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Cortisol release, heart rate, and heart rate variability in transport-naive horses during repeated road transport

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Abstract

Domestic animals are often repeatedly exposed to the same anthropogenic stressors. Based on cortisol secretion and heart rate, it has been demonstrated that transport is stressful for horses, but so far, changes in this stress response with repeated road transport have not been reported. We determined salivary cortisol concentrations, fecal cortisol metabolites, cardiac beat-to-beat (RR) interval, and heart rate variability (HRV) in transport-naive horses (N = 8) transported 4 times over a standardized course of 200 km. Immunoreactive salivary cortisol concentrations always increased in response to transport ($P < 0.001$), but cortisol release decreased stepwise with each transport ($P < 0.05$). Concentrations of fecal cortisol metabolites increased from 55.1 ± 4.6 ng/g before the first transport to 161 ± 17 ng/g the morning after ($P < 0.001$). Subsequent transport did not cause further increases in fecal cortisol metabolites. In response to the first transport, mean RR interval decreased with loading of the horses and further with the onset of transport (1551 ± 23, 1304 ± 166, and 1101 ± 123 msec 1 d before, immediately preceeding, and after 60–90 min of transport, respectively; $P < 0.05$). Decreases in RR interval during subsequent transports became less pronounced ($P < 0.001$). Transport was associated with a short rise in the HRV variable standard deviation 2 ($P < 0.001$ except transport 1), indicating sympathetic activation. No consistent changes were found for other HRV variables. In conclusion, a transport-induced stress response in horses decreased with repeated transport, indicating that animals habituated to the situation, but an increased cortisol secretion remained detectable.

Keywords: Horse; Transport; Cortisol; Heart rate variability

1. Introduction

Companion animals are increasingly exposed to anthropogenic stressors that are unlikely to occur in the natural surroundings of a species. The animals often have to adapt to situations or tasks they would naturally avoid. Transport is a potential stressor in most large domestic animals. The horse is a species that has been transported for centuries, first by ship and by train, and today mainly by road, but also by air. Based on increased cortisol secretion and changes in heart rate and heart rate variability (HRV), it has been clearly demonstrated that transport is stressful for horses [1–13]. Cortisol may improve the animals' stress response by energy mobilization and behavioral changes [14,15]. HRV, that is, short-term fluctuations in heart rate, reflects the balance of sympathetic and parasympathetic tone and is used as an indicator for the stress response of the autonomic nervous system. In general, increases in the values of the HRV variables standard deviation of beat-to-beat (RR) interval (SDRR) and root mean square of successive RR differences (RMSSD) reflect a shift

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toward parasympathetic dominance, whereas reduced values indicate a shift toward more sympathetic dominance [16–18].

In this study, we determined immunoreactive salivary cortisol, fecal cortisol metabolites, cardiac RR interval, and HRV in transport-naive horses that were transported repeatedly by road over a standardized route of 200 km. Cortisol concentrations in saliva reflect acute changes in cortisol release [6,19], whereas cortisol metabolites in feces owing to intestinal passage time [20] increase only 24 h after an increase in the blood and mainly reflect prolonged stressful situations [21].

2. Material and methods

2.1. Animals

Eight 3-yr-old geldings of the Brandenburg State Stud at Neustadt (Dosse), Germany, were available for the study. All horses were of the German Sport Horse breed. They were kept in a group stable on straw together with 8 additional geldings of the same age and had access to an outdoor paddock for 4 to 5 h per day. The animals were fed concentrates and hay twice daily and had free access to water. The group of horses had been together since weaning at the age of 6–9 mo. They had never been transported before and were not trained for riding or other equestrian activities. The horses had been handled regularly for grooming, feeding, and routine procedures such as hoof care, vaccinations, and deworming. The animals were well accustomed to humans and trained to lead and to stand when tied.

2.2. Experimental design

The group of horses was transported 4 times by road over the same 200-km route. Transport time was approximately 4 h. Recovery time between the first and second transport was 4 d and between all other transports was 2 d. The different transport interval was because of unavailability of the transport vehicle and driver for 2 d between transport 1 and transport 2. Transport was performed as described [6]. In brief, horses were transported in a van (6 horses) and trailer (2 horses) combination. The horses were loaded in individual stalls parallel to the axis of the vehicle and were tied to the wall facing the driving direction. The individual horses were always placed at the same location for each transport. Neither feed nor water was provided on the vehicle. Transport was started immediately after loading and followed 2-lane national roads. The region is flat and sparsely populated, thus transport led through neither cities nor hilly terrain. All 4 transports started in the morning between 8:00 and 8:30 AM. Stops for saliva sampling were made every 60 min. After the transport, horses were unloaded and returned to their stable. The study was approved by the Ethics and Animal Experimentation Committee of the Vienna University of Veterinary Sciences.

2.3. Experimental procedures

2.3.1. Loading time

Loading time was defined as the period from leading the horse out of the stable until the animal reached its final position on the transport vehicle. The transport vehicle was always located at the gate of the stable (about 5 m from the stable door), and loading was always performed by the same 3 persons.

2.3.2. Salivary cortisol

Saliva samples for determination of basal, pretransport cortisol concentrations were taken 1 d before each transport in the morning (8:00–9:30 AM, 4 samples at 30-min intervals) and on each transport day at 60 and 30 min before loading, which corresponds to 7:00 and 7:30 AM. During transport, samples were taken at 60-min intervals. Further samples were taken immediately after transport (time 0) and at 5, 15, 30, 60, 90, 120, and 180 min thereafter. Saliva was collected using cotton rolls (Salivette, Sarstedt, Nümbrecht-Rommelsdorf, Germany). A Salivette was grasped with a surgical arterial clamp, inserted at the angle of the lips into the mouth, and placed gently onto the tongue of the horse for 1 min until the cotton was well soaked with saliva. This procedure was well tolerated by all horses and was performed by 1 person without restraint of the animals except holding the horse loosely by its halter. The Salivette tube was centrifuged for 10 min at 1000 g. At least 1 mL saliva per sample was obtained and frozen at −20 °C until analysis. Concentrations of cortisol were determined with a direct enzyme immunoassay without extraction [21] validated for equine saliva [19]. The antiserum cross-reacts with cortisone and several corticosterone metabolites. As cortisone is present in large amounts in the saliva of horses [5,6], the values measured have to be interpreted as cortisol immunoreactivity (IR). The intra-assay coefficient of variation was 5.0%, the interassay variation was 6.7%, and the minimal detectable concentration was 0.3 pg/well.

2.3.3. Fecal cortisol metabolites

Fecal samples for analysis of cortisol metabolites were collected 3 times a day (approximately 6:00 AM, 2:00 PM, and 10:00 PM, directly after defecation) for

2 d before transport, on the day of transport, and for 2 d thereafter. Feces were collected from the floor of the stable after defecation was observed. Samples were frozen at −22 °C and analyzed as described [22]. The assay is directed against 11-oxoetiocholanolone-CMO linked to bovine serum albumin. The standard curve ranged from 2–500 pg/well, and the 50% intercept was at 20 pg. The interassay and intra-assay coefficients of variation were 11.2% and 8.7%, respectively.

2.3.4. Beat-to-beat interval and HRV

Cardiac RR interval was recorded as described [5,6] using a mobile recording system (S810i, Polar, Kempele, Finland) attached to a girth around the thorax of the horse. The positive electrode was located at the right shoulder and the negative electrode at the middle of the left thorax. Beat-to-beat interval was recorded on the day before each transport (from 8:00–10:00 AM), for 1 h directly before transport, continuously during transport, and for 2 h thereafter. The horses were prepared for recordings by putting on a girth with a nonactivated recording device for 2–3 h each day for 5 d before the actual experiment. During these periods, the horses were also acclimated to saliva sampling.

HRV was analyzed with Kubios HRV software, version 2.0 (Biomedical Signal Analysis Group, Department of Applied Physics, University of Kuopio, Finland, 2008). To remove trend components, data were detrended, and an artefact correction was made [5,6] following established procedures [23]. In our study, the RR interval was recorded and the variables SDRR, RMSSD, and the geometric means standard deviation 1 (SD1) and 2 (SD2) were calculated. The means for all HRV variables were determined for subsequent periods of 30 min each. The RMSSD is determined by calculating the difference between consecutive RR intervals before squaring and summing them; the values are then averaged, and the square root is obtained. The RMSSD is used to estimate high-frequency beat-to-beat variations that represent mainly vagal regulatory activity [16–18]. For calculation of the geometric means, the duration of each RR interval is plotted against the duration of the preceeding RR interval (Poincaré plot). To parameterize the shape of the plot, the Kubios HRV software fits an ellipse to the plot. The ellipse is oriented according to the line-of-identity (RRj = RRj+1) at 45 ° to the x-axis. The SD of the points perpendicular to the line-of-identity (SD1) describes short-term HRV caused mainly by parasympathetic activity. The standard deviation along the line-of-identity (SD2) describes long-term variability [18].

Because of technical problems with a recording device, HRV variables could not be calculated for the data obtained with this device. Because horses were carrying the same recording device on each transport, data from 1 horse had to be excluded. Results are thus based on N = 7 for HRV variables and N = 8 for salivary cortisol and fecal cortisol metabolites.

2.3.5. Statistical analysis

Statistical analysis was done with the SPSS statistics package (SPSS, version 17.0, Chicago, IL, USA,). All data were normally distributed (Kolmogorov-Smirnov test). Changes in salivary cortisol concentrations and heart rate variables over time were analyzed for each transport, including pretransport baseline values by analysis of variance (ANOVA) using a general linear model for repeated measures. In addition, the deviation from the mean baseline obtained 1 d before each transport was calculated as the area under the curve (AUC) for the actual transport time, and AUC values between transports were compared by repeated-measures ANOVA. For salivary cortisol concentrations and RR interval, the differences in AUC values between different transports were calculated as absolute values and percentage of the previous response. To analyze possible effects of the transport interval (4 d and 2 d), these values were then compared using ANOVA (general linear model for repeated measures with the Bonferroni correction for multiple comparisons). For fecal cortisol metabolite concentrations, mean values from 1 d before transport were taken as baseline and compared to values from the day of transport and 1 d after the respective transport using repeated-measures ANOVA. In case of overall significant effects, values differing from the pretransport baseline were identified by testing for least significant differences. A P value < 0.05 was considered significant. All data given are means ± SEM.

3. Results

3.1. Loading time

Mean time required for loading the horses onto the transport vehicle was longest for the first transport and shortest for the last transport (see Table 1). Differences in loading time among transports did not reach statistical significance (transport 1 vs transport 4: $P = 0.085$).

3.2. Salivary cortisol

On the day before transport, basal cortisol-IR concentrations in saliva were < 0.4 ng/mL. Compared to

Table 1
Cortisol release into saliva and changes in beat-to-beat interval and heart rate variability variables calculated as area under the curve for transports 1–4 (values are mean ± SEM; no units are given for values expressed as area under the curve).

Transport	Cortisol in saliva	RR interval	SDRR	RMSSD	SD1	SD2	Loading time (s)
1	17.9 ± 1.8[a]	−2287 ± 513[a]	−20 ± 48	−57 ± 51	−47 ± 36	575 ± 174	22.9 ± 8.3
2	10.4 ± 0.5[b]	−1442 ± 403[a,b]	97 ± 33	28 ± 45	21 ± 31	1192 ± 202	12.9 ± 4.7
3	8.1 ± 0.8[c]	−492 ± 568[a,b]	96 ± 40	50 ± 33	38 ± 24	948 ± 263	16.1 ± 5.6
4	5.2 ± 0.8[d]	333 ± 299[b]	−69 ± 42	−86 ± 52	−59 ± 36	733 ± 195	5.5 ± 1.1

Note: Values with different superscript letters differ significantly ($P < 0.05$).
Abbreviations: RMSSD, root mean square of successive RR differences; RR, beat-to-beat; SD1, standard deviation 1; SD2, standard deviation 2; SDRR, standard deviation of RR interval.

cortisol-IR concentrations on the day preceding transport, concentrations had increased slightly but significantly at 60 and 30 min before transport. A further, marked increase in cortisol IR concentrations occurred during each transport ($P < 0.001$ over time; Fig. 1). After transport, salivary cortisol concentrations decreased continuously and reached pretransport baseline values between 90 and 180 min after unloading. When transport-induced cortisol release was calculated as AUC, values for all 4 transports differed significantly from each other ($P < 0.001$). Greatest cortisol concentrations for transports 1–4 were 5.9 ± 0.6, 3.9 ± 0.2, 2.9 ± 0.2, and 2.2 ± 0.6 ng/mL, respectively (Fig. 1). In contrast, basal cortisol IR concentrations on the day before transport increased slightly but significantly ($P < 0.05$ for overall comparison; transport 1, 0.28 ± 0.02; transport 2, 0.35 ± 0.03; transport 3, 0.44 ± 0.05; transport 4, 0.42 ± 0.03 ng/mL; transport 1 vs 4, $P < 0.05$).

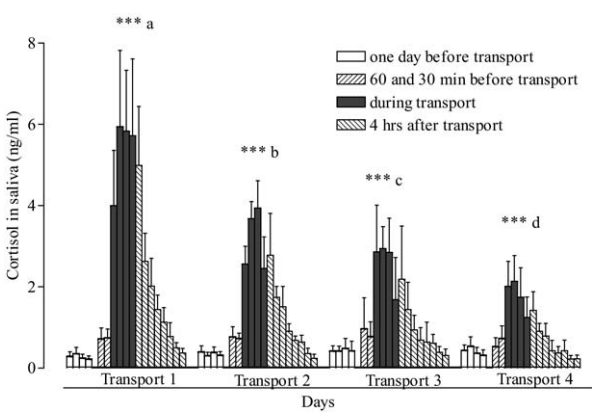

Fig. 1. Cortisol concentrations in saliva of horses (N = 8) 1 d before, 60 and 30 min before, during, and 3 h after repeated road transport. ***Significant changes over time versus baseline for the respective transport ($P < 0.001$; individual time points differing from baseline are not indicated). Different superscript letters indicate differences in area under the curve values between transport days ($P < 0.05$).

The difference in cortisol release calculated as AUC (ng/mL × h) was 7.5 ± 1.7 between transports 1 and 2 (4-d interval), 2.2 ± 0.8 between transports 2 and 3 (2-d interval), and 2.9 ± 0.6 between transports 3 and 4 (2-d interval). The difference between transports 1 and 2 was significantly more pronounced than the differences between transports 2 and 3 and transports 3 and 4, respectively ($P < 0.05$). When cortisol release was calculated as percentage of the previous transport, transport 2 values were 61.6% ± 5.7% of transport 1, transport 3 values 78.7% ± 7.4% of transport 2, and transport 4 values 63.5% ± 6.9% of transport 3. The decrease did not differ significantly between transport intervals.

3.3. Fecal cortisol metabolites

On the 2 d before the first transport, mean cortisol metabolite concentrations in feces ranged between 55 ± 5 and 85 ± 9 ng/g. On the evening of the first transport day, that is, approximately 10 h after unloading, cortisol metabolite concentrations increased to 121 ± 11 ng/g, and a maximum concentration of 161 ± 17 ng/g was reached the next morning (both $P < 0.001$ vs mean pretransport baseline). Only transport 4 caused another significant increase in cortisol metabolite concentrations in feces ($P < 0.01$; Fig. 2).

3.4. Beat-to-beat interval and HRV

In response to the first transport, mean RR interval decreased significantly with loading of the horses and further with the onset of transport ($P < 0.05$). The RR interval remained below pretransport baseline values until 90 min after unloading at the end of transport. The RR interval was 1551 ± 23 msec 1 d before the first transport and 1304 ± 166 msec during the 30 min immediately preceeding the transport, and it decreased to 1101 ± 123 msec in the second h of transport (n.s.). Decreases in RR interval during transports 2–4 were statistically significant ($P < 0.05$) but less pronounced

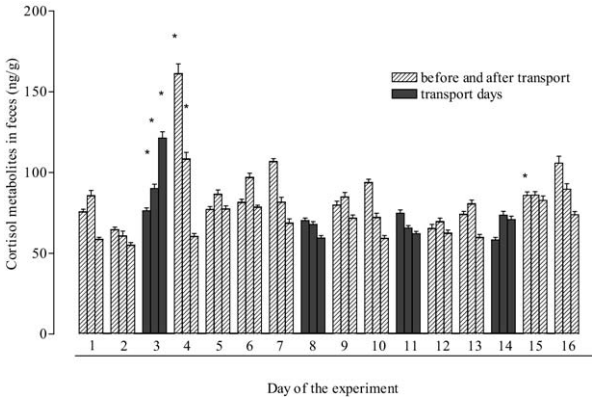

Fig. 2. Fecal cortisol metabolite concentrations in horses (N = 8) before, during, and after repeated road transport. Changes over time for each transport (day before vs day of transport and day after transport): transport 1, $P < 0.001$; transports 2 and 3, n.s.; transport 4, $P < 0.01$.

than during the first transport (comparison between AUC values: $P < 0.001$). The lowest RR rate for transports 2–4 was found during the first 30-min interval (Fig. 3a). When the RR interval was calculated as AUC, with the mean value from the day before transport as baseline, the decrease in RR interval differed significantly between transport days ($P < 0.001$; Fig. 3a). Mean basal RR interval on the day before each transport differed slightly but significantly between days ($P < 0.05$ for overall comparison; transports 1–4, 1545 ± 21, 1617 ± 22, 1514 ± 30, and 1450 ± 40 msec, respectively; transports 1 vs 2, $P < 0.05$).

The difference in RR interval AUC (msec × h) was −150 ± 311 between transports 1 and 2 (4-d interval), −1237 ± 263 between transports 2 and 3 (2-d interval), and −620 ± 391 between transports 3 and 4 (2-d interval; $P < 0.05$). The difference between transports 1 and 2 was less pronounced than the differences between transports 2 and 3 ($P < 0.05$). When the AUC for each transport was calculated as percentage of the previous transport, transport 2 values were 106% ± 24% of transport 1, transport 3 values 3% ± 32% of transport 2, and transport 4 values 93% ± 60% of transport 3 (n.s.).

The HRV variable SDRR changed in response to transport 2 ($P < 0.05$; Fig. 3b). In response to the last transport, a short but significant decrease in SDRR was found toward the end of transport time ($P < 0.001$). Changes over time in RMSSD were similar to SDRR but reached statistical significance for transport 4 only ($P < 0.01$, Fig. 3c).

Neither SDRR nor RMSSD calculated as AUC differed between transport days. We observed changes for the geometric HRV variable SD2 but no clear changes in SD1 over time; SD1 remained largely stable throughout the experiment, with significant deviations from the pretransport baseline only at 2 times after transports 2 and 4 ($P < 0.05$; Fig. 4a). Values for SD2 increased in the immediate pretransport period, reached a maximum during the first 30–60 min of transport, and decreased rapidly thereafter (Fig. 4b). A second transient increase was found in response to unloading of the horses and

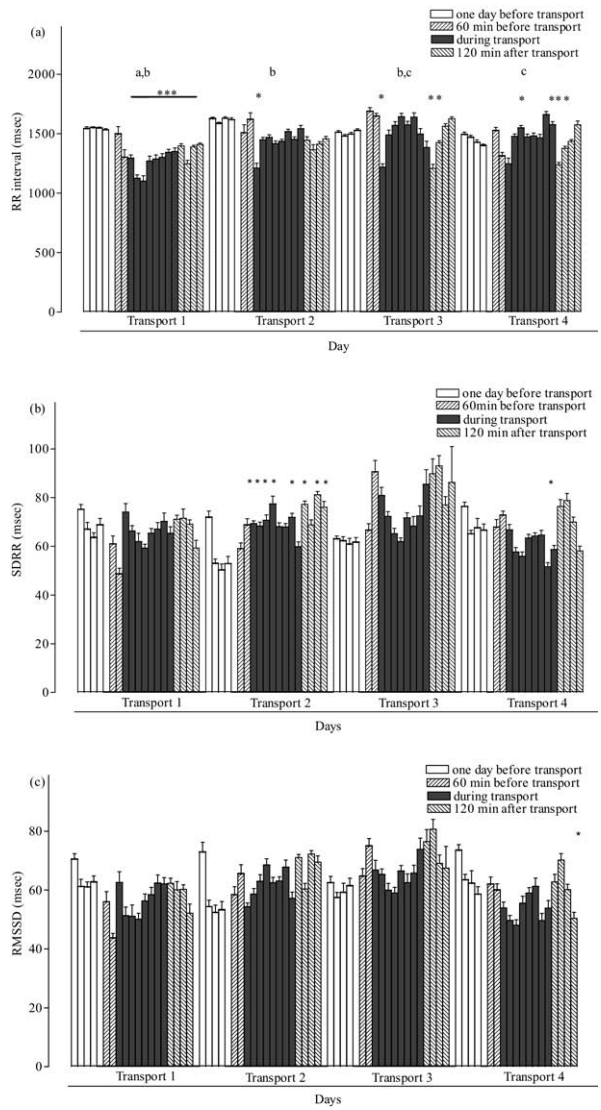

Fig. 3. (a) Beat-to-beat interval, (b) standard deviation of beat-to-beat interval, and (c) root mean square of successive RR differences in horses (N = 7) for 2 h on the days before repeated transport, 60 min directly before transport, and 2 h after repeated road transport. Columns represent 30-min recording intervals. *Values differ significantly from baseline (day before transport) for respective transport ($P < 0.05$). Different superscript letters indicate differences in area under the curve values between transport days ($P < 0.05$).

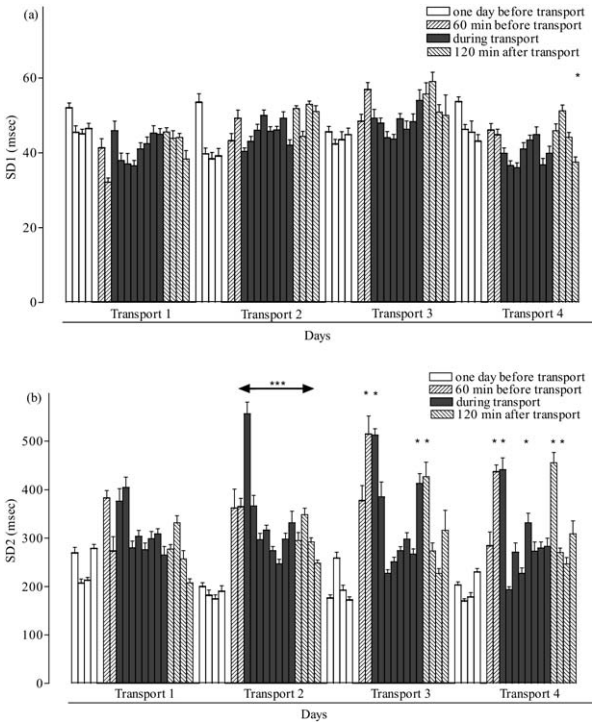

Fig. 4. Geometric heart rate variability variables (a) standard deviation 1 and (b) standard deviation 2 in horses (N = 7) for 2 h on the day before repeated transport, 60 min directly before transport, and 2 h after repeated road transport. Columns always represent 30-min recording intervals. *Values differ significantly from baseline (day before transport) for respective transport, $P < 0.05$. Changes in standard deviation 1 and standard deviation 2 calculated as area under the curve do not differ between transport days.

return to the stable for transports 3 and 4. Changes in SD2 over time were statistically significant for transports 2, 3, and 4 ($P < 0.001$) and tended to reach statistical significance for transport 1 ($P = 0.084$). The SD2 response did not differ between transport days.

When baseline values obtained 1 d before transport were compared, there was a significant effect of time ($P < 0.05$) for SDRR, but post hoc tests revealed no differences between pairs of data. No differences in baseline values existed for RMSSD and SD1. For SD2 baseline values, a significant effect of time existed ($P < 0.05$), and post hoc tests revealed a higher baseline before transport 1 (242 ± 11 msec) than before transport 4 (196 ± 9 msec; $P < 0.05$).

4. Discussion

Domestic animals are often repeatedly exposed to the same environmental and anthropogenic challenges and usually adapt successfully to such stressors. In this study, the stress response of horses during repeated exposure to the same challenge at short intervals was determined. The animals had never been transported before. They had grown up under traditional European stud farm conditions, they had been handled regularly since foal age, and all horses had an identical handling experience. The stress response was studied by analysis of salivary cortisol concentrations, fecal cortisol metabolites, RR interval, and HRV. These noninvasive procedures have been used previously by our group to investigate the response of horses to different transport situations [5,6].

As expected, results confirm that transport elicits a stress response in horses [3,5,6,9,24,25]. As in a previous study performed under identical conditions [5], transport caused an immediate and marked increase in salivary cortisol concentration. Cortisol remained elevated throughout the transport time and decreased to baseline values within 2 h thereafter. Under the conditions of our study, activation of the hypothalamo-pituitary-adrenal axis thus persisted throughout transport.

The cortisol increase in saliva is a surrogate parameter for non-protein-bound cortisol concentration in plasma [26]. Our study demonstrates that the transport-induced stress response decreases rapidly with repeated transports. Although the pattern of salivary cortisol concentrations was similar on all 4 transport days, cortisol release decreased significantly with each transport. The horses thus apparently rapidly learned to cope with the transport situation. The increase in cortisol concentration may even help the animals to adapt to the demands of transport. If transport had been realized as a primarily negative experience by the young horses, an enhanced cortisol release and an increase in loading time or a resistance to loading would have occurred with repeated transport.

Although cortisol release decreased with repeated transport, an increase was still clearly detectable in response to the last transport. Adaptation thus did not reach a point where the cortisol response became undetectable. This finding is in agreement with results from a previous study in older and experienced sport horses, which still showed an increase in salivary cortisol concentration in response to a long-distance transport [5]. In our study, the interval between transports 1 and 2 was longer than subsequent intervals. One might argue that a 2-d interval was too short to allow full recovery. However, when the cortisol response to transport was calculated as percentage of cortisol release during the previous transport, the difference between repeated transports was constant. Comparing absolute

values, the difference was most pronounced between transports 1 and 2, that is, the 4-d interval. In the case of incomplete recovery of adrenocortical function, the most pronouced decrease in transport-induced cortisol release would have been expected in association with the shorter but not the longer resting time. In addition, in a previous study, an overnight rest during long-distance transport was sufficient to allow cortisol release and HRV variables to return to normal, indicating that horses recover rapidly after transport [5]. This interpretation is further strengthened by results from pigs given ACTH at 6-h intervals over 2 d [27]. In these animals, although plasma cortisol release decreased in response to ACH, the ACTH-induced increase in salivary cortisol concentrations remained consistent over time, and endogenous cortisol release had fully recovered within 36 h after the end of ACTH administration. The increase in basal cortisol concentrations in our horses—that is, when only days before transport were compared—was small compared to transport-induced cortisol release and might be explained by the horses associating the presence of the investigators with transport.

Cortisol release in horses follows a diurnal rhythm, with highest values in the morning and a gradual decrease throughout the day [28–30]. Baseline values in our study were measured 1 d before transport and in the morning of each transport day, that is, at the time of greatest endogenous cortisol release. Sampling on the day before each transport was done at the same time of the day as transport. It can thus be excluded that diurnal changes caused the increase in salivary cortisol concentrations. Baseline cortisol concentration in the last hour before transport was slightly greater than that measured 1 d before transport. A difference between the 2 d was the preparation of the transport vehicle close to the stable, which the horses might have perceived as an unknown procedure.

Horses were transported in a van and a trailer attached to the van. Orientation of transported horses on the transport vehicle has been shown to influence the animals‘ stress response [2]. The number of horses in our study does not allow comparisons between locations of animals during transport, and such effects cannot be excluded. However, the aim of the study was not determination of absolute values during individual transports, but analysis of changes over time and habituation of the animals.

A clear increase in fecal cortisol metabolite concentrations was detectable only in response to the first transport. In agreement with results from previous studies [5,6], this response was delayed for approximately 24 h, reflecting intestinal passage time in the horse [20]. The lack of an increase in fecal cortisol metabolites in response to all transports except the first confirms the decrease in cortisol release also found in saliva.

The interpretation of salivary cortisol concentrations is supported by changes in RR interval. Comparable to the results of our experiment, a decrease in RR interval, reflecting an increase in heart rate, has been found in other studies [2,4,6,8,11–13]. Whereas most studies report only a transiently decreased RR interval with the onset of transport, we recently found a reduced RR interval throughout transport in transport-inexperienced horses [6]. In the current study, the reduction in RR interval became less and less pronounced with repeated transport. This result explains, at least in part, differences in the findings of this study and those of previous studies. Although in transport-inexperienced animals the RR interval remains at a lower level for a longer time, this effect becomes less pronounced with growing transport experience of the horses. A decrease in RR interval indicates increased sympathetic activity, decreased parasympathetic activity, or a combination of both [31]. As no pronounced physical activity was requested from the horses during transport, the decrease in RR interval can be interpreted as largely stress induced.

The unchanged SDRR is surprising, as stress in horses has been associated with a reduction in RR interval [17]. In this study, consecutive 30-min intervals were analyzed, and the RR interval—despite an overall reduction—might have changed transiently, for example, in response to factors such as decelerations and accelerations of the vehicle within the 30-min recording intervals. Such transient changes would lead to an increased overall SDRR for each 30-min period. Movements of the animals on the transport vehicle might in part also have masked stress-induced changes in HRV. Ideally, HRV recordings are made when the animal is totally quiet [18,32], a condition that is not achieved during transport.

Geometric analysis of HRV showed significant changes for SD2, representing long-term changes in HRV caused predominantly by changes in sympathetic regulation [18]. Each time, the beginning of transport was associated with a short, but marked, rise in SD2. At rest, horses have a high parasympathetic tone, and sympathetic activity plays little role in determining heart rate [33,34]. The onset of transport might be associated with increased sympathetic activity, which rapidly returns to normal. This increased activity might also

cause the decrease in RR interval with the onset of transport. An increase in SD2 versus the baseline was already seen in association with the loading of the horses. It has been suggested previously that loading can be the most stressful part of transport [35]. Interestingly, SD2 also increased with unloading of the horses after transports 3 and 4, suggesting that not only loading, but also unloading can be perceived as a transient stressor.

For HRV variables, within-group variability was high, indicating differences between individual horses. Road transport is not a continuous constant stressor, and coping with changes in speed, road surface, and turns will require some form of physical activity from the animals, which affects HRV and may contribute to the high variability. The use of young horses with no transport experience might further increase the sensitivity to such factors.

In conclusion, transport of horses leads to increased cortisol release and to changes in RR interval, indicating stress in the animals. The stress response decreases with repeated transport. Analysis of HRV might be restricted to study challenges that do not require physical activity by the horses, because otherwise it may not always be possible to differentiate between effects of stress and activity.

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