activity in horses. The ACTH challenge was chosen in order to standardise an adrenal stimulation.

Materials and methods

The Animal Care and Use Council of the University of Liège (Belgium) approved the use and treatment of animals in this study.

Horses

Five mature horses, 3 mares and 2 geldings, ranging in age from 9–17 years and weighing mean ± s.d. 486 ± 38 kg, were used. All horses were healthy, not pregnant and accustomed to handling and sampling sessions. They were housed on straw in their usual stable (individual stalls), at the Faculty of Veterinary Medicine of Liège. Water was freely available in automatic drinking troughs. Horses were fed with pellets and hay. Throughout the study, they were always handled by the same experimenter.

Experimental design and sampling

A catheter (large bore i.v. catheter 14 gauge × 13.4 cm)[1] was placed in the jugular vein of each horse on Day 1. This placement was performed by an experienced veterinarian. The standardised dose, 1 µg/kg of ACTH (Synacthen tetracosactidum 0.25 mg/1 ml)[2] was chosen according to the work of Bousquet-Mélou *et al*. (2006). ACTH was injected through the catheter, which was then flushed. During Day 1, 10 samples (5 saliva and 5 blood samples) were collected. First samplings were performed before catheter placement, and the others at 5, 10, 30 and 60 min after. During Day 2, 42 samples (21 blood and 21 saliva samples in parallel) were collected. First samplings were performed 30 and 15 min before ACTH injection and the following at 10, 20, 30, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 240, 280, 320, 260, 400 and 500 min after ACTH injection. At the end of Day 2, the catheter was removed.

Blood and saliva sampling

The first blood sample was collected by venipuncture before catheter placement. All other blood samples were drawn by i.v. catheter. Every blood sampling was followed by a cleaning injection of 1 ml of physiological liquid containing heparin (0.05%). Blood was collected in a clotting tube (Monovette)[3] and immediately stored at 4°C. Saliva was sampled using Salivette[3]. A swab on a metal clamp was maintained in the horse's mouth for 30–40 s, over and under the tongue and replaced in Salivettes and stored at 4°C. At the end of each day, the Salivettes and clotting tubes were centrifuged 10 min at 1500 ***g***. Saliva and serum samples were stored at -20°C until assayed (Garde and Hansen 2005).

Development of salivary cortisol RIA

In saliva, cortisol was assayed by a direct RIA. Minimum detection limits (MDL) were determined in each assay according to the protocol described by Skelley *et al*. (1973). The precision and reproducibility of the RIAs were calculated by the intra- and interassay coefficients of variation (CV). The buffer (pH 7.0) used throughout the procedure contained $C_6H_8O_7.H_2O$ (0.206%)[4], $Na_2HPO_4.2H_2O$ (3.211%)[5], NaCl (0.9%)[6] and NaN_3 (0.1%)[6]. The buffer was ready to use after the addition of bovine serum albumin (BSA 0.2%)[7]. Cortisol[8]was used as standard. Five µg of Cortisol-3CMO-histamine[9] in 10 µl of phosphate buffer (0.5 mol/l pH 7.5) were labelled with 2 mCi Na-^{125}I[10] by the usual chloramine-T method (Greenwood *et al*. 1963). The labelled solution was purified by high performance liquid chromatography (HPLC) on a C-18 reverse phase using an acetonitrile-water gradient. The primary antibody raised against cortisol-3-CMO-BSA was previously described by Sulon *et al*. (1978). The second antibody precipitation system consisted of a mixture of sheep anti-rabbit IgG (0.83%, v/v), normal rabbit serum (0.17%, v/v), polyethylene glycol 6000 (4%)[6], BSA 0.4% and microcrystalline cellulose (0.05%)[6].

At 300 µl of buffer, 50 µl of saliva were dispensed, in duplicate, in crystal polystyrene (75 × 12 mm) tubes. 100 µl of diluted tracer solution (about 30,000 counts/min) and 100 µl of antiserum (dilution 1/25,000) were added. A standard curve, ranging from 0.22–55.17 nmol/l was prepared in buffer and used in parallel. All tubes were incubated overnight at room temperature. After that, 1 ml of the second antibody precipitation system was added to all tubes and incubated 30 min at room temperature. After addition of 2 ml of buffer, all tubes were centrifuged at 2890 ***g*** for 20 min, in order to separate bound (pellet) and free (supernatant) ligands. The radioactivity of the dry pellet was counted using a gamma counter (126 Multigamma counter)[11]. Logit-log transformation was used to obtain a linear standard curve in order to estimate the salivary cortisol concentration (Banga-Mboko *et al*. 2003). As no chromatographic testing of the validity of the assay was done, we notice that cortisol concentration assayed is the immunoreactive cortisol concentration.

Development of serum cortisol RIA

In serum, dilutions were necessary before RIA. All the reagents and materials described above were also used for serum cortisol determination. A 1 mg/ml solution of 8-anilino-1-naphthalene sulphonic acid (ANS)[12] prepared in buffer was used to avoid nonspecific binding of cortisol to serum proteins such as cortisol

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binding globulin (CBG) or albumin (Ronayne and Hynes 1990). Aliquots of 50 µl of diluted (1/40) serum sample, were dispersed in polystyrene tubes in duplicate. A 300 µl aliquot of tracer prepared in ANS solution (30,000 counts/min) and 100 µl of primary antiserum were added. A calibration curve of cortisol, ranging from 8.6–2200 nmol/l, was obtained with a charcoal stripped cortisol free serum. The subsequent steps were as described for saliva samples.

Statistics

Continuous variables are reported as the mean ± s.d. We used repeated measures ANOVA to analyse cortisol kinetic in serum and saliva. Variable normality was assessed using the Shapiro-Wilk test. Transformation was applied for variable normalisation when necessary. Since the observations available to assess the link between salivary and serum cortisol were data from multiple subjects, i.e. several measures were made per horse, independence of the observation could not be assumed. We therefore had to apply methods for pooling data from multiple subjects for linear analysis. This was done using the standardisation procedure for individual response curves described by Poon (1988). After this standardisation, the classical linear regression model was used to assess the association between salivary and serum cortisol and to propose a prediction model for salivary cortisol concentration.

Statistical analyses were performed using R version 2.8.0[13]. A 2-tailed P value <0.05 was considered statistically significant.

Results

Characterisation of cortisol RIAs

The MDL were 0.2 and 8 nmol/l, respectively, in saliva and serum. In each case, 3 pools of saliva or serum containing low, moderate and high concentrations of cortisol were tested. Intra- and interassay CV determined at different ranges in the assays were <5.55% (4.04 ± 1.54%) and <10.40% (9.13 ± 1.33%) in saliva and, respectively, <6.53% (4.32 ± 1.92%) and <11.27% (8.22 ± 2.69%) in serum. Parallelism was assessed by serial dilutions (1:1, 1:2, 1:4, 1:8 and 1:16) of saliva (CV 5.91%) or serum (CV 4.43%) samples, selected with a high cortisol concentration.

Physiological validation of salivary and serum cortisol RIAs

Cortisol concentrations at rest: Saliva was collected with the Salivette without any problem. Horses accepted easily the metal clamp in their mouth. No increase in cortisol concentration appeared after saliva sampling (neither in serum nor saliva). Horses stayed calm and learned quickly (after 2 trials) to accept the swab. Horses did not need any special restraint to sample saliva, which was performed in the horse's stable. At 08.55 h on Day 1, before catheter placement, serum cortisol concentration varied from 137.84–256.92 nmol/l (188.81 ± 51.46 nmol/l). At the same time, salivary cortisol concentration varied from 0.58–1.77 nmol/l (1.19 ± 0.54 nmol/l).

During i.v. catheter placement: At 09.00 h on Day 1, the i.v. catheter was placed and serum cortisol concentrations showed no significant variation during the following hour (repeated measures ANOVA). At 10.00 h, serum cortisol concentrations varied from 146–222 nmol/l (182.98 ± 31.49 nmol/l). At the same time, salivary cortisol concentrations varied from 0.59–2.21 nmol/l (1.27 ± 0.60 nmol/l), values that are also not significantly different from those measured before the catheter placement (repeated measures ANOVA).

During an ACTH stimulation test: Figure 1 illustrates both the serum and the saliva cortisol responses, after ACTH administration. Thirty minutes earlier, the serum cortisol concentration (baseline) varied from 125–224 nmol/l (148.14 ± 42.69 nmol/l). Ten minutes after ACTH administration, the cortisol concentration was higher than baseline (repeated measures ANOVA, P<0.01). In the mean for the 5 horses, the peak was reached in serum after 96 ± 16.7 min; this peak was 356.98 ± 55.29 nmol/l. Thereafter, serum cortisol concentration decreased regularly to return to baseline at 280 min after ACTH injection (repeated measures ANOVA, P<0.05). We noticed that at 400 min after ACTH administration, the serum cortisol concentration dropped below the baseline (repeated measures ANOVA, P<0.01), to reach a concentration of 62.39 ± 16.14 nmol/l, at 500 min after ACTH injection. Thirty minutes before stimulation, salivary cortisol concentrations (baseline) varied from 0.46–3.14 nmol/l (1.23 ± 1.08 nmol/l). In saliva, cortisol concentration was significantly higher than baseline after 30 min (repeated measures ANOVA, P<0.05) and after 40 min (P<0.01), 20–30 min later than in blood. The mean peak was 21.79 ± 7.74 nmol/l, appearing about 124 ± 8.9 min after ACTH administration. The salivary cortisol concentration came back to baseline after 180 min (P>0.05), 100 min earlier than in blood (Fig 1).

On average, the peak was reached in serum after 96 ± 16.7 min and in saliva after 124 ± 8.9 min. Times needed to reach the peak are significantly different (paired Student *t* test, P<0.05). Times to come back to baseline were also different: about 280 min in serum and 180 min in saliva.

The mean percentage of discharge in serum was 225% (148.14–358.08 nmol/l), while it was 2150% in saliva (1.23–21.79 nmol/l) (significantly different, paired Student *t* test, P<0.05).

Regression analysis

During the study, 127 sera and 127 salivas were sampled in parallel on the 5 horses. These results show a time lag between serum cortisol and saliva cortisol variation. As expected, saliva cortisol increasing appears 20–30 min later than in serum. Because this time lag is changeable, depending on intensity and duration of stressor, statistical analysis was based on values gathered simultaneously. There was a strong association between salivary and serum cortisol as shown by a Pearson correlation coefficient of 0.90 and an adjusted r^2 of 0.80 (P<0.0001). This means that 80% of the salivary cortisol concentration variability could be explained by the serum cortisol concentration and reciprocally. The obtained classical linear regression (Fig 2) model is shown below:

$$Cortisol_{serum} = 159 + 56.7 \log_e cortisol_{saliva}$$

Discussion

In previous studies in man (Walker *et al*. 1978; Lac 2001) and in dogs (Beerda *et al*. 1996), the RIA method was found to be sensitive and reproducible for cortisol concentration determination

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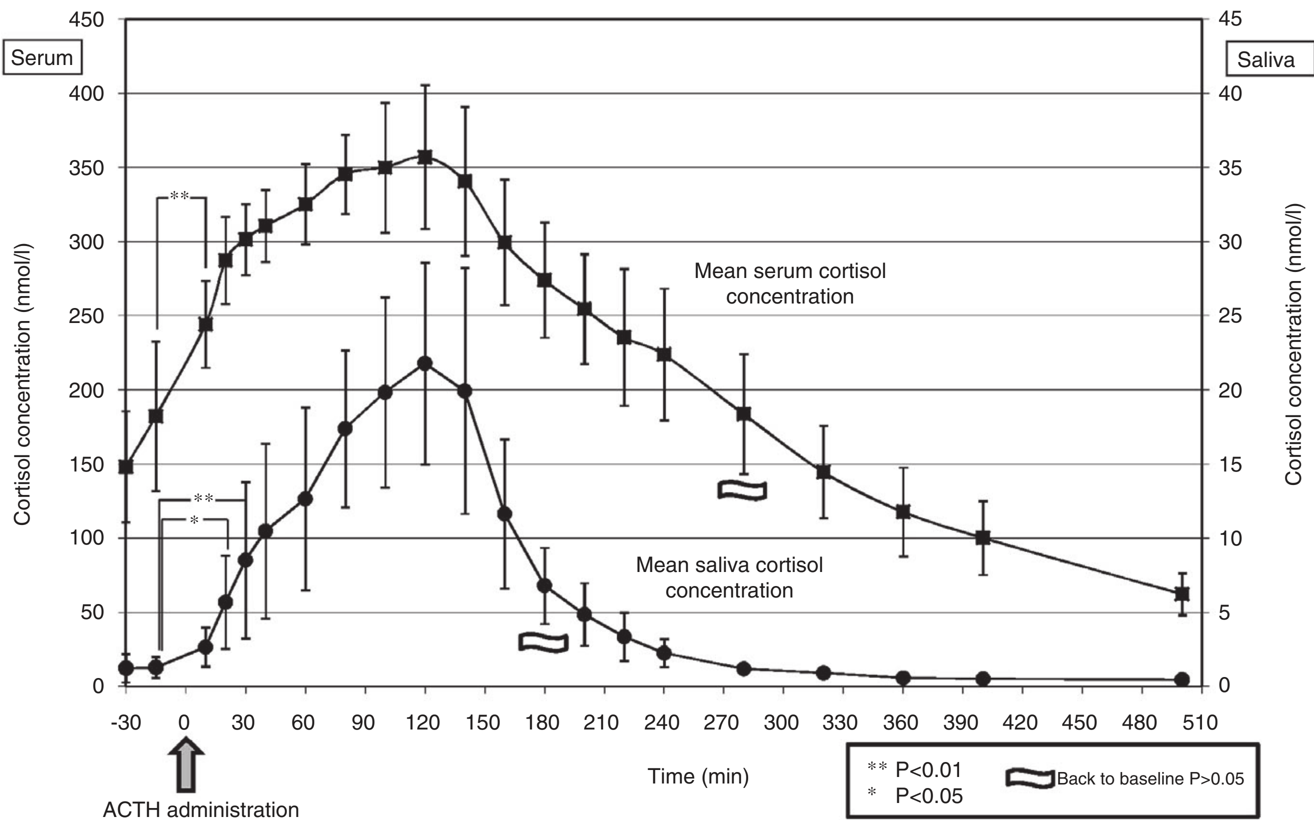

Fig 1: Means of total serum cortisol concentration and saliva cortisol concentration during an ACTH challenge for the 5 horses.

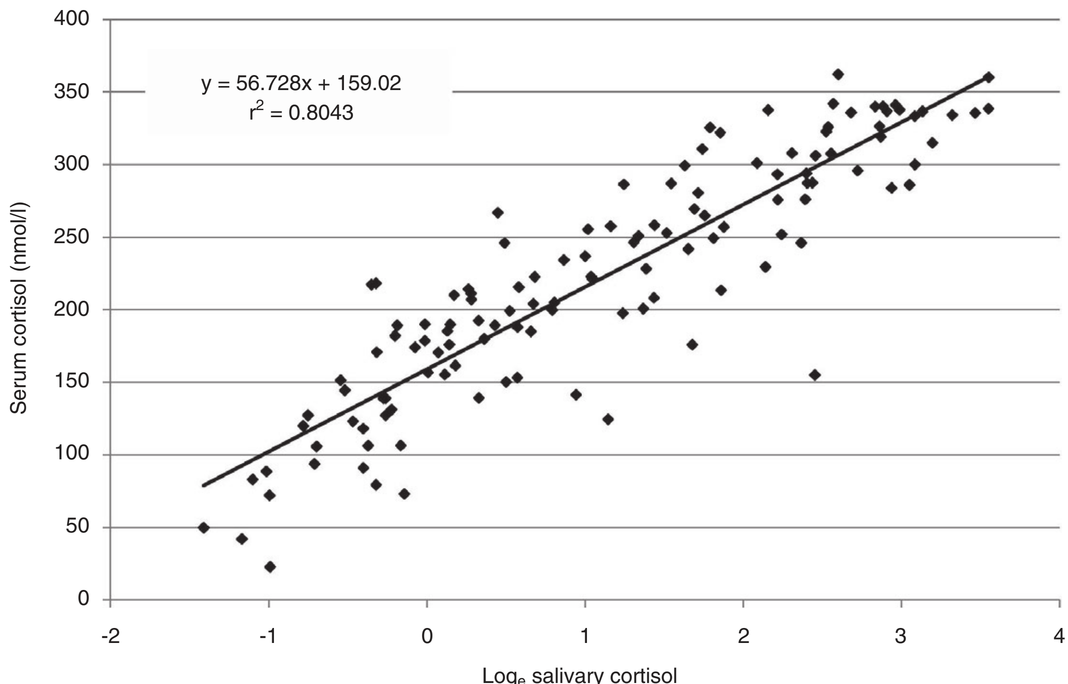

Fig 2: Linear regression found between total serum cortisol concentration and saliva cortisol concentration, for the 5 horses, during an ACTH challenge.

in both serum and saliva. In horses, there are few studies about the validation of salivary cortisol as an indicator of HPA activity. Saliva and blood samples on a small range of value were collected (Pell and McGreevy 1999; Creighton *et al*. 2004). If an ACTH stimulation test was used, the authors collected insufficient samples to find a significant regression (van der Kolk *et al*. 2001). The i.v. injection of ACTH to horses stimulates the HPA axis and induces a wide range of cortisol values in serum and saliva. This technique

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was used to compare salivary and serum cortisol rises, around a standardised physiological stress and the physiological response of cortisol after ACTH injection validated this RIA study from low (0.24 nmol/l in saliva and 45 nmol/l in serum) to high values of cortisol (35 nmol/l in saliva and 420 nmol/l in serum). In the present study, RIA was a sensitive and specific method to measure cortisol concentration in horses precisely, in 2 very different fluids, serum and saliva, as previously validated by Hughes *et al*. (2006) in saliva.

Cortisol concentrations obtained during resting time are in agreement with results from other studies, for both serum (Alexander *et al*. 1991; Anderson *et al*. 1999; Cavallone *et al*. 2002) and saliva (Pell and McGreevy 1999; van der Kolk *et al*. 2001) concentrations. Cortisol concentrations during venous catheter placement increased, but did not differ significantly from baseline, in both serum and saliva. This means that catheter placement should not involve as high a stress to horses as transport (Fazio *et al*. 2008; Schmidt *et al*. 2010b) or competition (Cayado *et al*. 2006).

In serum, the cortisol response to ACTH challenge was in accordance with previous studies in horses, with the same timing and peak amplitude (van der Kolk *et al*. 2001; Bousquet-Mélou *et al*. 2006). Bousquet-Mélou *et al*. (2006) obtained, for the same ACTH quantity, the same timing and amplitude for serum cortisol concentrations. Van der Kolk *et al*. (2001) also used an ACTH challenge to compare serum and salivary cortisol concentrations. They took 3 saliva samples and 3 blood samples after ACTH administration (after 2, 4 and 6 h). Their peak on the first sample (ACTH + 2 h) and concentration correspond to our peak (20.40 ± 5.50 and 413.0 ± 91.6 nmol/l in saliva and serum).

Many studies have found a significant positive correlation between serum and salivary cortisol, for example in man (Demey-Ponsart *et al*. 1986), guinea pigs (Fenske 1996) and cattle (Negrao *et al*. 2004). In a previous study (Peeters *et al*. 2008), a positive correlation of 0.55 ($P<0.05$) was found between mean cortisol concentrations (5 samples/day) in serum and saliva in horses. Some previous studies found no such correlation (Pell and McGreevy 1999) while others found a correlation, but only with a few samples (Lebelt *et al*. 1996). In the present study, the saliva/serum correlation was positive and significant ($r^2 = 0.90$; $P<0.001$; $n = 127$) and there was a strong association between salivary and total serum cortisol concentrations as shown by an adjusted r^2 of 0.80 ($P<0.001$), meaning that 80% of the salivary cortisol concentration variability could be explained by the total serum cortisol concentration and reciprocally. The regression relationship between these concentrations was therefore calculated and the relation can be more precisely illustrated with the following formula: $cortisol_{\mathrm{serum}} = 159 + 56.7 \log_e cortisol_{\mathrm{saliva}}$. The total serum cortisol concentration can be deduced from this formula, for salivary cortisol concentrations 0.24–34.83 nmol/l. Bousquet-Mélou *et al*. (2006) and Dorin *et al*. (2009) found that free cortisol in serum is not proportional to total serum cortisol. This explains the nonlinearity of the relationship between total serum and salivary cortisol concentrations found in the present study. This nonlinearity might be due to the variable saturation state of CBG and albumin. The maximal binding capacity of CBG is reached during an ACTH test (Bousquet-Mélou *et al*. 2006). In stress research, a biologically active cortisol form gives us more information about HPA axis activity. Studies comparing free serum cortisol to salivary cortisol in horses may lead to a linear relation as found for man (Arafah *et al*. 2007). Under stress conditions, the CBG becomes saturated, leading to an increase in free cortisol. This fact explains why the relative increase of cortisol in saliva is higher than in serum (about 10 times more). This high increase in salivary cortisol has already been found in previous studies on dogs (Vincent and Michell 1992) and horses (van der Kolk *et al*. 2001).

Cortisol concentrations came back to baseline faster in saliva than in serum (100 min faster). In horses, saliva sampling should therefore be preferred to blood sampling for stress level assessment, as Hellhammer *et al*. (2009) indicated for human individuals. In human research, le Roux *et al*. (2003) also found that free cortisol index is a better indication of HPA axis activity than serum total cortisol, all the more in acute stress situation, when CBG fall dramatically (le Roux *et al*. 2003). However, the time lag varies between serum and saliva cortisol. The regression found does not mean that saliva cortisol concentration can be used to calculate serum cortisol concentration but saliva cortisol concentration can be used to detect HPA axis activity as with serum cortisol. Moreover, salivary cortisol concentration seems to be more expressive and more specific to assess an increase of the HPA axis activity.

In others species, authors have already postulated that saliva sampling is a good alternative to blood sampling for free cortisol measurements. They explain this by various factors endorsed in the present study: 1) saliva is easy and noninvasive to collect; 2) in saliva, there is only free cortisol, so no separation manipulation is needed; 3) salivary cortisol concentration is independent of flow rate (Riad-Fahmy *et al*. 1982); according to the present results, also 4) cortisol in saliva has a higher increase when stress occurs (10 times more than in serum) and 5) salivary cortisol concentrations come back to baseline faster than serum concentrations; these points permit a better determination of stress occurring. In the present study a regression formula was also calculated to compare results from studies that used serum cortisol concentration to assess HPA axis activity.

In conclusion, as expected, saliva sampling and saliva RIAs are valid and noninvasive techniques for cortisol measurement in horses. The study agrees with Lebelt *et al*. (1996) that saliva may become the body fluid of choice for monitoring stress. The kinetics of salivary cortisol concentrations around a standardised stress are further revealed and this information is useful to determine good timing for saliva sampling in future stress and/or welfare studies in horses, as reported by Peeters *et al*. (2010).

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Manufacturers' addresses

[1]Jorvet, Loveland, Colorado, USA.
[2]Novartis, Vilvoorde, Belgium.
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[5]BDH, Leuven, Belgium.
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[7]INC Biochemicals, Aurora, Ohio, USA.
[8]Sigma, St Louis, Missouri, USA.
[9]Research Plus, Bayonne, New Jersey, USA.
[10]PerkinElmer, Zaventem, Belgium.
[11]LKB Wallac, Turku, Finland.
[12]Eastman Kodak Co., Rochester, New York, USA.
[13]http://www.R-project.org

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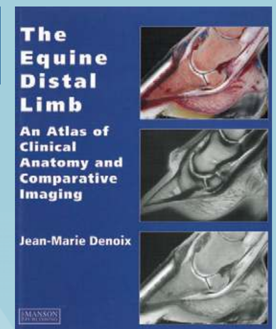

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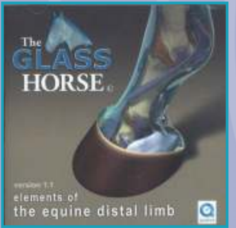

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