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Cortisol release and heart rate variability in horses during road transport

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ABSTRACT

Based on plasma cortisol concentrations it is widely accepted that transport is stressful to horses. So far, cortisol release during transport has not been evaluated in depth by non-invasive techniques such as analysis of salivary cortisol and faecal cortisol metabolites. Transport also causes changes in heart rate and heart rate variability (HRV). In this study, salivary cortisol, faecal cortisol metabolites, heart rate and HRV in horses transported by road for short (one and 3.5 h) and medium duration (8 h) were determined. With the onset of transport, salivary cortisol increased immediately but highest concentrations were measured towards the end of transport (4.1 ± 1.6, 4.5 ± 2.6, 6.5 ± 1.8 ng/ml in horses transported for one, 3.5 and 8 h, respectively). Faecal cortisol metabolite concentrations did not change during transport, but 1 day after transport for 3.5 and 8 h had increased significantly ($p<0.01$), reflecting intestinal passage time. Compared to salivary cortisol, changes in faecal cortisol metabolites were less pronounced. Heart rate increased and beat-to-beat (RR) interval decreased ($p<0.05$) with the onset of transport. Standard deviation of heart rate increased while root mean square of successive RR differences (RMSSD) decreased in horses transported for 3.5 (from 74 ± 5 to 45 ± 6 ms) and 8 h (from 89.7 ± 7 to 59 ± 7 ms), indicating a reduction in vagal tone. In conclusion, transport of horses over short and medium distances leads to increased cortisol release and changes in heart rate and HRV indicative of stress. The degree of these changes is related to the duration of transport. Salivary cortisol is a sensitive parameter to detect transient changes in cortisol release.

Introduction

Domestic animals are routinely exposed to a variety of anthropogenic stressors. During short-term stress, glucocorticoids improve fitness by energy mobilisation (Raynaert et al., 1976) and may change behaviour (Korte et al., 1993). Non-invasive endocrinological techniques have become the method of choice in wildlife and zoo animal research. Because reactions of the animals to repeated venipuncture are avoided, these techniques clearly have a place in domestic animal studies as well, especially for studies on stress.

Transport is a potential stressor in large domestic animal species and also other large mammals in captivity. The horse is a species, which has been transported for centuries, first by ship and by train. The increase in equestrian sports recently has led to a rapid increase in horse transported by road and by air (Cregier, 1982; Friend, 2001). Between 1997 and 2007, the number of international equestrian events has increased worldwide from approximately 550 to more than 2100 (International Equestrian Federation, 2007) and parallel increases have taken place at national level. To national and regional competitions, horses are mainly transported by road. Transport time is usually less than 8 h and it is not legally required in most countries that horses receive food or water during such short-or medium-distance transport.

Based on increases in plasma cortisol concentrations it has been demonstrated repeatedly that transport is stressful for horses (Baucus et al., 1990a,b; Clark et al. 1993; Smith et al., 1996; Friend, 2000; Stull and Rodiek, 2000; Fazio and Ferlazzo, 2003; Stull et al., 2004; Fazio et al., 2008). Cortisol concentration during transport has been suggested to be positively correlated with transport time (Fazio and Ferlazzo, 2003).

So far, cortisol release in response to transport has been mainly evaluated in blood plasma. Non-invasive techniques such as cortisol analysis in saliva and analysis of cortisol metabolites in faeces avoid stress reactions of the animals to repeated venipuncture. Studies determining cortisol concentrations in plasma of horses during transport ideally should include a control group subjected to venipuncture at the same intervals while remaining in familiar surroundings (Stull et al., 2008). In addition, plasma cortisol is mainly bound to carrier proteins, while salivary cortisol mirrors unbound, i.e. free cortisol (Riad-Fahmy et al., 1983; Kirschbaum, 2000; Möstl and Palme, 2002). Salivary cortisol concentrations have been determined in conjunction with loading horses onto a trailer. Cortisol levels

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increased in response to loading but with adequate preparation of the animals the stress-response decreased (Shanahan, 2003).

While cortisol in saliva and in plasma of horses reflects acute changes in cortisol release (Schmidt et al., 2009), cortisol metabolites in faeces–due to intestinal passage time (Palme et al., 1996)–increase only 24 h after an increase in blood and reflect mainly prolonged stressful situations (Palme and Möstl, 1997; Merl et al., 2000). Cortisol metabolites have not been used in studies on transport stress in horses so far.

Transport of horses also causes changes in heart rate (Clark et al., 1993; Waran, 1993; Smith et al., 1994; Waran and Cudderford, 1995; Waran, 1996) and heart rate variability (HRV; Ohmura et al., 2006). Heart rate variability, i.e. short-term fluctuations in heart rate, is essentially based on the antagonistic oscillatory influences of the sympathetic and parasympathetic nervous system on the nodus sinuatrialis of the heart. It thus reflects the prevailing balance of sympathetic and parasympathetic (vagal) tone. Heart rate variability is used as an indicator for the response of the autonomic nervous system to stress. In general, decreases 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 towards more sympathetic dominance, while increased values indicate a shift towards parasympathetic dominance (Von Borell et al., 2007).

In this study, we have determined salivary cortisol, faecal cortisol metabolites, heart rate and HRV in transport-unexperienced horses before, during and after transport by road. Stress responses to different transport times (one, 3.5 and 8 h, corresponding to 50, 200 and 500 km) were compared.

Materials and methods

Animals and experimental design

A total of 24 horses of the Brandenburg State Stud (Neustadt/Dosse, Germany, 52°, 52′N, 12°, 26′E) were transported by road over distances of 50, 200 and 500 km, corresponding closely to 1 (group T1), 3.5 (group T3.5) and 8 h (group T8) of transport ($n=8$ horses per group). Details on the horses are summarized in Table 1. The horses had no recent transport experience and each horse was only used once in the experiment. The experiment was performed in March 2008.

Experimental procedures

Horses were transported in a van (6 horses of each group) and trailer (2 horses of each group) combination. Horses were loaded in individual stalls parallel to the axis of the vehicle and were facing the driving direction. Neither feed nor water were provided during transport. Transport was started immediately after loading of the horses. Transport followed two-lane national roads in Brandenburg State, Germany. The region is predominantly flat and sparsely populated, thus transport neither led through cities nor through hilly or undulating terrain. No stops were made on the 1-h transport. The 3.5- and the 8-h transport were stopped every 60 min for collection of saliva samples. Immediately after the end of transport, horses were unloaded and returned to their stables. The study was approved by the Ethics and Animal Experimentation Committee of the Vienna University of Veterinary Sciences.

Table 1
Horses used for the experiment ($n=8$ per group).

Group	Age (years) means ± SEM (range)	Mares	Geldings	Stallions
T1	13.9 ± 1.3 (8–19)	3	5	–
T3.5	11.4 ± 1.8 (7–22)	2	6	1
T8	7.6 ± 0.7 (5–11)	2	6	–

Salivary cortisol

Saliva samples for cortisol determination were taken at 30-min intervals for 2 h before loading of the horses, every hour during transport, immediately after transport and at 30-min intervals for 6 h thereafter. Saliva was collected with specific cotton rolls (Salivette, Sarstedt, Nümbrecht-Rommelsdorf, Germany) placed onto the tongue of the horses with the help of a surgical arterial clamp for 1 min until the cotton was well soaked with saliva. The cotton roll was then returned to the Salivette polypropylene tube and stored at 4 °C until centrifugation at the end of transport or centrifuged within 10 min at 1000*g* for 10 min. At least 1 ml saliva per sample was obtained and frozen at −20 °C until analysis. Cortisol was determined by a direct enzyme immunoassay without extraction (Palme and Möstl, 1996) validated for equine saliva (Schmidt et al., 2009). The assay was described by Palme and Möstl (1996). The antiserum shows cross-reactivity with cortisone and several corticosterone metabolites. Thus values obtained have to be interpreted as cortisol immunoreactivity (IR). The intra-assay coefficient of variation was 5.0%, the inter-assay variation was 6.7% and the minimal detectable concentration was 0.3 pg/well.

Faecal cortisol metabolites

Faecal samples for analysis of cortisol metabolites were collected on 2 days before transport three times daily (6:00, 14:00 and 22:00 h), on the day of transport at 6:00 and 22:00 h and on 3 days following transport three times daily (6:00, 14:00 and 22:00 h). Samples were frozen at −20 °C until analysis as described by Möstl et al. (2002). The assay is directed against 11-etiocholanolone-CMO coupled to bovine serum albumin. The standard curve ranged from 2 to 500 pg/well, the 50% intercept was at 20 pg. The inter-assay and intra-assay coefficients of variation were 11.2% and 8.7%, respectively.

Heart rate and heart rate variability

Heart rate was recorded with a mobile recording system (f810i, POLAR, Kempele, Finland) attached to the thorax of the horse with an elastic girth. The positive electrode was located at the right shoulder and the negative electrode at the mid of the left thorax. The electrodes were fixed with a second girth which also contained a pocket for the recording watch.

Heart rate was recorded on the day before transport (from 6 to 8 a.m.), during transport and for 12 h thereafter. In group T8 because of limited storage capacity, data were transferred to a computer and watches were restarted after 4 h of transport and during the 12-h post-transport recordings watches were restarted after 6 h. Mean heart rate and HRV were calculated for 30-min intervals. The Kubios HRV software (Biomedical Signal Analysis Group, Department of Applied Physics University of Kuopio, Finland) was used for HRV analysis. To remove trend components, data were detrended and, in addition, an artefact correction was made. Detrending follows the procedure described by Tarvainen et al. [26]. HRV is usually non-stationary and slow linear or more complex trends in the HRV signal can cause distorsion of HRV analysis. The Kubios HRV programme uses a detrending procedure based on smoothness priors approach (Tarvainen et al., 2002; Tarvainen and Niskanen, 2008). The smoothness parameter was set at 500 ms. For artefact correction, the custom filter of the programme was set at 0.3, identifying RR intervals differing from the previous RR interval by more than 30% as artefacts. After abnormal interval removal, the programme's algorithm substitutes detected errors with interpolated intervals calculated from differences between previous and next accepted RR intervals.

In our study, heart rate (HR) was recorded and the HRV variables RR interval, standard deviations of HR and RR interval for a given

recording time (SDHR and SDRR), RMSSD (root mean square of successive RR differences) as well as the geometric means standard deviation 1 (SD1) and 2 (SD2) were calculated. The means for all HRV variables were then 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 obtained. The RMSSD is used to estimate high frequency beat-to-beat variations that represent vagal regulatory activity. For calculation of the geometric means, the duration of each RR interval is plotted against the duration of the preceeding RR interval (Poincaré plot). In order 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 standard deviation (SD) of the points perpendicular to the line-of-identity (SD1) describes short-term HRV mainly caused by parasympathetic activity. The standard deviation along the line-of-identity (SD2) describes long-term variability (Von Borell et al., 2007; Tarvainen and Niskanen, 2008).

Statistical analysis

Statistical analysis was performed with the SPSS statistics package (SPSS, Chicago, Illinois, USA). Data were analysed for normal distribution by Kolmogorov–Smirnov-Test. Because data were not normally distributed for all parameters, non-parametric tests were used throughout. Comparisons within groups were made by Friedman-Test (>2 time points) and Mann–Whitney *U*-Test (2 time points), comparisons between groups were made by Kruskal–Wallis *H*-Test. In addition to comparisons between groups at individual time points, changes in salivary cortisol concentrations, heart rate and HRV parameters were calculated as area under the curve (AUC) for the transport times of one, 3.5 and 8 h, respectively, taking into account the 30-min HRV recording interval, and for changes in faecal cortisol metabolite concentration for the time period from 22:00 on the day of transport until 32 h later. The mean of the pre-transport baseline values was subtracted from each transport or post-transport value. A p-value <0.05 was considered significant.

Results

Salivary cortisol

Before loading and transport, mean cortisol-IR levels in saliva were below 1 ng/ml with only minor individual variations. With the onset of transport, salivary cortisol-IR concentrations increased. In horses transported for 1 h (group T1; no sample taken during transport), highest cortisol-IR concentrations were measured at the end of transport (4.1 ± 1.6 ng/ml), in horses transported for 3.5 and 8 h (groups T3.5 and T8), highest values were reached at 3 h (4.5 ± 2.6 ng/ml) and 7 h (6.5 ± 1.8 ng/ml), respectively. Cortisol-IR release into saliva calculated as area under the curve for the duration of transport differed significantly between groups (T1 versus T3.5 versus T8: $p<0.01$, Table 2). After unloading, cortisol-IR concentrations in saliva decreased rapidly and irrespective of the duration of transport, pre-transport baseline values were reached within 2 h (Fig. 1).

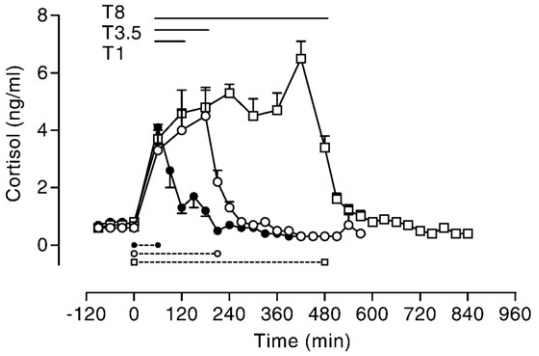

Fig. 1. Cortisol concentration in saliva of horses before, during and after road transport for 1 h (T1), 3.5 (T3.5) and 8 h (T8), $n = 8$ per group. Data are means ± SEM. Error bars are presented either above or below mean values. Dotted lines parallel to *X*-axis indicate transport times. Bars at top of graph indicate times with significant ($p<0.05$) increases in cortisol concentrations versus the mean pre-transport baseline values for the respective groups. Cortisol release calculated as area under the curve differs significantly between groups (T1 versus T3.5 versus T8: $p<0.01$).

Table 2
Changes in salivary cortisol concentrations, heart rate and heart rate variability parameters calculated as area under the curve (AUC) for the respective transport times of one, 3.5 and 8 h ($n = 8$ per group) and changes in faecal cortisol metabolite concentration calculated as AUC for the time period from 22:00 on the day of transport until 32 h later (values are means ± SEM).

Parameter	Group T1	Group T3.5	Group T8
Cortisol IR in saliva	5.9 ± 0.9[a]	20.0 ± 4.6[b]	63.0 ± 6.9[c]
Cortisol metabolites in faeces	40.7 ± 22.0[a]	114.5 ± 14.0[b]	152.7 ± 27.8[b]
Heart rate	51 ± 17[a]	156 ± 27[b]	376 ± 175[b]
Beat-to-beat (RR) interval	−1024 ± 168[a]	−3846 ± 447[b]	−6802 ± 1958[b]
SDHR	8.7 ± 1.7[a]	14.0 ± 4.6	49.0 ± 13.7[b]
SDRR (standard deviation of RR interval)	35.7 ± 19.4	−80.6 ± 56.8	−186.0 ± 128.2
RMSSD (root mean square of successive RR intervals)	19.6 ± 21.8[a]	−178.5 ± 56.5[b]	−296.5 ± 134.6[b]
Standard deviation 1 (SD1) (Poincaré analysis)	15.8 ± 17.8[a]	−131 ± 41[b]	−222.1 ± 96.8[b]
Standard deviation 2 (SD1) (Poincaré analysis)	257 ± 106	683 ± 137	513 ± 883

a, b, c: values with different superscript letters differ significantly ($p<0.05$).

Faecal cortisol metabolites

Mean cortisol metabolite concentrations before transport did not differ between groups (e.g. values in the morning before transport were 76.1 ± 13.6, 80.4 ± 7.9 and 77.6 ± 10.4 ng/g in horses of groups T1, T3.5 and T8, respectively). On the day of transport, neither changes within groups nor differences between groups were found. On the day following transport, faecal cortisol metabolite concentrations had increased significantly ($p<0.01$ versus pre-transport baseline) in horses of groups T3.5 and T8 and statistical significance was nearly reached in horses of group T1 ($p = 0.061$). Highest values were reached in group T8 (136.2 ± 26.7 ng/g), followed by groups T3.5 (110.8 ± 13.1 ng/g) and T1 (90.5 ± 4.7 ng/g). Values differed significantly between groups on the day after transport at 22:00 (T8 and T3.5 versus T1: $p<0.05$). Also, AUC values differed significantly between groups (T1 versus T3.5 and T8: $p<0.05$; Table 2). Two days after transport, faecal cortisol metabolite concentrations had declined to pre-transport baseline values and no longer differed between groups (Fig. 2).

Heart rate and heart rate variability

Mean heart rate increased significantly with the onset of transport and remained elevated above pre-transport baseline values for the duration of transport in groups T1 and T8 and for about double the transport time in group T3.5. Baseline heart rate was 40 ± 2, 35 ± 1 and 37 ± 1 beats/min in horses of groups T1, T3.5 and T8, respectively. Highest values (group T1: 66 ± 13, group T3.5: 60 ± 6, group T8: 60 ± 13 beats/min) were reached between 90 and 150 min after the start of transport and thus after the end of transport in horses of group T1. When the increase in heart rate over time was calculated as the area under the curve for the respective transport

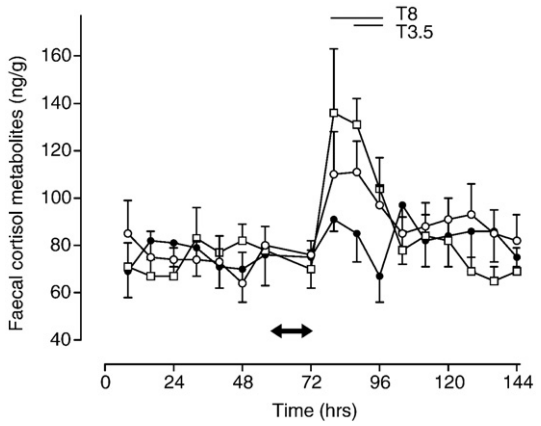

Fig. 2. Faecal cortisol metabolite concentrations in horses before, during and after road transport for one h (t1), 3.5 (t3.5) and 8 h (t8), $n = 8$ per group. Data are means ± SEM. ↔ = day of transport. Error bars are presented either above or below mean values. Bars at top of graph indicate times with significant ($p<0.05$) increases in cortisol concentrations versus the mean pre-transport baseline values for the respective groups. Cortisol metabolite concentrations calculated as area under the curve differ significantly between groups (T1 versus T3.5 and T8: $p<0.05$).

period, values for group T1 were significantly lower than for groups T3.5 and T8 ($p<0.01$; Fig. 3a, Table 2).

The mean RR interval decreased significantly ($p<0.05$) in horses of all groups and remained at a lower level throughout the transport period (Fig. 3b). Minimal values reached did not differ between groups and for groups T1, T3.5 and T8 were 1103 ± 133 (baseline 1605 ± 107), 1089 ± 77 (baseline 1742 ± 64) and 1239 ± 143 ms (baseline 1675 ± 55). When the deviation from baseline values was calculated as the area under the curve for the respective transport period, values for group T1 were significantly lower than for groups T3.5 and T8 ($p = 0.01$).

Standard deviation of heart rate (SDHR) increased with the onset of transport and remained elevated for at least the duration of transport. Mean pre-transport baseline values were 3.0 ± 0.1, 3.9 ± 0.9 and 4.3 ± 0.4 beats/min for groups T1, T3.5 and T8, respectively. At 60 min after the onset of transport, values of 7.1 ± 1.1 (group T1), 7.7 ± 0.7 (group T3.5) and 7.9 ± 0.6 (group T8) were reached ($p<0.05$ versus respective baseline). When the increase in SDHR over time was calculated as the area under the curve for the respective transport period, values for group T1 were significantly lower than for group T8 ($p<0.05$; Fig. 3c). Mean SDRR only in group T1 at individual time points after transport was higher than pre-transport baseline values (Fig. 3d).

Mean RMSSD values in horses of group T1 at individual time points after transport were higher than the pre-transport baseline ($p<0.05$, Fig. 3e). In horses of groups T3.5 and T8, RMSSD decreased after the onset of transport (group T3.5 from 74 ± 5 to 45 ± 6 ms, group T8 from 89.7 ± 7 to 59 ± 7 ms for the time period 30–60 min of transport). RMSSD values calculated as area under the curve for the transport time were significantly reduced in groups T3.5 and T8 versus group T1 ($p<0.01$; Fig. 3e).

Mean SD1 values did not change significantly over time in horses transported for 1 h (group T1) but showed marked individual variation from about 1 h to 5 h after transport. SD1 decreased during transport over 3.5 and 8 h (groups T3.5 and T8). SD1 calculated as area under the curve for the transport time was significantly lower for groups T3.5 and T8 compared to group T1, $p<0.01$, Fig. 3f). Mean SD2 values were significantly ($p<0.05$) elevated in all groups for the first 30-min interval of transport. No differences between groups were found. Baseline and peak values in groups T1, T3.5 and T8 were 266 ± 60 and 492 ± 55, 226 ± 14 and 589 ± 31, 289 ± 33 and 639 ± 56 ms, respectively (Fig. 3g).

Discussion

In this study, salivary cortisol, faecal cortisol metabolites, heart rate and HRV were analysed in horses with no recent transport experience and exposed to road transport over different duration. It was the aim of the experiment to assess the stress experienced by the animals during short and medium time transport. As transport time over a given distance depends on the vehicle, driving speed and road quality, transport time rather than distance was used to differentiate between groups in our study. Stress was analysed with non-invasive procedures which are not restricted to the horse but are also applicable to a range of research topics in other large animal species.

Irrespective of its duration, transport caused an immediate and marked increase in salivary cortisol concentration. Cortisol remained elevated throughout the transport time, and decreased to baseline values rapidly after unloading of the horses. In both the 3.5 and 8 h transport groups horses showed a decline in salivary cortisol concentrations already during the last measurement period. The last transport sample was taken as close as possible after unloading of the horses and return to the stable and the delay between stopping of the van and sampling was approximately 5 min. This time apparently was sufficient to allow a decrease in cortisol levels. The 1-h transport was not stopped for sampling and actual cortisol concentrations when the van moved may have been even higher than concentrations determined in the study. In agreement with studies determining plasma cortisol (Friend, 2000; Stull and Rodiek, 2000, 2002; Stull et al., 2008), changes in salivary cortisol in our study confirm that transport in horses stimulates cortisol release.

Although salivary cortisol concentrations, after the initial increase at the onset of transport, tended to increase further with ongoing transport time, no significant differences between peak levels at different times were found. While most studies have focussed on long-distance transport, recently, increased cortisol concentrations in plasma have been reported for horses transported over distances comparable to our study. Cortisol levels at the end of transport did not depend on the distance the horses had travelled (Fazio et al., 2008). In that study, blood samples were taken before and after transport only and thus changes in cortisol release during the transport time could not be determined. Our data show that cortisol release is stimulated early during transport and thus, short-time transport stimulates cortisol release nearly as effectively as transport over longer distances. Activation of the hypothalamo-pituitary-adrenal axis persists throughout transport and is linked to the duration of transport. Cortisol determination has been judged a crude and highly variable parameter for stress analysis (Ohmura et al., 2006). This might be true when samples are taken at long intervals, e.g. before and after transport only. With repeated sampling and at least hourly sampling intervals, salivary cortisol concentrations allow a detailed analysis of the stress response in horses exposed to transport.

Horses in our study did not receive water and feed during transport. The stress determined in the experiment thus might be considered a combined action of transport and of temporary feed and water withdrawal. This may explain in part the slight increase in cortisol during transport that followed the pronounced initial increase. However, major effects of food and water withdrawal during transport on salivary cortisol levels are unlikely. During the experiment, ambient temperatures did not exceed 15 °C and the transport vehicle was not exposed to direct sun. In addition, the schedule for feeding concentrates to the horses was not changed for the experiment. In Germany it is neither legally required nor common to offer feed and water to horses during transport not exceeding 8 h. Thus, the situation in our study closely mirrors the conditions during routine short- and medium-time road transport of sport horses but, nevertheless, combined effects of different transport-associated stressors were determined. Major losses in body weight and

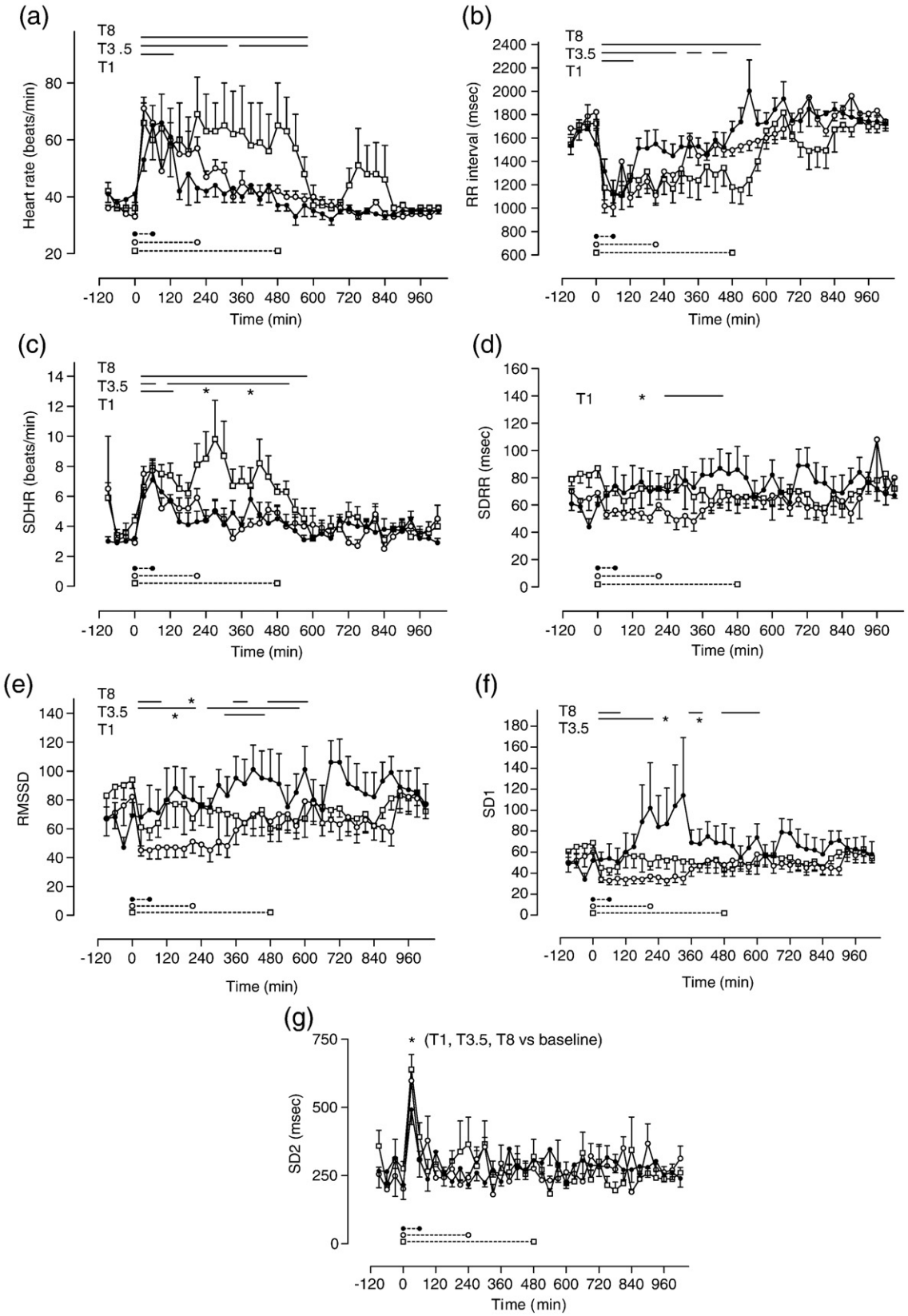

Fig. 3. (a) Heart rate, (b) RR interval, (c) standard deviation of heart rate (SDHR), (d) Standard deviation of RR interval (SDRR), (e) root mean square of successive RR differences (RMSSD) and geometric means (f) SD1 and (g) SD2 in horses before, during and after road transport for one h (T1), 3.5 (T3.5) and 8 h (T8), $n = 8$ per group. Data are means ± SEM. Error bars are presented either above or below mean values. Dotted lines parallel to *X*-axis indicate transport times. Bars at top of graph indicate times with significant ($p<0.05$) changes versus the mean pre-transport baseline values for the respective groups. For significant differences between groups for heart rate and HRV parameters calculated as area under the curve (AUC) see Table 2.

dehydration of horses were found mainly when horses were transported for 24 h or longer and under climatic conditions with higher ambient temperatures and direct exposure of transport vehicles to the sun (Smith et al., 1996; Friend, 2000; Stull and Rodiek, 2000).

Basal salivary cortisol levels in our experiment were in the same range as reported from studies on loading (Shanahan, 2003) and isolation of horses (Harewood, 2005) and in normal horses (Pell and McGreevy 1999; van der Kolk et al., 2001). Compared to plasma cortisol concentrations in other studies (Baucus et al., 1990a,b; Clark et al., 1993; Smith et al., 1996; Stull and Rodiek, 2000, 2002; Fazio and Ferlazzo, 2003), salivary cortisol levels are clearly lower. In saliva, only free, i.e. unbound cortisol occurs, while in plasma both free and protein-bound cortisol is measured. Because only 2% to 15% of cortisol is not protein-bound (Riad-Fahmy et al., 1983; Kirschbaum, 2000), absolute cortisol concentrations are higher in plasma than in saliva.

Changes in faecal cortisol metabolite concentrations confirmed data for salivary cortisol but were less pronounced and delayed for 1 day. With longer transport times, higher faecal cortisol metabolite concentrations were found. However, only after the 3.5- and 8-h transport but not the 1-h transport, faecal cortisol metabolite concentrations showed a clear increase reaching statistical significance. Highest cortisol metabolite concentrations in faeces were found on the day following transport, starting 16 h after the end of transport and values remained elevated for approximately 12 h. This time delay is in agreement with previous reports in ponies and reflects intestinal passage time in the horse (Palme et al., 1996). While cortisol determination in saliva allows detection of small and transient changes in cortisol release, faecal cortisol metabolite levels increase only in response to marked or prolonged cortisol release. Overall, our data show that non-invasive endocrinological techniques can be used successfully for stress analysis.

Heart rate, beat-to-beat interval and HRV were affected by transport. Heart rate increased and RR interval decreased during transport and remained at this level until horses were unloaded and returned to their stables. An increase in heart rate indicates increased sympathetic activity, decreased parasympathetic (vagal) activity or a combination of both (Von Borell et al., 2007). Rapid changes in heart rate are mostly caused by shifts in vagal regulation and occur within less than 5 s while heart rate responses to sympathetic regulation occur more slowly (Hainsworth, 1995). Initial changes in heart rate comparable to our experiment have been found in other studies (Clark et al., 1993; Waran, 1993; Smith et al., 1994; Waran and Cuddeford, 1995; Waran, 1996; Ohmura et al., 2006), however, in these studies heart rate first increased but decreased again during transport. It has thus been suggested that loading of the horses may be the most stressful part of transport (Shanahan, 2003). Also in our study, the highest heart rate was found at the beginning of transport, but values remained elevated throughout transport. This might be due to the short transport time and heart rate may have decreased if transports had been continued. In addition, our horses were not used to being transported and thus might calm down later during transport than experienced horses in other studies. It thus would be of interest to determine the stress response of horses to controlled repeated transport.

The HRV variables RMSSD and SD1 decreased in response to transport over 3.5 and 8 h but not 1 h while long-term variability increased in all groups. RMSSD and SD1 are the primary HRV variables used to estimate high frequency beat-to-beat variations that represent parasympathetic activity. The decrease in RMSSD and SD1 reflects a reduced parasympathetic (vagal) tone in reaction to transport (Von Borell et al., 2007). A reduced vagal tone in transported horses is in agreement with reduced high frequency power during transport when HRV was determined by power spectrum analysis (Ohmura et al., 2006).

The increase in SDHR and unchanged SDRR at first appear to be surprising as stress in horses (Visser et al., 2002) or calves (Mohr et al., 2002) has been reported to lead to a reduction in variability of the beat-to-beat interval. In our study, HRV was analysed for consecutive 30-min intervals. Within these intervals, heart rate and beat-to-beat intervals might change transiently in response to external factors such as decelerations and accelerations of the vehicle. This will affect HRV parameters and lead to an increased overall SDHR and SDRR for each 30-min period which might have not occurred with shorter recording intervals. Ideally, HRV recordings are made with the animal quietly standing (Rietmann et al. 2004, Von Borell et al., 2007). This cannot be achieved during transport and balancing movements and changes in muscle tonus cannot be avoided.

Geometric analysis of heart rate changes also showed significant changes for SD2 (standard deviation 2), representing long-term changes in HRV that are predominantly caused by changes in sympathetic regulation (Von Borell et al., 2007). In horses of all groups the beginning of transport was associated with a short, but marked rise in SD2, i.e. a transient activation of the sympathetic branch of the autonomous systems. This might cause the dramatic increase in heart rate with the onset of transport. At rest, horses have a high parasympathetic tone and sympathetic activity plays little role in determining heart rate (Hamlin et al., 1972; Kuwahara et al., 1996). The onset of transport might be associated with increased sympathetic activity which rapidly returns to normal while vagal activity remains reduced.

Although, heart rate and HRV changed significantly over time, within-group variability was high, indicating differences between individual horses. Because only little physical activity was requested from the animals during transport, changes in heart rate were primarily caused by the need to adapt to the transport situation. However, road transport is not a continuous constant stressor and coping with changes in speed, road surface and turnings will require some form of physical activity which affects heart rate and HRV and thus may contribute to the high variability.

Although all horses were mature, at least 6-year-old sport horses and kept in the institution since birth, horses transported for 8 h tended to be younger than those transported for 1 h and 3.5 h. However, when horses were grouped by age (6–8, 9–12 and 13–22 years) no significant difference in the cortisol and heart rate response to transport was found between groups (data not shown).

In conclusion, transport of horses over short and medium distances leads to increased cortisol release and to changes in heart rate and HRV indicative of stress in the transported animals. The degree of these changes was related to the duration of transport. Longer transport thus appear to be more stressful for horses than short-time transport. Cortisol concentrations in saliva are a sensitive parameter to detect even small and transient increases in plasma cortisol levels while cortisol metabolite concentrations in faeces detect only marked or prolonged increases in cortisol release. Changes in HRV during transport indicate increased sympathetic activity at the onset of transport and decreased vagal tone throughout the duration of transport.

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References

Baucus, K.L., Ralston, S.L., Nockels, C.F., McKinnon, A.O., Squires, E.L., 1990a. Effects of transportation on early embryonic death in mares. J. Anim. Sci. 68, 345–351.

Baucus, K.L., Squires, E.L., Ralston, S.L., McKinnon, A.O., Nett, M.T., 1990b. Effects of transportation on the estrous cycle and concentrations of hormones in mares. J. Anim. Sci. 68, 419–426.

Clark, D.K., Friend, T.H., Dellmeier, G., 1993. The effect of orientation during trailer transport on heart rate, cortisol and balance in horses. Appl. Anim. Behav. Sci. 38, 179–189.
Cregier, S.E., 1982. Reducing equine hauling stress: a review. J. Equine Vet. Sci. 2, 186–198.
Fazio, E., Ferlazzo, A., 2003. Evaluation of stress during transport. Vet. Res. Commun. 27 (Suppl. I), 519–524.
Fazio, E., Medica, P., Aronica, V., Grasso, L., Ferlazzo, A., 2008. Circulating β-endorphin, ACTH and cortisol levels of stallions before and after short road transport: stress effect of different distances. Acta. Vet. Scand. 50. doi:10.1186/1751-0147-50-6.
Friend, T.H., 2000. Dehydration, stress and water consumption of horses during long-distance commercial transport. J. Anim. Sci. 78, 2568–2580.
Friend, T.H., 2001. A review of recent research on the transportation of horses. J. Anim. Sci. 79, E32–E40.
Hainsworth, R., 1995. The control and physiological importance of heart rate. In: Malik, M., Camm, A.J. (Eds.), Heart rate variability. Futura Publishing Company, Armonk, NY, pp. 3–19.
Hamlin, R.L., Klepinger, W.L., Gilpin, K.W., Smith, C.R., 1972. Autonomic control of heart rate in the horse. Am. J. Physiol. 222, 976–978.
Harewood, E.J., 2005. Behavioral and physiological responses to stabling in naïve horses. J. Equine Vet. Sci. 25, 164–170.
International Equestrian Federation, 2007. Annual Report 2007 of the FEI (Fédération Equestre Internationale). http://www.fei.org (accessed 21 July 2008).
Kirschbaum, C., 2000. Salivary cortisol. In: Fink, G. (Ed.), Encyclopedia of stress. Academic Press, San Diego, CA, pp. 379–383.
Korte, S.M., Bouws, G.A.H., Bohus, B., 1993. Central actions of corticotropin-releasing hormone (CR-H) on behavioral, neuroendocrine and cardiovascular regulation: brain corticoid receptor involvement. Horm. Behav. 27, 167–183.
Kuwahara, M., Hashimoto, S., Ishii, K., Yagi, Y., Hada, T., Hiraga, A., Kai, M., Kubo, K., Oki, H., Tsubone, H., Sugano, S., 1996. Assessment of autonomic nervous function by power spectral analysis of heart rate variability in the horse. J. Auton. Nerv. Syst. 60, 43–48.
Merl, S., Scherzer, S., Palme, R., Möstl, E., 2000. Pain causes increased concentrations of glucocorticoid metabolites in horse faeces. J. Equine Vet. Sci. 20, 586–590.
Mohr, E., Langbein, J., Nürnberg, G., 2002. Heart rate variability—a noninvasive approach to measure stress in calves and cows. Physiol. Behav. 75, 251–259.
Möstl, E., Palme, R., 2002. Hormones as indicators of stress. Domest. Anim. Endocrinol. 23, 67–74.
Möstl, E., Maggs, J.L, Schrötter, G., Besenfelder, U., Palme, R., 2002. Measurement of cortisol metabolites in faeces of ruminants. Vet. Res. Commun. 26, 127–239.
Ohmura, H., Hiraga, A., Aida, H., 2006. Changes in heart rate and heart rate variability in Thoroughbreds during prolonged road transport. Am. J. Vet. Res. 67, 455–462.
Palme, R., Möstl, E., 1996. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Proceedings, 1st Intern. Symposium Physiology and Ethology of Wild and Zoo Animals, Berlin, Germany, September 1996, p. 84.
Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of ^{14}C-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. 43, 43–63.
Palme, R., Möstl, E., 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int. J. Mammal. Biol. 62 (Suppl. II), 192–197.
Pell, S.M., McGreevy, P.D., 1999. A study of cortisol and beta-endorphin levels in stereotypic and normal Thoroughbreds. Appl. Anim. Behav. Sci. 64, 81–90.
Raynaert, R., De Paepe, M., Peeters, G., 1976. Influence of stress, age and sex on serum growth hormone and free fatty acids in cattle. Horm. Metab. Res. 8, 109–114.
Riad-Fahmy, D., Read, G.F., Walker, R.F., 1983. Salivary steroid assays for assessing variation in endocrine activities. J. Steroid. Biochem. 19, 265–272.
Rietmann, T.R., Stuart, A.E.A., Bernasconi, P., Stauffacher, M., Auer, J.A., Weishaupt, M.A., 2004. Assessment of mental stress in warmblood horses: heart rate variability in comparison to heart rate and selected behavioural parameters. J. Appl. Anim. Welf. Sci. 88, 121–136.
Schmidt, A., Aurich, C., Neuhauser, S., Aurich, J., Möstl, E., 2009. Comparison of cortisol levels in blood plasma, saliva and faeces of horses submitted to different stressors or treated with ACTH. Proceedings, 5th Intern. Symposium Equitation Science, Sydney, July 2009, p. 53.
Shanahan, S., 2003. Trailer loading stress in horses: behavioural and physiological effects of nonaversive training (TTEAM). J. Appl. Anim. Welf. Sci. 6, 263–274.
Smith, B.L., Jones, J.H., Carlson, G., Pascoe, J.R., 1994. Effect of body direction on heart rate in trailered horses. Am. J. Vet. Res. 55, 1007–1011.
Smith, B.L., Jones, JH., Hornof, W.J., Miles, J.A., Longworth, K.E., Willits, N.H., 1996. Effects of road transport on indices of stress in horses. Equine Vet. J. 28, 446–454.
Stull, C.L., Spier, S.J., Aldridge, B.M., Blanchard, M., Stott, J.L., 2004. Immunological response to long-term transport stress in mature horses and effects of adaptogenic dietary supplementation as an immunomodulator. Equine Vet. J. 36, 583–589.
Stull, C.L., Rodiek, A.V., 2000. Physiological responses of horses to 24 hours of transportation using a commercial van during summer conditions. J. Anim. Sci. 78, 1458–1466.
Stull, C.L., Rodiek, A.V., 2002. Effects of cross-tying horses during 24 h of road transport. Equine Vet. J. 34, 550–555.
Stull, C.L., Morrow, J., Aldridge, B.A., Stott, J.L., McGlone, J.J., 2008. Immunophysiological responses of horses to 12-hour rest during 24 hours of road transport. Vet. Rec. 162, 609–614.
Tarvainen, M.P., Niskanen, J.-P., 2008. Kubios HRV Version 2.0 User's Guide. Department of Physics, University of Kuopio, Kuopio, Finnland.
Tarvainen, M.P., Ranta-aho, P.O., Karjalainen, P.A, 2002. An advanced detrending method with application to HRV analysis. IEEE Trans. Biomed. Eng. 49, 172–175.
Van der Kolk, J.H., Nachreiner, R.F., Sonott, H.C., Refsal, K.R., Zanella, A.J., 2001. Salivary and plasma concentration of cortisol in normal horses and horses with Cushing's disease. Equine Vet. J. 33, 211–213.
Visser, E.K., van Reenen, C.G., van der Werf, J.T.N., Schilder, M.B.H., Knaap, J.H., Barneveld, A., Blokhuis, H.J., 2002. Heart rate and heart rate variability during a novel object test and a handling test in young horses. Physiol. Behav. 76, 289–296.
Von Borell, E., Langbein, J., Després, G., Hansen, S., Leterrier, C., Marchant-Forde, J., Marchant-Forde, R., Minero, M., Mohr, E., Prunier, A., Valance, D., Veissier, I., 2007. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals—a review. Physiol. Behav. 92, 293–316.
Waran, N.K., 1993. The behaviour of horses during and after transport by road. Equine Vet. Education 5, 129–132.
Waran, N.K., 1996. Effects of transporting horses facing either forwards or backwards on their behaviour and heart rate. Vet. Rec. 139, 7–11.
Waran, N.K., Cuddeford, D., 1995. Effects of loading and transport on the heart rate and behaviour of horses. Appl. Anim. Behav. Sci. 43, 71–81.