

Salivary cortisol concentrations changes in horses during daily routine

Martin Massányi^{1*}, Marko Halo¹, Eva Mlyneková¹, Marko Halo Jr.², Peter Massányi²

¹Slovak University of Agriculture in Nitra, Faculty of Agrobiological and Food Resources,
Department of Animal Husbandry, Slovak Republic

²Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences,
Department of Animal Physiology, Slovak Republic

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The aim of the study was to observe the salivary concentrations of cortisol in horses during daily routine over the course of four weeks lasting experiment in three different stages of day to monitor the changing values of cortisol in horse saliva during the day. Saliva was obtained from 12 Slovak warmblood horses – 1 stallion, 6 mares and 5 geldings. In the experiment we focused purely on changes of salivary cortisol concentrations over the course of the daily routine. The results shown us multiple significant changes between individual sample collections and thus, we can state that according to our results the highest concentration of cortisol in horse saliva is in the morning and it decreases throughout the day with lowest measured concentrations being in the final sample collection of the day (at 22:00). These changes had no visible effect on horses' organism throughout entirety of the experiment and were caused due to horses daily rhythm.

Keywords: horse, cortisol, saliva, rest, daily routine

1 Introduction

Our study aims to observe the changing concentrations of cortisol over the course of the day. This data has high importance as it can be used in further experiments as basal values to compare the stress which animals undergo during daily schedule (including training, transportation etc.). In sport, there is a high demand for the best individuals, both physically but also psychically stable and calm when it comes down to competition. It is important to individualize the training programme for each animal to suit their needs. There are multiple parameters that affect cortisol levels in horse saliva – the way how individuals are housed, nutrition is important to maintain horses' health (Halo et al., 2009) as well as training to maintain their physical condition. Commonly known fact is that with increasing performance of the organism it becomes more vulnerable to the effects of environment. It can be affected via study of animal's behaviour and physical constitution but it is not to completely eliminated (Halo et al., 2008). Another aspect that affects the well-being and health state of horses is environmental pollution – coming from various sources such as herbicides used in plant production, logistics as well as increased industrial production (Massányi et al., 2014). Observing circadian rhythm occurs in some studies that spectate stress levels in horse but not all and it develops when horses are accustomed to management routine, stabling, exercise and feeding (Irvine and Alexander, 1994). Several contraindications on this topic appear in the literature (Bohák et al., 2013) Therefore, it is important to make repeated monitors and observations to reach consensus in scientific community. Observation of horse salivary cortisol to follow the stress levels in horse is a model that has been used commonly in research following this subject (Kang and Yun, 2016; Strzelec et al., 2011; Ille et al., 2013). It has been repeatedly spotted that i.e. transportation is stressful based on rising concentrations of cortisol. Observing cortisol in saliva as it is much less-invasive method compared

***Corresponding Author:** Martin Massányi, Slovak University of Agriculture in Nitra, Faculty of Agrobiological and Food Resources, Department of Animal Husbandry, Tr. A. Hlinku 2, 949 76, Nitra, Slovak Republic; e-mail: martinmassanyi@yahoo.com. ORCI: <https://orcid.org/0000-0002-9281-4495>

to blood sample collections and analysing blood plasma as blood sample collections themselves might rise cortisol concentrations, especially when repeated (Stull et al., 2008; Schmidt et al., 2010).

2 Material and methods

Saliva samples were obtained from 12 Slovak warmblood horses stabled (1 stallion, 6 mares and 5 geldings) in separate boxes in Experimental centre of Department of Animal husbandry. Animals were aged between 5–9 years of age and weighed 550 ± 50 kg. Animals were fed thrice a day at 7:00, 12:00 and 18:00 in ratio of 25 : 25 : 50 percent. Feed dose consisted of 10 kg of hay per horse a day, 3.5 kg of oats per horse a day and 1 kg of supplementary compound feed per horse a day. All tested horses were treated by same personnel, have been trained to maintain medium performance level. They have been trained by uniform stable rider pool and the trainings have been done in the same times of day. Horses were lounged for 20 minutes during the day as a regeneration part of training. Samples were gathered over the course of four weeks and collected in precisely determined times (8:00, 15:00, 22:00) using a sterile gauze tampon. The tampon was later inserted into a plastic 15 mL tube and centrifuged for 5 minutes at 7000 rpm to separate the saliva from the tampon into the tube. Entire volume of saliva was afterwards pipetted into Eppendorf tube and stored in deep freezer (-80 °C) until further analysis using a diagnostic ELISA Cortisol kit (DIALAB GmbH, Austria). Management of animals and experimental procedures were executed following the EU Directive 2010/63/UE (EU 2010) regarding the animals for experimental purpose and all of the sample collections were performed with unanimous consent of all animals' owners.

2.1 Statistical analysis

Statistical analysis were carried on using GraphPad Prism 6.1 (6.1 version for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Values were compared using ordinary one-way ANOVA and the significance differences between groups were set to $p < 0.5$; $p < 0.01$; $p < 0.001$; $p < 0.0001$ and column statistics were calculated.

3 Results and discussion

Observing concentration of cortisol in saliva of horses during different times of day, our results ranged from 1.347 ng/mL to 0.892 ng/mL. We have seen multiple significant changes during the day. In the morning the levels of cortisol were significantly higher than in the afternoon as well as in the sample collection done during night. The levels measured in the afternoon were significantly elevated in comparison with samples collected during night-time (Table 1). Thus, we can state that the values of cortisol in horse saliva are highest in the morning and continually decline over the course of the day and are lowest in the evening/night.

Table 1 Basal values of cortisol during the day and after lounge

Parameter	Morning	Afternoon	Night	Lounge	P-value (+)
Cortisol (ng/mL)	$1.347 \pm 0.344^{A,B}$	$1.191 \pm 0.283^{A,C}$	$0.892 \pm 0.154^{B,C}$	3.592 ± 0.403	[*] A, ^{****} B, ^{***} C

+ within the same row, means with different letters differ significantly ($p < 0.5$; $p < 0.01$; $p < 0.001$; $p < 0.0001$)

^{*} $p < 0.5$; ^{**} $p < 0.01$; ^{***} $p < 0.001$; ^{****} $p < 0.0001$ – significances between individual groups

Kang and Lee (2016) observed changes in salivary cortisol concentrations in tourist-riding horses, resting group and education horse-riding group of horses. They found out that basal concentrations of cortisol in horse saliva in resting group were 1.113 ng/mL which is in the middle of our measured values. Interestingly, their measured values in education horse-riding group were much higher, rising all the way to 1.925 ng/mL which can indicate that it puts much more stress on the animal. On the other hand, the group of tourist-riding horses, cortisol level was only 0.972 ng/mL which is only slightly higher than our lowest measured results from the "night" collection. Peeters et al. (2011) also measured basal concentrations of salivary cortisol while comparing concentrations between blood serum and salivary cortisol levels in horses using adrenocorticotrophic hormone challenge. Their measured values ranged from 0.210 ng/mL to 0.642 ng/mL which is in contradiction with our measured values as they were much higher. Values of cortisol after horse lounging rose all the way to 3.592 ng/mL therefore the levels of cortisol points to that it would be suitable to set the regeneration phase of training (lounging) in the morning times of the day to keep the cortisol circadian cycle more uniform.

Van der Kolk et al. (2001) observed the concentrations of salivary cortisol in horses with Cushing's disease and healthy horses. The levels of cortisol have been artificially decreased overnight by application of dexamethasone from 1.85 ng/mL to 1.60 ng/mL. Whereas in the normal horses, the concentrations ranged from 0.69 to 0.76 ng/mL. These measure values are slightly lower than our results from night collection.

Another research spectated the cortisol circadian rhythm ratio and its influence on risk of colic in horses. Sample collections were done in the morning (6 to 7 AM) and 8 hours after the first sample. It has been noticed that the serum concentrations levels of cortisol were 66.9 ± 30.0 ng/mL in the morning and 58.6 ± 29.7 ng/mL (Leal et al., 2011), therefore we can state that this correlates with our findings. Where the levels of salivary cortisol were also decreasing over the course of the day. Study by Bohák et al. (2013) also monitored the concentration of cortisol during circadian rhythm in horses and according to their observation, the highest concentration of cortisol was at lowest in night time, then it started rising during the early mornings and the highest values were at 12 PM (noon). Their results do not completely consent with our results as we measured the highest concentrations of cortisol in the morning but the night time values correlated with ours. This can also be caused by extensive sample collecting over the entirety of the day which itself is stressful for the horse but may also be caused by different daily routine of the horses used in the experiment.

Contreras-Aguilar et al. (2020) have studied changes in salivary analytes of horses based on circadian rhythm and season – one of the observed parameters in their experiment was cortisol. They found out that the concentrations of cortisol were approximately similar in the morning (6:30) and in the afternoon (12:30) and the values continually decreased towards evening. Lowest measured value was at 20:30 which was the last measured time in their experiment. Trend was very similar as in our experiment, though we found that the concentrations were slightly higher in the morning.

4 Conclusions

To summarize, this study observes the levels of horse salivary cortisol in rest state. Based on obtained data we can state that there have been statistically significant changes between levels of salivary cortisol over the different stages of the day. The concentrations in saliva were decreasing over the course of the day with the highest values obtained being in the morning and lowest in the sample collection in the night at 22:00. Data we collected will be used in further experiments to observe the training stress in jumping horses as well as can be used by other researchers to compare gathered data.

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