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Effects of transportation procedures on salivary and plasma cortisol concentrations in cold-blood horses

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Summary: Horses are often transported. Road transport is stressful for horses and induces various rates of cortisol release related to breed and age. The aim of this study was to evaluate the stress level in cold-blood horses of different ages, in response transport over two different distances. The stress level was evaluated on the basis of cortisol concentrations in plasma and saliva samples taken during transportation procedures. A total of 36 cold-blood mares in the process of being sold and bought were included in the study. The horses were divided into four age- and distance-related groups. Twelve adult mares and 12 fillies were transported 50 km. The other two groups (six fillies and six adult mares) were transported 550 km. Samples were taken at rest, two times during the transportation procedure and 12 hours after transport 50 km or 24 hours after transport 550 km. Salivary and plasma cortisol concentrations increased during transportation procedures and remained elevated 12 and 24 hours after the end of transport. In conclusion, in cold-blood horses subjected to transportation, even 24 hours post-transport rest is insufficient to lower plasma and salivary cortisol concentrations to baseline values. Cold-blood, naive horses seem to be more sensitive to changes in location than to road transport. Saliva samples are useful for the determination of cortisol concentration in transported cold-blood horses.

Keywords: cold-blood horses, cortisol, transport, animal welfare, equine

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Introduction

Horses are often transported various distances by road for breeding, competitions, sales and, unfortunately, also for slaughter. It is well known that road transport is stressful and induces cortisol release, especially in naïve horses (*Foreman* and *Ferlazzo* 1996, *Schmidt* et al. 2010b, *Fazio* et al. 2013). Transport conditions, such as water deprivation and trailer design, affect the stress level of transported horses (*Friend* et al. 1998, *Stull* 1999). Just cross-tying horses individually in stalls during transport significantly increases the serum cortisol concentration, in comparison to loose traveling horses (*Stull* and *Rodiek* 2002). The distance of road transport also influences horse response. Transit from 50 to 500 km significantly changed the cortisol release ratio, however, it did not influence plasma or salivary cortisol levels determined immediately after unloading (*Fazio* et al. 2008, *Schmidt* et al. 2010c). A 24 hour resting period after 24 hours of transport was generally sufficient to restore the plasma cortisol concentration to initial levels, even on a summer day, with maximal environmental temperature exceeding 38 °C (*Fazio* et al. 2008b, *Stull* et al. 2008, *Stull* and *Rodiek* 2000). Moreover, *Schmidt* et al. (2010a,b,c) stated that salivary cortisol concentration in experienced horses decreased after transport to baseline values within 30 min, and in naïve horses, within three hours. However, the exercise performed by sport horses, which were transported a day before, induced a higher increase in serum cortisol levels than in untransported horses (*Medica* et al. 2010).

Cortisol concentration can be measured in blood plasma, saliva and faeces (*Schmidt* et al. 2010 a,b,c). Saliva cortisol concentrations correlate well with those in plasma, and across the diurnal rhythm (*Dorn* et al. 2007, *Bohák* et al. 2013). Cortisol, like other steroids, is poorly soluble in water. In circulating blood, steroids are bound with plasma protein carriers, and only 2%–15% of cortisol is free and biologically active (*Riad-Fahmy* et al. 1983). Only this non-protein-bound fraction of cortisol reaches the saliva. Therefore, salivary cortisol concentration represents this free and biologically active fraction. Therefore, nowadays, the measuring of salivary cortisol level is a commonly accepted method of assessing stress levels in horses (*Peeters* et al. 2011, *Strzelec* et al. 2011, 2013). However, there are breed and age related differences in cortisol release in response to transportation (*Foreman* and *Ferlazzo* 1996, *Tischner* and *Niezgoda* 2000, *Söder* et al. 2012). To the best of our knowledge, only light-bred horses have been examined in the context of transporting. In general, cold-blood horses are less excitable than warm-blood horses. On the other hand, their high body mass requires constant balancing acts, as the vehicle changes speed and direction. Thus, transport can be more challenging for them than for light-bred horses.

The aim of this study was to evaluate the stress level in cold-blood horses of different ages, in response to transport over two different distances. The stress level was evaluated on the basis of cortisol concentrations determined in plasma and saliva samples taken before and after transport, and after post-transport rest. The usefulness of saliva and plasma samples for the determination of cortisol concentrations, indicating the stress level in transported cold-blood horses was also compared.

Materials and methods

This study was part of a larger project designed to investigate how stress caused by procedures associated with the use of horses affects horses. The study was conducted according to European Community regulations concerning the protection of experimental animals and in accordance with the rules of the Local Ethic Review Committee for Animal Experiments.

A total of 36 cold-blood mares, being sold and brought was chosen for the study. The authors' availability to study the horses was time-limited. Prior to the study, the horses had been pastured in familiar groups and brought to stables at night during the whole grazing period. They were not trained for work in harness or other equestrian sports. All horses were acquainted with humans and trained to stand and to be led with a rope. The horses had been handled frequently for routine veterinarian and breeding procedures such as grooming, hoof care etc. All horses were naïve and, they had never been transported before. During the study, they were moved from their stables to a local holding pen for temporary housing, which was unfamiliar for these horses. Some horses were moved on foot, while others were transported short distances in two-horse trailers. In the holding pen, they were kept in 3 x 4 m box stalls with straw bedding. Some other, unfamiliar horses were also moved to this stable. The studied horses spent at least five days in this place while waiting for transport. Then they were loaded onto a horse-truck and transported to another, unfamiliar place.

The horses were divided into four groups according to their age and the distance of transportation (Table 1). Twelve adult mares and 12 fillies were transported 50 km, which took one hour. The other two groups (six fillies and six adult mares) were transported 550 km, which took 12 hours. For transport, a commercial horse-truck with trailer was used. Both of the vehicles carried up to 12 horses in individual stalls. The horses were cross-tied individually in stalls during transport. Food and water were not available during transport. The horses were transported at night at a mean temperature of 13 °C. The horses were transported to new holding pens for temporary housing, where they were kept in stables and tied in individual stalls.

Four saliva samples and four blood samples were collected from each horse. All horses were sampled by the same operators: one person sampled saliva and the other collected blood samples. Both types of samples were taken according to the following protocol: 1) at rest, in the morning, in the stable, before moving the horses to the holding pens, 2) after five days spent in the holding pens, immediately before loading onto the truck, 3) immediately after unloading from the truck, 4) after restitution in the new place. This restitution lasted 12 hours for horses transported 50 km, and 24 hours for those transported 550 km. In each case, first saliva was sampled and then blood was taken. The saliva samples were collected with a small piece of sponge which was inserted into the horse's mouth and then, after soaking in saliva, it was placed in a plastic tube, as described previously (*Strzelec* et al. 2011). Blood samples were collected by jugular venipuncture into EDTA K3 tubes. The obtained blood was immediately centrifuged at 2000 × g for 10 min and the plasma was stored at -20 °C until assayed.

Before laboratory analysis, the saliva samples were centrifuged at 500 × g for 15 min at room temperature. Next, the sponge with the straw was removed and the saliva was transferred to test tubes. The concentrations of cortisol in saliva and plasma samples were measured by enzyme-immunoassay using the Cortisol Elisa kits (DRG Instruments GmbH, Marburg, Germany). All samples were analyzed in duplicate. The absorbance was measured by Multiscan reader (Labsystem, Helsinki, Finland) using a Genesis V 3.00 software program. The intra- and interassay CV for salivary cortisol determined in the laboratory amounted to 9% and 11% and for plasma cortisol concentration 5% and 8%, respectively. The results were expressed in ng/ml.

The results are presented as means ± standard deviation (SD). Statistical analyses were performed using the statistical software package GraphPad Prism™ (Graph Pad Software, La Jolla, CA, USA). Comparisons between the results obtained at rest, immediately before transportation and after unloading, and after post-transport rest in the studied groups were made by the Tukey test (two-way ANOVA). Statistical significance was accepted at $P < 0.05$.

Results

Mean plasma cortisol concentrations obtained in the study are presented in Table 2. A statistically significant increase in plasma cortisol concentration was found in all groups of horses after five days spent in the holding pens, as compared to values obtained at rest. Values of this hormone did not differ significantly in samples obtained immediately after unloading as compared to samples collected immediately before transport. In horses transported 50 km, cortisol concentrations determined after 12 hours of post-transport restitution were significantly higher than those obtained before transportation. There were no statistically significant differences between the results obtained in subsequent samples in fillies and adult mares (Table 2). In both distance related groups, a significant increase in salivary cortisol concentration was found in sam-

Table 1 Mean age and body weight of studied horses and technical conditions of their transport

Groups of horses	n	Age of horses (years)	Body weight (kg)	Duration of transport	Duration of rest after transport
			50 km		
Adult mares	12	11.3 ± 2.75	654 ± 26.1	1 hour	12 hour
Fillies	12	1.07 ± 0.21	402 ± 26.8	1 hour	12 hour
			550 km		
Adult mares	6	12.1 ± 2.37	641 ± 30.6	12 hours	24 hours
Fillies	6	1.18 ± 0.18	393 ± 23.5	12 hours	24 hours

ples taken immediately before and after loading, and after the post-transport rest, in comparison to samples taken at rest (Table 3). Moreover, salivary cortisol concentrations in fillies after 24 hours of post-transport restitution were significantly higher than the values found immediately before loading. There were no differences between the results obtained in subsequent samples in the age related groups and distance related groups.

Discussion

Transport induced increased plasma and salivary cortisol concentrations in the studied cold-blood horses and was similar to that described by other researchers in warm-blood horses (*Stull* and *Rodiek* 2000, *Fazio* et al. 2008a, 2013, *Schmidt* et al. 2010a,b,c). The means of plasma cortisol concentrations were characterised by high SD values. The large variations among individuals, reflected by high SD values, can be explained by the genetic conditioning of the horse and partially by their various response to transport. This phenomenon can also be the result of individual response to manipulation of blood collection. However, cortisol release is a time-dependent process; it takes almost 20 minutes to reach peak values in plasma after the stress stimulation (*Thompson* et al. 1988).

There were no distance-related differences in the obtained results. The relatively low increase in salivary and plasma cortisol concentrations and the lack of distance-related differences were surprising especially because the used transport conditions were disadvantageous for horses. It is known that water deprivation, cross-tying the horses during transport, and horses being transported for the first time increase stress levels and cortisol release (*Stull* 1999, *Stull* and *Rodiek* 2002, *Schmidt* et al. 2010b). The studied horses were transported under these conditions one hour or 12 hours, without significant differences in cortisol values measured immediately after transport. It should be mentioned that in other studies, the distance of transport also did not influence plasma or saliva cortisol concentrations determined just after the end of transport (*Fazio* et al. 2008a, *Schmidt* et al. 2010a,b,c). In fact, in a number of studies there was a significant increase in salivary or plasma cortisol concentrations only during the early stage of transport (*Stull* and *Rodiek* 2000, *Schmidt* et al. 2010a,b,c).

Results from cold-blood horses after post-transport rest clearly indicated that plasma and saliva cortisol levels did not decrease during the restitution period. The cortisol values determined 12 hours after transport 50 km as well as 24 hours after transport lasting 12 hours, were still on the same level as immediately after the end of transport. It is also known that three hours of post-transport rest is sufficient to return elevated salivary cortisol level to baseline value in naïve horses (*Schmidt* et al. 2010a, b, c). Some studies showed that the effect of transport can be seen even after 24 hours (*Stull* and *Rodiek* 2000, *Fazio* et al. 2008b, *Stull* et al. 2008, *Medica* et al. 2010). Nevertheless, the fact that plasma and salivary cortisol concentrations did not decrease during 12 and 24 hours after the end of transport indicates that this period was insufficient to restore the homeostasis of the studied horses. The horses were not studied for a longer time. Continuing the study was impossible for reasons independent of the authors. However, it can be stated that in contrast to light-bred horses (*Stull* et al. 2008, *Söder* et al. 2012), in cold-blood horses plasma and salivary cortisol concentrations, and thus stress levels, remained elevated 12 to 24 hours after transport. There could be at least two reasons for this phenomenon: 1) the horses were still under the influence of tiring and stressful transport, and/or 2) the new environment in the holding pen was stressful for the studied horses, which were still affected by unknown surroundings, such as unfamiliar stables, other unknown horses, caretakers, etc. It is not known if moving horses to an unfamiliar environment could be the reason for increased cortisol release, nevertheless, it involved their emotional arousal (*Janczarek* and *Kędzierski* 2011a,b).

It is worth noting that the important increase in cortisol concentrations in examined samples took place just before loa-

Table 2 Plasma cortisol concentrations in transported horses (Means ± SD; ng/ml)

Groups of horses	At rest	Just before loading	Just after unloading	After post-transport rest
		50 km		
Adult mares	65.4 ± 13.2^{a}	111 ± 85.2^{b}	131 ± 120^{bc}	134 ± 103^{c}
Fillies	59.5 ± 17.0^{a}	97.4 ± 56.7^{b}	121 ± 64.0^{bc}	153 ± 82.3^{c}
		550 km		
Adult mares	59.7 ± 21.3^{a}	83.4 ± 41.0^{b}	126 ± 98.5^{b}	141 ± 130^{b}
Fillies	42.6 ± 10.4^{a}	75.4 ± 21.3^{b}	119 ± 107^{b}	156 ± 151^{b}

Table 3 Salivary cortisol concentrations in transported horses (Means ± SD; ng/ml)

Groups of horses	At rest	Just before loading	Just after unloading	After post-transport rest
		50 km		
Adult mares	1.86 ± 1.21^{a}	7.49 ± 1.93^{b}	9.26 ± 2.47^{b}	8.81 ± 2.02^{b}
Fillies	1.56 ± 0.37^{a}	6.18 ± 3.53^{b}	7.51 ± 3.59^{bc}	9.84 ± 4.15^{c}
		550 km		
Adult mares	1.69 ± 0.96^{a}	6.49 ± 3.72^{b}	7.72 ± 3.86^{b}	11.2 ± 4.53^{b}
Fillies	2.14 ± 1.50^{a}	9.50 ± 3.81^{b}	12.1 ± 5.17^{b}	12.3 ± 5.39^{b}

ding the horses onto a horse-truck, after five days in an unfamiliar environment. This observation indicates that keeping the studied horses in a new, unknown holding pen resulted in increased cortisol release.

Cortisol concentrations did not differ between fillies and adult mares. The results of other studies indicate that cortisol release rate was higher in young horses than in older horses in response to exercise or ACTH stimulation (*Malinowski* et al. 2006, *Liburt* et al. 2013). On the other hand, *Tischner* and *Niezgoda* (2000) reported that transportation on day 9 after parturition was a stronger stress-causing factor for mares than for their foals.

In the study, both plasma and saliva samples were used for determination of cortisol concentration. While keeping the horses in a stable before transportation, cortisol concentration in plasma samples increased about 50–60%, whereas the concentration of this hormone in saliva samples increased about 4-fold. This means that cortisol concentration in saliva samples increased over a larger range than in plasma samples. Therefore, saliva samples seem to be better material than plasma samples for determination of the changes in cortisol release. Many other researchers used saliva samples instead of collecting plasma for cortisol determination as a measure of stress level in horses (*Schmidt* et al. 2010a,b,c, *Strzelec* et al. 2011, 2013, *Kędzierski* et al. 2014). Moreover, there is universal agreement that cortisol levels are one, easy measurable parameter for the judgement of animal welfare.

Conclusions

In naïve, cold-blood horses submitted to transportation, even 24 hours post-transport rest is insufficient to lower plasma and salivary cortisol concentrations to baseline values. The studied horses were more sensitive to changes of location than to transport. Saliva samples are more reliable material for determination of changes in cortisol concentration in transported cold-blood horses than plasma samples.

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Erweiterte Zusammenfassung

Einfluss des Transports über Land auf Speichel- und Plasmacortisol-Konzentratioinen beim Kaltblutpferd

Pferde werden oft zu Zuchtzwecken, für Wettkämpfe oder Verkauf und ebenso zum Schlachthof über unterschiedliche Distanzen transportiert. Es ist bekannt, dass der Transport der Pferde auf der Straße für diese Stress bedeutet und vermehrte Kortisolausschüttung hervorruft. Die Kortisolkonzentration im Speichel korreliert dabei mit den Blutwerten. Im zirkulierenden Blut sind Steroide an Plasmaproteine gebunden und nur 2% bis 15% des Kortisols ist ungebunden sowie biologisch aktiv. Nur diese nicht proteingebundene Kortisol-Fraktion erreicht den Speichel. Somit entspricht der Kortisolwert im Speichel der ungebundenen und biologisch aktiven Fraktion. So ist heute die Bestimmung des Kortisolwertes im Speichel eine allgemein akzeptierte Methode zur Beurteilung des Stresslevels beim Pferd. Allerdings sind rasse- und altersbedingte Unterschiede bei der Kortisolfreisetzung zu beachten. Im Allgemeinen sind Kaltblutpferde weniger leicht erregbar als Warmblüter. Allerdings könnte der Transport für diese Pferde aufgrund ihres hohen Körpergewichtes und des damit erforderlichen konstanten Ausbalancierens während des Transportes anstrengender sein als für Pferde leichter Rassen. Ziel dieser Studie war die Beurteilung des Stresslevels aufgrund von Über-Land-Transport über zwei verschiedene Distanzen bei Kaltblütern unterschiedlichen Alters.

Der Stresslevel wurde auf Basis der Kortisolkonzentrationen von Plasma- und Speichelproben genommen vor und nach Transport sowie nach einer Ruhephase nach dem Transport beurteilt. Insgesamt standen 36 Kaltblutstuten zur Verfügung. Die Pferde waren an den Menschen gewöhnt und trainiert am Strick geführt zu werden. Es handelte sich um Zuchtstuten, die an veterinärmedizinische Vorgänge gewöhnt, jedoch nie zuvor transportiert worden waren. Während der Studie wurden sie von ihrem Stall zu einem anderen gebracht, wo sie vorübergehend untergestellt wurden. Manche legten die Strecke zu Fuß zurück, andere wurden mit einem Pferdehänger transportiert. Dort wurden die Pferde in einer 3×4 m großen Box mit Stroheinstreu gehalten. Zusätzlich zu der Studienpopulation wurden weitere unbekannte Pferde in den Stall gebracht. Bis zu dem ersten längeren Transport verbrachten die Pferde 5 Tage in der Unterbringung. In Hinblick auf das Alter der Tiere und die Länge des Transportes (Tabelle 1) wurden die Pferde auf 4 Gruppen aufgeteilt. Zwölf adulte Stuten und 12 Jungstuten unterlagen einem Transport von 50 km, welcher sich über eine Stunde belief. Pferde der anderen zwei Gruppen(6 Jungstuten und 6 adulte Stuten) wurden 550 km transportiert. Dieser Transport dauerte 12 h. Für den Transport wurde ein kommerzieller Transporter mit Anhänger eingesetzt. Beide Fahrzeuge transportierten bis zu 12 Pferde in individuelle Ställe. Die Pferde waren im Transporter einzeln angebunden und hatten keinen Zugang zu Wasser oder Futter. Die Kaltblutstuten wurden bei Nacht und einer mittleren Temperatur von 13°C transportiert. Die Tiere wurden in der neuen Unterbringung aufgestallt und von jedem Pferd vier Speichel- und Blutproben genommen. Dies erfolgte von zwei Personen, von denen eine Person immer die Speichelproben und eine Person immer die Blutproben entnahm. Beide Probennahmen folgten einem Protokoll: 1) Ruhezeit, morgens, im Stall, vor dem Transport, 2) nach den fünf Tagen im neuen Stall direkt vor dem erneutem Transport, 3) direkt nach dem Abladen, 4) nach einer Erholungszeit im neuen Stall. Dieser Vorgang umfasste 12 h für diejenigen Pferde, die 50 km und 24 h für Tiere, die 550 km gefahren wurden. Die Konzentration des Kortisols in Speichel und Plasma wurde mittels Enzymimmunoassay bestimmt. Die Ergebnisse wurden in ng/ml erhoben. Die Vergleiche der Ergebnisse zum Ruhezeitpunkt, direkt vor dem Transport und danach sowie nach einer Ruhephase nach Transport erfolgten mit dem Tukey Test (ANOVA). Eine statistische Signifikanz galt ab $p<0,05$.

Ein statistisch signifikanter Anstieg des Plasmakortisols im Vergleich zum Ruhewert lag bei allen Gruppen nach fünf Tagen in der neuen Umgebung vor(Tabelle 2). Bei den über 50 km transportierten Pferden waren die Kortisolkonzentrationen erhoben 12 h nach Beendigung des Transportes signifikant höher als vor dem Transport. In beiden Transportgruppen wurde ein signifikanter Anstieg der Kortisolwerte im Speichel direkt vor sowie nach dem Transport als auch nach der Ruhephase nach Transport im Vergleich zu der ersten Probe in Ruhephase (Tabelle 3) festgestellt. Des Weiteren waren die Speichelwerte bei den Jungstuten 24 h nach dem Transport signifikant höher als direkt vor dem Transport. Die nachfolgend genommenen Proben unterschieden sich nicht aufgrund des Alters der Pferde. Auch konnten keine Unterschiede aufgrund der unterschiedlichen Transport-Distanz nachvollzogen werden. Der relativ geringe Anstieg der Kortisolkonzentrationen im Speichel und Plasma sowie der fehlende Einfluss der Transportlänge auf die Werte war überraschend, insbesondere da die Transportbedingungen für die Pferde ungewohnt waren. Die Ergebnisse zeigen, dass die Kortisolwerte während der Erholungsphase nach dem Transport nicht sanken. Die Tatsache, dass die Kortisolkonzentrationen im Plasma und Speichel 12 h und 24 h nach Beendigung des Transports nicht absanken, impliziert dass diese Zeitperiode für eine Wiederherstellung der Homöostase unzureichend ist. Entweder die Pferde standen weiterhin unter dem Einfluss des Transportstress und/oder es war die neue unbekannte Umgebung mit fremden Pferden und fremden Personal, die eine neu Stresssituation darstellte. Diese Beobachtung indiziert, dass der Aufenthalt in einem neuen Stall mit einer Kortisolfreisetzung einhergeht. In der Studie wurden zum einen Plasma als auch Speichel für die Bestimmung des Kortisols verwendet. Während des Stallaufenthaltes vor dem Transport stiegen die Kortisolwerte im Plasma um 50%–60%, wogegen die Konzentration des Kortisols im Speichel um das vierfache anstieg. Das bedeutet, dass die Kortisolkonzentration in Speichelproben im Vergleich zum Plasma auf einen höheren Level ansteigt. Somit scheint Speichel im Vergleich zum Plasma das bessere Probenmaterial zur Erfassung von Veränderungen der Kortisol-freisetzung zu sein. Die Ergebnisse lassen den Schluss zu, dass bei Kaltblutstuten nach einem Transport eine vierundzwanzigstündige Ruhephase nach dem Transport nicht ausreicht, um die Plasma- und Speichelkortisolkonzentrationen auf das Basisniveau absinken zu lassen. Die hier beurteilten Pferde reagierten sensibler auf Veränderungen der Unterbringung als auf den Transports. Speichelproben sind für die Beurteilung von Veränderungen der Kortisolkonzentrationen bei zuvor noch nie transportierten Kaltblütern das geeignetere Probenmaterial.

Schlüsselwörter: Kaltblutpferde, Cortisol, Transport, Tierschutz, Speichelprobe, Pferd