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Effects of season, age, sex, and housing on salivary cortisol concentrations in horses

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ABSTRACT

Analysis of salivary cortisol is increasingly used to assess stress responses in horses. Because spontaneous or experimentally induced increases in cortisol concentrations are often relatively small for stress studies, proper controls are needed. This requires an understanding of the factors affecting salivary cortisol over longer times. In this study, we have analyzed salivary cortisol concentration for 6 mo in horses (n = 94) differing in age, sex, reproductive state, and housing. Salivary cortisol followed a diurnal rhythm with the highest concentrations in the morning and a decrease throughout the day ($P < 0.001$). This rhythm was disrupted in individual groups on individual days; however, alterations remained within the range of diurnal changes. Comparison between months showed highest cortisol concentrations in December ($P < 0.001$). Cortisol concentrations increased in breeding stallions during the breeding season ($P < 0.001$). No differences in salivary cortisol concentrations between nonpregnant mares with and without a corpus luteum existed. In stallions, mean daily salivary cortisol and plasma testosterone concentrations were weakly correlated ($r = 0.251$, $P < 0.01$). No differences in salivary cortisol between female and male young horses and no consistent differences between horses of different age existed. Group housing and individual stabling did not affect salivary cortisol. In conclusion, salivary cortisol concentrations in horses follow a diurnal rhythm and are increased in active breeding sires. Time of the day and reproductive state of the horses are thus important for experiments that include analysis of cortisol in saliva.

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1. Introduction

Analysis of salivary cortisol concentrations is increasingly used to assess the adrenocortical response of horses to potentially stressful situations, and several cortisol assays have been validated for equine saliva [1–4]. From horses accustomed to routine handling by humans, saliva can be collected easily, repeatedly, and without restraint of the animal. Salivary cortisol mirrors the unbound, that is, biologically active fraction of total plasma cortisol while plasma cortisol is largely bound to carrier proteins [5].

In horses, as in other species including humans [6] and rhesus monkeys [7], cortisol release into blood follows a diurnal rhythm with the highest concentrations in the morning and a nadir in the late afternoon and evening [8–10]. This rhythm can be disrupted by even minor perturbations resulting in a damping of the daily oscillations and elevated cortisol concentrations, especially around the time of the daily nadir [10]. The diurnal rhythm in cortisol release is well reflected by concentrations in saliva [11–14]. However,

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salivary cortisol concentrations have previously been determined in horses during specific experiments only, not allowing the comparison of undisturbed cortisol release patterns among different horse groups or times of the year. Cortisol release into blood or saliva may be physiologically influenced also by reproductive state and the sex of the individuals (humans [15] and dog [16]). Although most phases of pregnancy were without effect on plasma cortisol in horses [17], salivary cortisol increased in mares shortly before foaling [18]. Elevated plasma cortisol concentrations have also been demonstrated in pregnant women [19].

Marked increases in salivary cortisol concentrations as occurring in mares at foaling [20], in horses transported by road [21–23], or in foals at weaning [11] clearly indicate increased adrenocortical activity. However, experimentally induced increases in salivary cortisol concentrations are often relatively small and hardly exceed the range of physiological diurnal changes. This is especially true for studies aimed at assessing stress in horses submitted to equestrian training [3,24–26]. Whether such increases, although statistically significant, indicate that horses perceive a particular challenge or procedure as an acute stressor often can be doubted, thus, for stress studies, proper controls are required. The accuracy of studies evaluating adrenocortical activity in horses could be improved by a better understanding of the factors affecting salivary cortisol concentrations.

In this study, we have analyzed salivary cortisol concentration in horses of the same breed and genetic background and kept on the same premises over a 6-mo period from December to May. Horses have a seasonal sexual activity (reviewed in [27]), and the study period included the nonbreeding season and the breeding season. Horses were divided by age, sex, sexual activity, and reproductive state. We hypothesized that basal cortisol concentrations not only follow a diurnal but also a circannual rhythm. We further tested if salivary cortisol is affected by age of the horses, their reproductive state, and by the housing system with increased cortisol release in single housed vs group-stabled horses.

2. Materials and methods

2.1. Animals

A total of 94 warmblood sport horses of the Brandenburg State Stud at Neustadt (Dosse), Germany, were included into the study. They were kept either in group stables on straw or in individual loose boxes on straw or wood chippings. Horses were fed oats, concentrates, and mineral supplements 3 times daily and hay twice daily. Water was freely available at all times. From December to April, all group-housed horses had daily access to a paddock for 4 to 5 h. Horses housed in individual boxes had either access to individual paddocks or were ridden for approximately 1 h every day. Except adult breeding stallions and adult geldings, all groups were turned out on pasture in May. On pasture, broodmares received oats, concentrates, and mineral supplements twice daily, whereas all other groups were not given any additional feed. Details on horses included into the study are summarized in Table 1. Adult breeding stallions were used exclusively for artificial insemination, and semen was collected once daily 4 to 6 times per week. Geldings had been orchidectomized at least 8 mo before the start of the study.

2.2. Experimental procedures

Saliva for cortisol analysis was taken from December to May on 1 d/mo at 6 AM, 12 noon, and 6 PM (sampling days: 17–20 December, 14–17 January, 11–14 February, 18–21 March, 15–18 April, and 13–16 May, all horses in 1 group were sampled on the same day). The sampling day was always kept apart from any management-related changes in the husbandry system (eg, start of pasture period). In contrast to all other groups, late pregnant mares were sampled only until February (19 December, 16 January, and 13 February). Mares foaled between February 16 and April 18; and thus, no homogenous group of late pregnant mares was available after February.

Saliva was collected with a cotton-based swab (Salivette cortisol; Sarstedt, Nümbrecht-Rommelsdorf, Germany). The Salivette was inserted at the angle of the lips into the mouth of the horse and placed gently onto the tongue for 1 min until it was well soaked. After centrifugation for 10 min at 1,000*g*, 1 mL of saliva was aspirated, transferred into polypropylene tubes (Sarstedt), and frozen at −20°C until analysis. Collection of saliva was tolerated by the horses of all groups without resistance. In addition, after the last saliva sample on each sampling day, 1 blood sample was taken from a jugular vein into

Table 1
Horse groups included in the study.

Group	Number (n)	Age (yr)	Housing	Remarks
1. Adult breeding sires	11	6.8 ± 0.7	Individual	Breeding via AI only
2. Nonpregnant, adult mares	6	9.5 ± 1.5	Group	
3. Pregnant, adult mares	7	8.8 ± 1.8	Group	
4. Adult geldings	12	12.8 ± 1.6	Individual	
5. 1-yr-old stallions	8	1[a]	Group	
6. 1-yr-old stallions	8	2[a]	Group	
7. 3-yr-old geldings	8	3[a]	Group	Castrated at 2 yr of age
8. 1-yr-old mares	11	1[a]	Group	
9. 2-yr-old mares	11	2[a]	Group	
10. 3-yr-old mares	12	3[a]	Group	

Abbreviations: AI, artificial insemination; SEM, standard error of the mean.
Values are mean ± SEM.
[a] Horses of groups 5 to 10 were born between January and May of their respective years of birth.

heparinized vacutainer tubes (Becton Dickinson, Heidelberg, Germany) for analysis of testosterone in stallions and progesterone in mares. Blood samples were centrifuged within 20 min at 1000*g*, and the supernatants were transferred into polypropylene tubes and frozen at −20°C until hormone analysis.

2.3. Hormone analysis

Cortisol concentrations were determined with a direct enzyme immunoassay as described [22,28]. Cross-reactivity of the antiserum determined at 50% binding was 100% with cortisol, 6.2% with corticosterone, 4.6% with 5α-dihydrocortisol, 0.8% with allotetrahydrocortisol, and <0.1% with all other steroids tested. The intra-assay coefficient of variation determined from duplicates of a control saliva in each assay was 4.5% and the interassay coefficient of variation was 11.7% (mean 1.04 ng/ml). The minimal detectable concentration defined as 2 standard deviations from zero binding was 0.05 ng/mL. Progesterone concentrations in plasma of mares were determined with a commercial enzyme immunoassay (ADI-900–011; Assay Designs, Ann Arbor, MI, USA) as described [18]. The intra-assay coefficient of variation was 7.3%, the interassay coefficient of variation 14.1%, and the minimal detectable concentration 0.01 ng/mL. Testosterone concentration in male horses was determined by direct enzyme immunoassay without extraction (Testosterone ELISA; Demeditec Diagnostics, Kiel, Germany) as described for bovine plasma [29]. The assay was validated for equine plasma in our laboratory. Recovery of testosterone standards added to plasma was 100%, and serial dilution of plasma samples resulted in changes in optical density parallel to the standard curve. All samples were assayed in duplicates. The intra-assay coefficient of variation was 7.4%, the interassay coefficient of variation was 8.4%; and the minimal detectable concentration was 0.01 ng/mL.

2.4. Statistical analysis

For statistical analysis the SPSS statistics package (version 20.0; IBM SPSS, Armonk, NY, USA) was used. All data were normally distributed (Kolmogorov–Smirnov test). Changes in salivary cortisol concentrations over time were analyzed by analysis of variance (ANOVA) using a general linear model for repeated measures with animal group as between subject factor and month and time of day as within subject factors. In the case of overall significant effects, post hoc individual comparisons between times were made by testing for least significant differences with Bonferroni correction for multiple comparisons. In addition, salivary cortisol concentrations at 6 AM on each sampling day were compared between months by general linear model ANOVA for repeated measures and comparisons between groups for individual months were made by 1-way ANOVA. For stallions, Pearson's coefficient of correlation was calculated for correlations between mean daily cortisol concentrations in saliva and testosterone concentrations in plasma. A P value < 0.05 was considered significant. All data given are means ± standard error of mean.

3. Results

When data from all 94 horses irrespective of group and housing system were combined, cortisol concentrations in saliva followed a diurnal pattern with the highest concentrations in the morning and a decrease throughout the day ($P < 0.001$). Comparison between months showed significant differences over time (overall effect $P < 0.001$) and post hoc tests revealed higher cortisol concentration in December vs all other months ($P < 0.01$) except March (Fig. 1).

Salivary cortisol concentrations in 1-, 2-, and 3-yr-old young mares differed slightly but significantly between groups with average values lower in 2-yr-old vs 1- and 3-yr-old mares ($P < 0.05$). An overall diurnal rhythm in salivary cortisol concentrations was evident ($P < 0.001$), although disrupted in individual groups on individual days (eg, 3-yr-old mares in March). Cortisol concentrations differed significantly between months with the lowest values in April (Fig. 2A).

Cortisol concentrations in saliva did not differ between 1-yr-old stallions, 2-yr-old stallions, and 3-yr-old geldings, but within these groups, decreased throughout the study period ($P < 0.01$). Post hoc tests revealed the highest values in December and the lowest in May. As in mares of the same age, a diurnal rhythm ($P < 0.001$) was disrupted in individual groups on individual days (eg, 1-yr-old stallions in April; Fig. 2B).

When data for 1- to 3-yr-old mares and 1- to 3-yr-old stallions and geldings were combined and peak daily cortisol concentrations at 6 AM compared between sexes, no significant differences between female and male young horses existed except for May ($P < 0.001$). Differences between months ($P < 0.001$) could again be demonstrated ($P < 0.001$; Table 2).

Cortisol concentrations in adult horses over 3 yr of age differed between months (at least $P < 0.01$; Table 2). Significant differences existed also between groups with

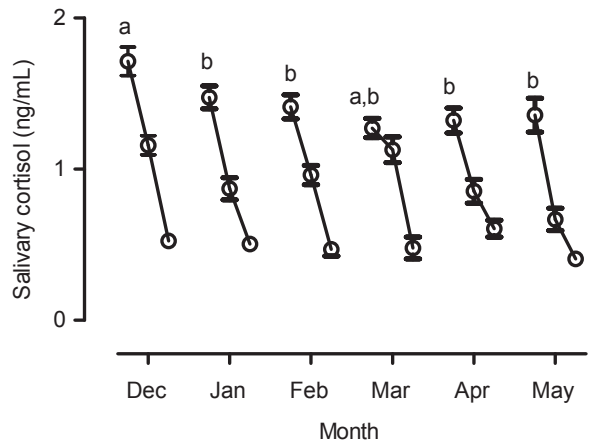

Fig. 1. Daily salivary cortisol concentrations (sampling at 6 AM, 12 noon, and 6 PM) in all horses included into the study (n = 94) from December to May. Values are means ± SEM. Significant differences within days ($P < 0.001$) and between months ($P < 0.001$, GLM). Different letters indicate individual differences between months ($P < 0.01$, post hoc comparisons). GLM, general linear model; SEM, standard error of the mean.

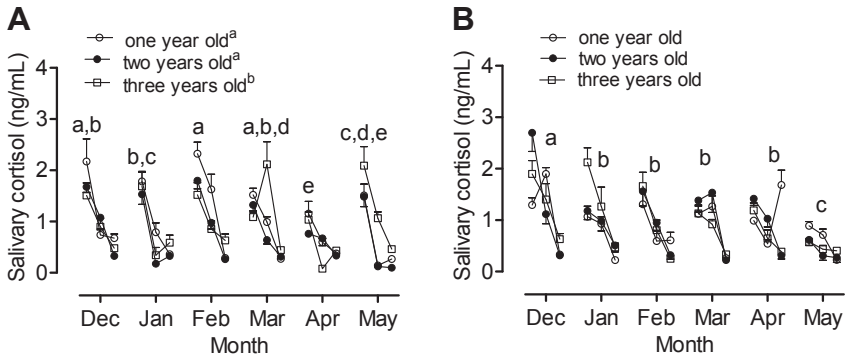

Fig. 2. Daily salivary cortisol concentrations (sampling at 6 AM, 12 noon, and 6 PM) in (A) 1- (n = 11), 2- (n = 12), and 3-yr-old mares (n = 11) and (B) 1- (n = 8) and 2-yr-old stallions (n = 8), and 3-yr-old geldings (n = 8) from December to May. Values are means and SEM. For (A) significant differences within days ($P < 0.001$), between months ($P < 0.001$), and between groups ($P < 0.05$), for (B) significant differences within days ($P < 0.001$) and between months ($P < 0.001$), not significant between groups (GLM). For (A) and (B) different letters indicate differences between individual months ($P < 0.01$) and groups ($P < 0.05$, post hoc comparisons). GLM, general linear model; SEM, standard error of the mean.

Table 2
Salivary cortisol concentrations (ng/mL) in the morning (6 AM) in horses of different groups from December to May.

Group	*P* over time	December	January	February	March	April	May
1- to 3-yr-old horses							
Female (n = 34)	<0.001	1.8 ± 0.2	1.7 ± 0.1	1.9 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.7 ± 0.2
Male (n = 24)	<0.001	2.0 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	0.7 ± 0.1
P between groups	—	NS	NS	NS	NS	NS	<0.001
Adult male horses							
Stallions (n = 11)	<0.001	1.3 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	1.1 ± 0.2	2.1 ± 0.3	2.6 ± 0.6
Geldings (n = 12)	<0.01	1.5 ± 0.2	1.7 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	0.9 ± 0.1
P between groups	—	NS	<0.05	<0.05	NS	<0.05	<0.05
Adult mares							
Pregnant (n = 7)	NS	2.0 ± 0.4	1.4 ± 0.3	1.0 ± 0.2	—	—	—
Nonpregnant (n = 6)	<0.01	1.1 ± 0.8	0.9 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	0.4 ± 0.1
P between groups	—	<0.05	NS	NS	—	—	—
Nonpregnant mares[a]							
Progesterone ≤1 ng/mL (n = 2–22)	—	1.8 ± 0.3	1.4 ± 0.2	1.7 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	1.9 ± 0.4
Progesterone >1 ng/mL (n = 18–38)	—	1.5 ± 0.1	1.7 ± 0.1	1.8 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.5 ± 0.2
P between groups	—	NS	NS	NS	NS	NS	NS
Adult geldings							
Individual housing (n = 12)	<0.01	1.5 ± 0.2	1.7 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	0.9 ± 0.1
Group housing (n = 8)	<0.001	1.9 ± 0.3	2.1 ± 0.3	1.7 ± 0.3	1.1 ± 0.1	1.2 ± 0.1	0.6 ± 0.1
P between groups	—	NS	NS	<0.05	NS	NS	NS

Abbreviations: NS, not significant; SEM, standard error of the mean.
Values are means ± SEM.
[a] No comparisons over time because of different numbers of animals in different months.

higher cortisol concentrations in sexually active stallions during the breeding season than in adult geldings (April and May $P < 0.05$ between groups) and in pregnant compared with nonpregnant mares in December ($P < 0.05$; Table 2).

For all nonpregnant mares irrespective of age (n = 40) grouped by plasma progesterone concentration (≤1 ng/mL and >1 ng/mL), significant differences between these 2 groups could be demonstrated at no time (Table 2). The number of mares with a plasma progesterone concentration >1 ng/mL was 29, 18, 18, 23, 24, and 38 in December, January, February, March, April, and May, respectively.

Plasma testosterone concentrations in yearling, 2-yr-old, and adult stallions showed seasonal variations ($P < 0.001$) with the lowest values from January to March, no differences between groups but significant interactions group × time ($P < 0.05$; Fig. 3). When mean daily salivary cortisol concentrations for all stallions irrespective of age (n = 27) were calculated and correlations with plasma testosterone concentration analyzed for all 6 mo (n = 162 data pairs), a weak, although significant positive correlation could be demonstrated ($r = 0.251$, $P < 0.01$).

Effects of type of housing were compared between adult geldings in single loose boxes and in group housing. Except for February ($P < 0.05$), no significant effect of the housing system existed, whereas differences between months were significant ($P < 0.01$; Table 2).

4. Discussion

In this study, effects of season, time of day, age, sex and reproductive stage, and group housing vs individual stabling on cortisol concentrations in saliva of horses were analyzed. As could be expected from nontreated control group horses

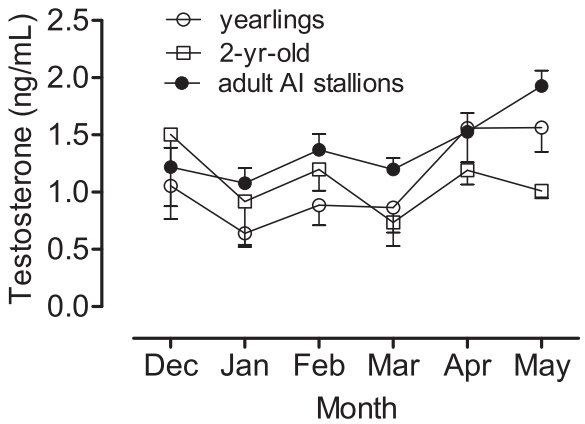

Fig. 3. Plasma testosterone concentrations from December to May in stallions of different age groups. Values are means ± SEM. Significant changes over time ($P < 0.001$) and interactions group × time ($P < 0.05$), not significant between groups (GLM). GLM, general linear model; SEM, standard error of the mean.

in previous studies [11–14], salivary cortisol concentrations showed a clear diurnal rhythm with the highest values in the morning and a nadir in the late afternoon and evening. A diurnal rhythm in salivary cortisol concentrations existed also in geldings. This is in contrast to results from rhesus macaques [30], indicating species differences between horses and rhesus monkeys. However, it cannot be excluded that the diurnal rhythm might have been subsided initially after orchidectomy but was re-established after several months. Deviations from this diurnal rhythm in individual horse groups indicate that the inherent diurnal rhythm can be easily disturbed by apparently minor challenges that were not evident to the investigators. Damping of the diurnal rhythm by even minor perturbations has previously been demonstrated for total cortisol in blood plasma of horses [10]. The management of horses in our study excluded known stressors that might affect cortisol release such as transport [21–23,31,32], change of stable or group mates [14,33], equestrian exercise [3,12,34–36], or veterinary procedures [37]. Factors such as weather, ambient temperature, insects, or interactions within the horse groups are apparently sufficient to cause transient alterations in the diurnal cortisol release pattern. Because these alterations remained within the range of diurnal changes we consider them variations within a physiological range and not indicative of a distress situation.

Cortisol in saliva of late pregnant mares in the present study remained clearly lower than that in mares in the immediate peripartum period [18,20], although in part, it was higher than that in nonpregnant mares. Cortisol in blood plasma of women [19] but not blood plasma of horse mares [17] is elevated during the last months of pregnancy compared with nonpregnant control subjects. This might not be a true species difference because in women, free, that is, unbound cortisol [19] and in horse mares, total cortisol [17] in plasma was measured.

Increased cortisol release in stallions at the height of the breeding season in April and May indicates a moderate chronic stress situation in these animals. In a previous study [38], semen collections were not perceived as an acute stressor in sexually experienced and well-trained stallions, based on a lack of acute changes in heart rate, heart rate variability, and salivary cortisol concentrations. We therefore suggest that not the regular semen collections to which the stallions were accustomed but the housing of sexually active stallions in adjacent boxes in the stable represents a stress-like challenge. Housing of adult stallions in boxes with visual, auditory, and olfactory contact to their neighbors resembles the situation in bachelor stallions, that is, stallions without contact to mares. In contrast, sexual activity at semen collections represents a situation as in harem stallions, which express a certain degree of aggressiveness to fight of rivaling other stallions and to round up their herd [39]. A discrepancy between semen collections, that is, sexual activity as in harem stallions and housing with only stallions in adjacent boxes, that is, a situation as in bachelor stallions might explain increased basal cortisol concentrations in these horses. It is unlikely that the increase in salivary cortisol concentrations is linked to a seasonal increase in testosterone release because between concentrations of these steroid hormones only a weak correlation existed.

No association could be demonstrated between progesterone and salivary cortisol concentrations in mares. It might be expected that estrus in group-housed mares increases locomotion leading to higher cortisol concentrations. Puberty in mares is attained at approximately 12 mo of age, that is, in the breeding season after the year of birth [40]. Thus, at least from March to May, all nonpregnant mares in our study should have been cyclic and those without a corpus luteum thus in estrus; however, this did not affect cortisol release. Our data do not exclude that male and female horses may respond differentially to explicit stressors; however, with regard to baseline cortisol release, data from geldings, nonpregnant mares, and nonsexually active stallions kept under identical conditions can be combined for many studies.

A decrease in salivary cortisol concentrations from December to May was evident in several groups and most pronounced in male young horses. Whether this is directed by photoperiod cannot be concluded from our data. Horses of the 1-, 2-, and 3-yr-old groups were together in their respective groups since weaning at approximately 6 mo of age. Because the decrease in cortisol concentrations occurred in all 3 age groups to the same extent, effects of habituation to the other group members causing less stimulation of cortisol release with ongoing time can be excluded.

Our data allow only a limited comparison of group housing and individual stabling with regard to cortisol release because groups were not evenly distributed over housing systems. To exclude effects of reproductive stage, comparisons between types of housing were restricted to geldings. Individually stabled and group-housed geldings did not differ with regard to salivary cortisol concentrations. The transfer of horses from group housing to individual boxes is associated with an acute and transient increase in salivary cortisol concentrations. With habituation of horses to their new stable, cortisol concentrations return to baseline values within a few days [14]. Geldings in the present study were kept in the respective stables and groups for several months before the start of the study and were thus habituated to their environment.

In conclusion, salivary cortisol concentrations in horses follow a clear diurnal rhythm. Time of the day is thus important for experiments that include analysis of cortisol in saliva. Experimentally induced increases in cortisol release that stay within this diurnal range should not be overinterpreted. Cortisol concentrations also depend on the season, but these changes were not evident in all groups. Although housing system and separation of horses from conspecifics acutely stimulate cortisol release in blood [33] and saliva [14], horses habituate to the new situation, and these factors do not have long-term effects on the diurnal cortisol rhythm. Rapid habituation of horses has also been found to repeated road transport with the cortisol response decreasing with each transport [22]. Sex and age of the horses was without apparent effect on basal cortisol concentrations in saliva. Basal cortisol concentrations were elevated in active breeding sires used for semen collection.

References

[1] Van der Kolk JH, Nachreiner RF, Schott HC, Refsal KR, Zanella AJ. Salivary and plasma concentration of cortisol in normal horses and horses with Cushing's disease. Equine Vet J 2001;33:211–3.

[2] Hughes T, Creighton E, Coleman R. Salivary and fecal cortisol as measures of stress in horses. J Vet Behav Clin Appl Res 2010;5:59–60.

[3] Schmidt A, Aurich J, Möstl E, Müller J, Aurich C. Changes in cortisol release and heart rate and heart rate variability during the initial training of 3-year-old sport horses. Horm Behav 2010;58:628–36.

[4] Peeters M, Sulon J, Beckers J-F, Ledoux D, Vandenheede M. Comparison between blood serum and salivary cortisol concentrations in horses using an adrenocorticotropic hormone challenge. Equine Vet J 2011;43:487–93.

[5] Riad-Fahmy D, Read GF, Walker RF. Salivary steroid assays for assessing variation in endocrine activities. J Steroid Biochem 1983;19:265–72.

[6] Weitzman ED, Fukushima D, Nogeire C, Roffwarg G, Gallagher TF, Hellman L. Twenty-four hour pattern of the episodic secretion of cortisol in normal subjects. Endocrinology 1971;33:14–22.

[7] Smith CJ, Norman RL. Influence of the gonads on cortisol secretion in female rhesus macaques. Endocrinology 1987;121:2192–8.

[8] Hoffsis GF, Murdick PW, Tharp VL, Ault K. Plasma concentrations of cortisol and corticosterone in the normal horse. Am J Vet Res 1970; 31:1379–87.

[9] Bottoms GD, Roesel OF, Rausch FD, Akins EL. Circadian variation in plasma cortisol and corticosterone in pigs and mares. Am J Vet Res 1972;33:785–90.

[10] Irvine CHG, Alexander SL. Factors affecting the circadian rhythm in plasma cortisol concentrations in the horse. Domest Anim Endocrinol 1994;11:227–38.

[11] Erber R, Wulf M, Becker-Birck M, Kaps S, Aurich J, Möstl E, Aurich C. Physiological and behavioural responses of young horses to hot iron branding and microchip implantation. Vet J 2012;191:171–5.

[12] Becker-Birck M, Schmidt A, Lasarzik J, Aurich J, Möstl E, Aurich C. Cortisol release and heart rate variability in sport horses participating in equestrian competitions. J Vet Behav Clin Appl Res 2013;8:87–94.

[13] Bohák Z, Szabó F, Beckers J-F, Melo de Sousa N, Kutasi O, Nagy K, Szenci O. Short communication: monitoring the circadian rhythm of serum and salivary cortisol concentrations in the horse. Domest Anim Endocrinol 2013;45:38–42.

[14] Erber R, Wulf M, Aurich J, Rose-Meierhöfer S, Hoffmann G, von Lewinski M, Möstl E, Aurich C. Stress response of three-year-old horse mares to changes in husbandry system during initial equestrian training. J Equine Vet Sci 2013;33:1088–94.

[15] Paris JJ, Franco C, Sodano R, Freidenberg B, Gordis E, Anderson DA, Forsyth JP, Wulfert E, Frye CA. Sex differences in salivary cortisol in response to acute stressors among healthy participants, in recreational or pathological gamblers, and in those with posttraumatic stress disorder. Horm Behav 2010;57:35–45.

[16] Mongillo P, Prana E, Gabai G, Bertotto D, Marinelli L. Effect of age and sex on plasma cortisol and dehydroepiandrosterone concentrations in the dog (Canis familiaris). Res Vet Sci 2014;96:33–8.

[17] Satué K, Domingo R, Redondo JI. Relationship between progesterone, oestrone sulphate and cortisol and the components of renin angiotensin aldosterone system in Spanish purebred broodmares during pregnancy. Theriogenology 2011;76:1404–15.

[18] Nagel C, Erber R, Bergmaier C, Wulf M, Aurich J, Möstl E, Aurich C. Cortisol and progestin release, heart rate and heart rate variability in the pregnant and postpartum mare, fetus and newborn foal. Theriogenology 2012;78:759–67.

[19] Abou-Samra AB, Pugeat M, Dechaud H, Nachury L, Bouchareb B, Fevre-Montange M, Tourniaire J. Increased plasma concentration of N-terminal beta-lipotrophin and unbound cortisol during pregnancy. Clin Endocrinol 1984;20:221–8.

[20] Nagel C, Erber R, Ille N, von Lewinski M, Aurich J, Möstl E, Aurich C. Parturition in horses is dominated by parasympathetic activity of the autonomous nervous system. Theriogenology 2014;82:160–8.

[21] Schmidt A, Biau S, Möstl E, Becker-Birck M, Morillon B, Aurich J, Faure JM, Aurich C. Changes in cortisol release and heart rate variability in sport horses during a long-distance road transport. Domest Anim Endocrinol 2010;38:179–89.

[22] Schmidt A, Hödl S, Möstl E, Aurich J, Müller J, Aurich C. Cortisol release, heart rate, and heart rate variability in transport-naive horses during repeated road transport. Domest Anim Endocrinol 2010;39:205–13.

[23] Schmidt A, Möstl E, Wehnert E, Aurich J, Müller J, Aurich C. Cortisol release and heart rate variability in horses during road transport. Horm Behav 2010;57:109–15.

[24] Becker-Birck M, Schmidt A, Wulf M, Aurich J, von der Wense A, Möstl E, Berz R, Aurich C. Cortisol release, heart rate and heart rate variability, and superficial body temperature, in horses lunged either with hyperflexion of the neck or with an extended head and neck position. J Anim Physiol Anim Nutr 2013;97:322–30.

[25] Peeters M, Closson C, Beckers J-F, Vandenheede M. Rider and horse salivary cortisol levels during competition and impact on performance. J Equine Vet Sci 2013;33:155–60.

[26] Smiet E, Van Dierendonck MC, Sleutjens J, Menheere PPCA, van Breda E, de Boer D, Back W, Wijnberg ID, van der Kolk JH. Effect of different head and neck positions on behaviour, heart rate variability and cortisol levels in lunged Royal Dutch Sport horses. Vet J 2014;202:26–32.

[27] Aurich C. Reproductive cycles of horses. Anim Reprod Sci 2011;124: 220–8.

[28] Palme R, Möstl E. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int J Mamm Biol 1997;62(Suppl 2):192–7.

[29] Pieler D, Peinhopf W, Becher AC, Aurich JE, Rose-Meierhöfer S, Erber R, Möstl E, Aurich C. Physiological and behavioral stress parameters in calves in response to partial scrotal resection, orchidectomy, and Burdizzo castration. J Dairy Sci 2013;96:6378–89.

[30] Smith CJ, Norman RL. Circadian periodicity in circulating cortisol is absent after orchidectomy in rhesus macaques. Endocrinology 1987;121:2186–91.

[31] Baucus KL, Ralston SL, Nockels CF, McKinnon AO, Squires EL. Effects of transportation on early embryonic death in mares. J Anim Sci 1990;68:345–51.

[32] Baucus KL, Squires EL, Ralston SL, McKinnon AO, Nett MT. Effects of transportation on the estrous cycle and concentrations of hormones in mares. J Anim Sci 1990;68:419–26.

[33] Alexander SL, Irvine CHG. The effect of social stress on adrenal axis activity in horses: the importance of monitoring corticosteroid-binding globulin capacity. J Endocrinol 1998;157:425–32.

[34] Ille N, von Lewinski M, Erber R, Wulf M, Aurich J, Möstl E, Aurich C. Effects of the level of experience of horses and their riders on cortisol release, heart rate and heart rate variability during a jumping course. Anim Welfare 2013;22:457–65.

[35] Von Lewinski M, Biau S, Erber R, Ille N, Aurich J, Faure JM, Möstl E, Aurich C. Cortisol release, heart rate and heart rate variability in the horse and its rider: different responses to training and performance. Vet J 2013;197:229–32.

[36] Ille N, Aurich C, Erber R, Wulf M, Palme R, Aurich J, von Lewinski M. Physiological stress responses and horse rider interactions in horses ridden by male and female riders. Comp Exerc Physiol 2014;10:131–8.

[37] Berghold P, Möstl E, Aurich C. Effects of reproductive status and management on cortisol secretion and fertility of oestrous horse mares. Anim Reprod Sci 2007;102:276–85.

[38] Pasing S, von Lewinski M, Wulf M, Erber R, Aurich C. Influence of semen collection on salivary cortisol release, heart rate, and heart rate variability in stallions. Theriogenology 2013;80:256–61.

[39] McDonnell S. Reproductive behavior of stallions and mares: comparison of free-running and domestic in-hand breeding. Anim Reprod Sci 2000;60-61:211–9.

[40] Wesson JA, Ginther OJ. Puberty in the female pony: reproductive behavior, ovulation, and plasma gonadotropin concentrations. Biol Reprod 1981;24:977–86.