

BEHAVIOURAL AND PHYSIOLOGICAL STRESS RESPONSES TO TRANSPORTATION IN A GROUP OF PRZEWALSKI'S HORSES (*EQUUS FERUS PRZEWALSKII*)

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ABSTRACT

A variety of stimuli such as transportation can cause stress to animal. Stress is expressed differently by free-ranging, captive wildlife and domestic animals, and the response can be characteristic of the species. Via observation of behaviour and measurement of faecal glucocorticoid metabolites (FGM), our research focused on the capture and reintroduction of Przewalski's horses (*Equus ferus przewalskii*). The results showed that: (1) compared to pre-transportation behavior, one stallion and 5 females moved significantly more and significantly decreased the time spent standing, stand resting, and drinking. The stallion mark excretion decreased significantly; and other behaviors showed no significant change. (2) The average FGM for female horses rose significantly from 22 hr pre-transportation to 24 hr post-transportation; the average FGM of the stallion also rose at respective times, and then declined. Seventy-two hours following transportation, all values returned to their respective baseline levels. In light of these results, we advise that the animal should be attentively cared for that the day of and the day following transportation. The recovering period length of FGM level was more similar to domestic animals than to other wildlife, which might be caused by nearly one hundred years of captive breeding of these horses. We believe that our results could be the basic data set, which can be used to compare with future monitoring data. Stress response of the Przewalski's horse to transportation, as an artificial stimulus, can be used as a monitoring rewinding process method, which is also an indicator of the ultimately "rewilding".

Key words: behaviour; faecal glucocorticoid metabolites; Przewalski's horse; stress response; transportation stimulus.

INTRODUCTION

The stress response is an important physiological index in animals. Stress in mammals is a complex and multistage syndrome that is orchestrated by the sympathetic nervous system and glucocorticoids (Sapolsky, 2001). Short-term stress responses might help an animal handle a harsh environment; but long-term consequences can harm health (Sapolsky, 1998). Plasma glucocorticoid is a widely used measure of physiological stress (McDonald, 1980; Broom and Johnson, 1993; Hierbert *et al.*, 2000). Unfortunately, it requires repeated capture and anaesthetization and blood sampling, which, in itself, causes stress, so this sampling process is often not feasible for field studies (Millspaugh and Washburn, 2004). Concentration changes in blood can be reflected by corresponding glucocorticoid metabolites in feces (Graham and Brown, 1996), which allows for collection and analysis of feces, without disturbing the animals. Faecal glucocorticoid metabolites (FGM) measurement could serve as a non-invasive tool for monitoring animal adrenocortical activity, recently has been used in making assessment for wildlife and conservation (Goymann *et al.*, 1999; Terio *et al.*, 1999; Dehnhard *et al.*, 2001).

Reintroduction is one of the most important aspects of saving endangered species (Wakefield *et al.*, 2002). Reintroduction requires capturing and transportation to an unfamiliar environment, all of which are stressful. Faecal glucocorticoid measurement (FGM) is the main strategy to study stress of wild animals subjected to capture and transportation (Fazio *et al.*, 2003; Dembiec *et al.*, 2004; Franceschini *et al.*, 2008; Viljoen *et al.*, 2008). Compared with the livestock (Palme *et al.*, 2000; Dixit *et al.*, 2001; Fisher *et al.*, 2010), wild animals express longer stress response during the capture, handling and transportation (Goymann *et al.*, 1999; Terio *et al.*, 1999; Dehnhard *et al.*, 2001; Turner *et al.*, 2002). Stress seems to reflect a "wild character" status on certain species, until now there has been no research on this aspect. One of the essential signs of successful reintroduction is breeding of the subsequent generation. However, in the short term, recovery of alertness is obviously an important signal of successful re-wilding. Although there are a lot of reintroduction efforts on different species, lack of monitoring data accumulation is still a problem (Stanley Price, 1991), not to mention the study about how to evaluate the species re-wilding level.

Przewalski's horse (*Equus ferus przewalskii*) is considered the only remaining truly wild 'horse' in the world and may be the closest living wild relative of the domesticated horse. The horse is native to the steppes of central Asia, specifically Mongolia, and at one time was extinct since the mid-20th century in the wild (Mohr, 1971). Since 1986, Chinese researchers have been cultivating a captive population of Przewalski's horses, and the program has seen over twenty years of successful population expansion. Since August 2001, researchers began to successively reintroduce the group of captive horses into Mt. Kalamaili Ungulate Nature Reserve. Przewalski's horses have been bred in captivity for nearly a century (Boyd and Houpt, 1994); studies have rarely investigated the horse's stress response to transportation conditions. Here, we apply non-invasive methods to investigate the physiological reaction on the transportation, and observe pre- and post-transportation behaviour. It is not only related to the follow-up management and protection, but also can accumulate the basic data on the restoration of "wild character", which can also provide the important scientific basis for a guidance of the Przewalski's horse reintroduction.

MATERIALS AND METHODS

Animals and transportation: Six horses from the same group were sampled, 1 male and 5 females (Table 1). They were bred in the Wild Horse Breeding Centre in Xinjiang Uyghur Autonomous Region, China (89° 14' - 89° 36' E, 45° 49' - 46° 04' N). These six Przewalski's horses belong to one stable family group that has been successfully bred and raised offspring after artificially selecting and grouping. For the study, the horses were transported together by truck to a new release enclosure, located in the middle of the Kalamaili Nature Reserve (the enclosure area was about 1 x 0.5 km², 89° 26' 30.5" E, 45° 29' 44.3" N). The whole process of transportation and release occurred between 1:00 p.m. and 6:15 p.m. Beijing Standard Time on June 3, 2011. The total time for transportation and release was 5 hours and fifteen minutes. The horses had no prior transportation experience and they traveled in individual transportation stalls. During travel, horses were given neither food nor drink, and the temperature in the stall was maintained below 30 °C. After the horses were released into the new enclosure, they had access to grass and drinking water that had been prepared in advance. The study was approved by the Ethics and Animal Experimentation Committee of the Beijing Forestry University of Veterinary Sciences.

Behaviour: Before capture in the Wild Horse Breeding Centre and after release at the new release enclosure, the six horses were observed from distances of 50 – 100 m. Observations were made with binoculars¹ and took place

between May 30 and June 7, 2011 except June 3 when transportation started. The observation periods were restricted to daytime hours between 8:00 am and 8:00 pm. (Beijing Standard Time). The observation periods generally lasted six consecutive hours with alternating morning and afternoon observation periods to allow an even coverage over all hours, accumulated 8 days, totally 48 hours. The day length at the reintroduction site lasts 14-16 hours, local and Beijing time is 2 hours' time difference. Therefore, there was sufficient light during the behaviour observation and feces collection. Individual animal identification was done using both coat color and hip number. Using the instantaneous scan sampling method, as described by Altmann (1974), individual behaviours were recorded every 10 min. According to Boyd's protocol (Boyd, 1991), only the behaviours that lasted more than 3 s were recorded. Behaviours were classified using definitions coined by Feist and McCullough (1976) and Boyd and Houpt (1994). All behaviours were grouped into nine main activities: grazing, drinking, grooming, stand resting, lying recumbent, moving, marking, standing, and other behaviours (Table 2).

Sample collection: Animals were observed through binoculars from a distance; defecation was noted and samples collected after the horses moved away. Feces were collected 24 hours before transportation, and then and at each defecation event over a period of 75 hours following the initial collection. No sample was collected while the study horses were in respective stalls, because the feces were stepped on and mixed in with urine. Forty-five samples were collected. The collection times were: 22 hours and 3 hours before transportation, 5, 24, 36, 48, and 72 hours after transportation. Fresh samples were thoroughly mixed. Subsequently, about 12 g of the mixed samples² were placed in 95 % ethanol (2.5 ml ethanol: 1 g feces), sealed in jars with vapor-proof screw-top lids, and preserved below 25 °C (Cavigelli, 1999, Curtis *et al.*, 2000).

Extraction of glucocorticoid (GC) and radioimmunoassay: Glucocorticoid measures were conducted following established methods (Möstl *et al.*, 1999; Wasser and Hunt 2003; Galama *et al.*, 2004; Morato *et al.*, 2004), with some modifications. The ethanol in the samples was evaporated in a fume hood overnight at room temperature prior to desiccation. The samples were then dried in a conventional oven at 95 °C for 4 hr; then 0.5 g pulverized sample was boiled in 10 ml of 80 % methanol for 20 min. Following centrifugation at 3,500 r/min for 20 min, the supernatants were obtained. Pellets were then re-suspended in an additional 5 ml of 80 % methanol, vortexed, and centrifuged again for 15 min. The combined supernatants were then evaporated in boiled water (60 °C) until dry, re-dissolved in 1 ml methanol and stored at -20 °C until assay.

Table 1. The family of Przewalski's horses for release

Identification	Sex	Birth
Z3311		June 2003
Z215		May 2000
Z213		May 2002
Z231		May 2003
Z174		May 2004
Z191		May 2004

Table 2: Ethogram based on Feist and McCullough (1976) and Boyd and Houpt(1994).

Behavior	Description
Grazing:	The horse has its head down, and shows chewing and biting mouth movements. The horse may stand still or walk slowly.
Drinking	There is ingestion of water, typically using lips, at or slightly below the surface of water; drawing water with sucking action through slightly parted lips and teeth, and swallowing.
Grooming	A number of behaviors directed at skin and coat care. These can be performed individually or in groups, and they usually including rolling, shaking, auto grooming, mutual grooming, and so on.
Stand Resting	Horse is standing and relaxed. The neck can be horizontal or low, the head relaxed and a little low or in the continuation of the neck, the nose is directed toward the ground, and the eyes may be closed or half-shut. The ears are in a lateral position or directed behind, and one of the three hind legs is flexed.
Lying Recumbent	The horse is resting or sleeping while lying down with head up or with legs and head outstretched.
Moving	The horse is walking, trotting, or galloping with a minimum duration of 10 s. The horse is not grazing.
Marking	This is usually performed by a stallion after he sniffs urine or feces. He typically steps over the excretion and marks it with urine or feces.
Standing	The horse is standing, with a sustained position. All legs are stretched. The head is high and the neck is held with tension. The ears are directed forward or are moving to locate surrounding sounds.

Other behaviors This includes all other behaviors, e.g. social behaviors, urination, defecation, and interaction with other Przewalski's horse groups or other species.

The radioimmuno assays of the sample supernatants were conducted by the BNIBT³. A modified Cortisol Kit for Rats and Mice, and a Model SN-682 RIA counter were used to assay the FGM concentration (Wasser *et al.*, 2000). The assay parameters were as follows: measuring range: 10 - 500 ng/ml; sensitivity: 2.0 ng/ml; intra-assay coefficients of variation < 10 %; inter-assay coefficients of variation < 15 %; and recovery: 98 %. All hormone values are expressed as ng/g of dry feces.

Data analysis: Statistical analyses were performed using the SPSS⁴11.5. The change between pre- and post-transportation faecal glucocorticoid metabolite concentrations was analyzed using the One-way ANOVA test. Significance levels for all tests were =0.05. Means and standard errors are given unless otherwise noted.

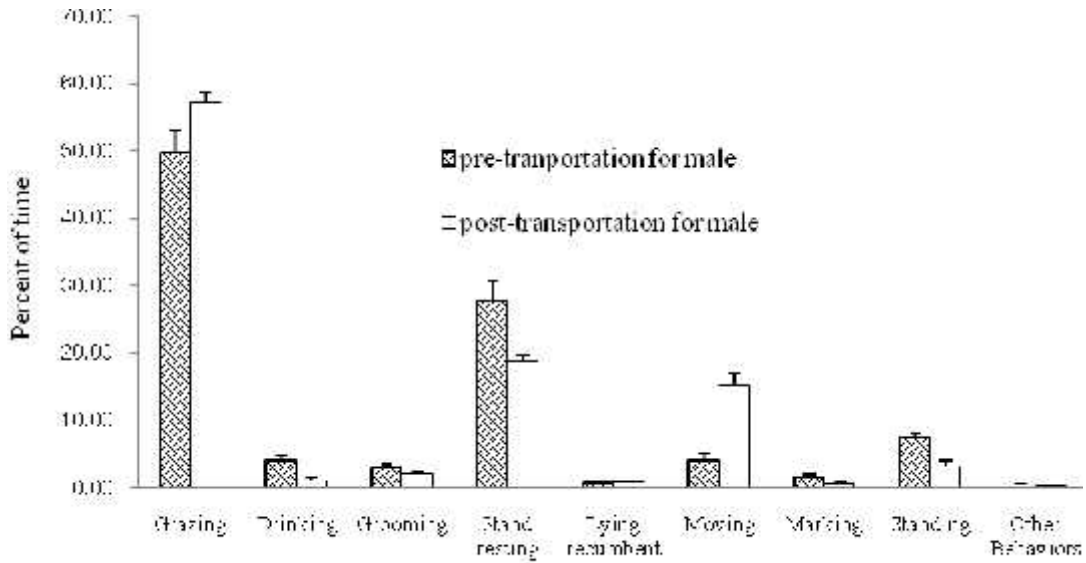
Because of their mating system, the family group allows just one male. For faecal glucocorticoid metabolite level, only the female horses were used to avoid pseudo replication. However, behavioural time budget belongs to time series data, which means that we can get enough data during observing period, 8 days. Therefore, all horses were observed.

RESULTS

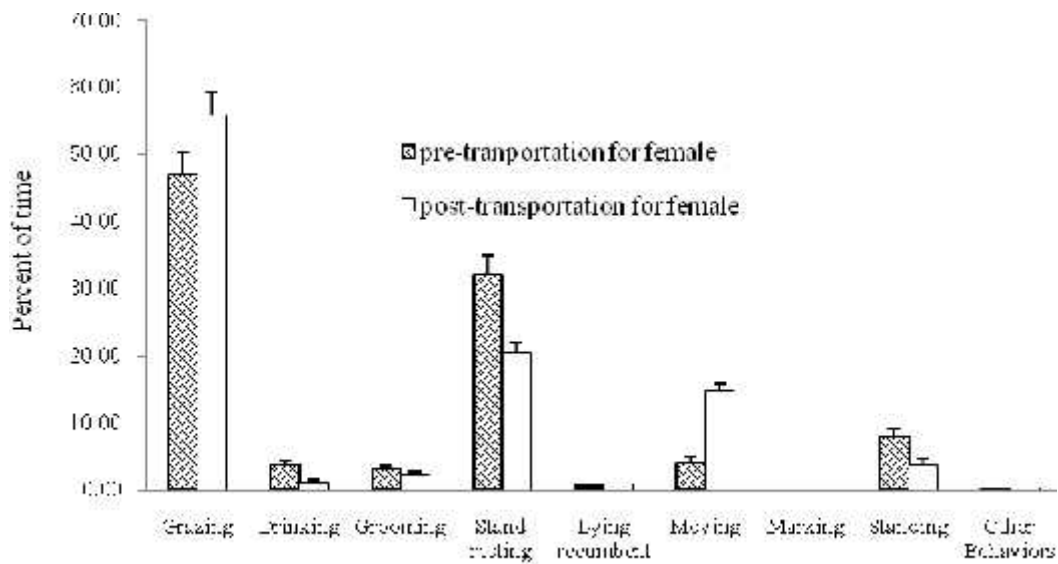
Time budget and activity patterns: Although the duration time and frequency of certain behaviours changed following transportation, we observed no new behaviours.

For the male, moving increased significantly ($p=0.005$), but grazing and lying recumbent did not ($p=0.100$ and $p=0.224$), respectively. There were significant decreases for standing ($p=0.012$), stand resting ($p=0.036$), drinking ($p=0.012$), and marking ($p=0.009$) behaviours. However, there were no significant differences for grooming ($p=0.205$) or other behaviours ($p=0.198$) (Figure 1. A).

In female horses, moving significantly increased ($p=0.002$), but no significant time difference for grazing ($p=0.127$) or lying recumbent ($p=0.175$). There were significant decreases for standing ($p=0.025$), stand resting ($p=0.020$), and drinking ($p=0.020$) and there were no significant differences for grooming ($p=0.228$) or other behaviours ($p=0.081$). (Figure 1. B).



(A) Behavioral Types for Male Horse



(B) Behavioral Types for 5 Female Horses

Fig1. The time budget changes of Przewalski’s horses’ behaviors during pre- and post-transportation (A) one male (B) 5 females.

Faecal glucocorticoid metabolites: For the stallion, the FGM was 22.94 ng/g dry feces 22hours before transportation, and following transportation, it began to increase (Figure 2). The maximum FGM value, 51.98 ng/g dry feces, occurred approximately 24 hours after transportation, a value nearly 2.3 times higher than that before transportation. Following this peak, the FGM began to decrease and approximately 72 hours following transportation, the stallion FGM was at a level similar to the pre-transportation value.

For the six females, there was a variation between the pre- and post-transportation mean FGM. The baseline FGM was 39.65±2.10 ng/g dry feces; a little higher than the corresponding value for the stallion. Twenty-four hours following transportation, FGM began to increase, and the average maximum value was 91.25±5.96 ng/g dry feces; this was also 2.3 times higher than the baseline value ($p < 0.01$). Following this peak, the FGM began to decrease and about 72 hours following transportation, FGM was 27.84±1.73 ng/g dry

feces, which was no significantly lower than the pre- transportation value.

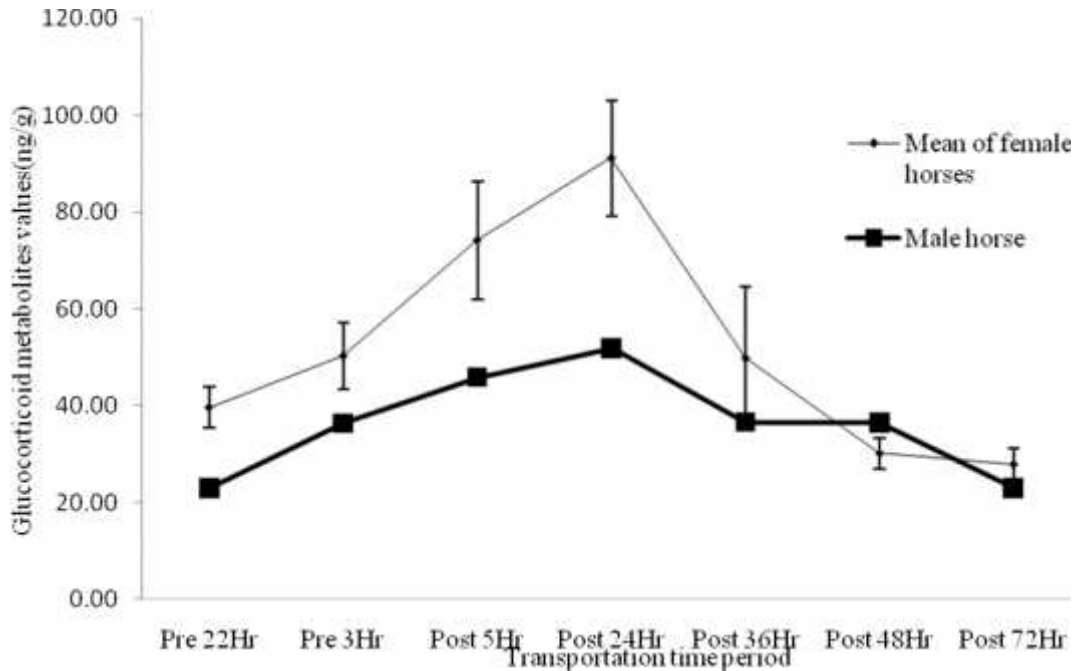


Fig2. The concentration changes of faecal GC metabolites of Przewalski's horse during pre- and post-transportation

DISCUSSION

The male and female Przewalski's horses demonstrated similar behavioural responses to transportation. Increased time allocation for post-transportation moving behaviour corresponded to a decreased time allocation for post-transportation standing and stand resting behaviour. The increased time allocation for moving behaviours was caused by transportation, and was likely a result of the decrease in drinking and marking (refer in particular to stallion). Transportation can result in animal fatigue (Dixit *et al.*, 2001; Friend, 2001; Dembiec *et al.*, 2004; Fisher *et al.*, 2010), and it appeared that the Przewalski's horses' grazing behavior increased slightly, perhaps as a result of transportation fatigue. On one hand, transportation stimulus may not have caused intense behavioural change. At the same time, energy consumption and fatigue experienced during transportation led to the increase in grazing and lying recumbent behaviours following transportation. Boyd (1998) observed that after Przewalski's horses were released into an enclosure, moving time increased; and our results demonstrated the same. We also found that after the Przewalski's horses entered an unfamiliar environment, such as the enclosure, that sharp decreases in standing, stand resting, and drinking were observed. This was likely due to the increased time spent on exploration of the new

environment. Overall, we concluded that following transportation, the Przewalski's horse expressed behavioural change, but only to a certain extent. The adaption to captivity may have weakened the behavioural response to transportation. So, as time following the reintroduction to the wild increases, the behavioural response of the Przewalski's horse to such an external stimulus might be enhanced. Thus, the behavioural response to external stimuli may be used as a parameter for evaluating the progress of animal reintroduction to the wild.

Both male and female horses showed similar faecal Glucocorticoid value changes, and both exhibited peak Glucocorticoid values 24 hours after transportation. Thus, all of the Przewalski's horses in this study expressed similar stress reactions. Plame *et al.* (2000) demonstrated that faecal Glucocorticoid in cattle significantly increased 8 to 16 hours following transportation; however, 26 to 48 hours following transportation faecal Glucocorticoid recovered to its normal level. Fisher *et al.* (2010) studied the physiological and behavioural responses of sheep to transportation, and also discovered that the concentration of glucocorticoid in blood sharply increased as transportation time increased and returned to normal 72 hours following transportation. Schmidt *et al.* (2010) studied cortisol release and heart rate variability among horses and showed an increase in the post-transportation period that lasted for 48 hours. Our results are similar.

We showed that faecal Glucocorticoid values of Przewalski's horses increased soon after they were transported, and the values returned to normal an average of 72 hours later.

The long term effects, however, may still linger. Dembiec *et al.* (2004) demonstrated that transportation as short as 30 min could affect tigers physiologically for up to 9 to 12 days. Viljoen *et al.* (2008) reported similar results on the translocation stress and FGM levels in free-ranging African savanna elephants (*Loxodonta africana*); FGM values remained at a higher level for up to 23 days following transportation. Turner *et al.* (2002) noted that the faecal Glucocorticoid of rhinoceros (*Diceros bicornis*) returned to the baseline value 4 to 6 weeks after transportation. Similar results were shown by Franceschini *et al.* (2008); for Grevy's zebra (*Equus grevyi*), the faecal Glucocorticoid returned to its baseline value 11 to 18 weeks following transportation and release into nature. Hence, it seems possible that wild life tend to be more physiologically sensitive to transportation stress than domestic animals. A lengthy captivity time may have weakened the stress response of the Przewalski's horse, so its reaction is similar to that of domestic animals. Our results could therefore, provide reliable and powerful data aid in the evaluation of the introduction of Przewalski's horse, particularly in relation to the transportation stress responses.

In this study, the peak FGM concentration occurred at 24 hours after transportation. An average time lag of 22 hours occurred in bear *Ursus arctos* (Wasser and Hunt, 2003), 23.8 hours for dog (Schatz and Palme 2001), 24.5 hours for giant panda (*Ailuropodamel anoleuca*) (Li, 2005), and 48 hours for cheetah (*Acinonyx jubatus*) (Terio *et al.*, 1999). The time lags, which ranged from a few hours to several days (Whitten *et al.*, 1998), revealed that there were significant differences among species, which is likely due to different gastrointestinal transit times. The time lag between the stressor and the maximum response for in this study was 24 hours. This is in agreement with the findings of Östl *et al.* (1999), who showed that in domestic horses the highest FGM concentrations occurred about 1 day after administration of ACTH.

The FGM concentration in female horses was higher than that in the stallion, both pre-transportation and 24 hours post-transportation. A possible reason for this difference is a higher sensitivity to transportation in female horses, which would lead to a higher peak FGM concentration in females. Following our research results, we propose that physiologically, the day and the day after transportation could be considered as the peak transportation stress response period, for the Przewalski's horse. During this period of sensitivity, care workers should attentively manage and protect the horse so that accidental injury to the animals is avoided.

It seems that the Przewalski's horses were well adapted to nearly 5 hours transportation stimulus. Cortisol concentration during transport has been suggested to be positively correlated with transport time (Fazio and Ferlazzo, 2003), it is worthy to do further research on prolonging the transportation time or the distance, whether the Przewalski's horse could have a resistance to the stimulus. This information could improve future reintroduction protocols.

Conclusions: We offer the following conclusions. (1) The evaluation of transportation stress for the Przewalski's horse, as observed by behaviour and FGM, showed that the Przewalski's horse had some behavioural and physiological responses to transportation stress. (2) We propose that the day of and the day after transportation should be considered of the period of peak physiological sensitivity to transportation stress; during this time, care workers should attentively manage and protect the horses. (3) Behavioural observation and non-invasive FGM measurement could be introduced as a monitoring method for the Przewalski's horse to evaluate reintroduction progress; (4) our results could be the basic data set, which can be used to compare with future monitoring data. Stress response of the Przewalski's horse to transportation, as an artificial stimulus, can be used as a monitoring rewinding process method, which is also an indicator of the ultimately "rewilding".

¹ Bushnell Nature view 8×42 mm Binoculars 220842, Bushnell Corporation, U.S.A.

² Mixed samples: We mixed the fecal samples every time (22 hours and 3 hours before transportation, 5, 24, 36, 48, and 72 hours after transportation) when we collected for each individual.

³ Beijing North Institute of Biological Technology.

⁴ SPSS 11.5, Chicago, Illinois, USA.

Acknowledgements: We are grateful to Prof. Dong Zhang (Beijing Forestry University, Beijing, China) and Yu-Nan Su (Renmin University of China, Beijing, China) for their invaluable assistance with this study. We thank the Wild Horse Breeding Centre in Xinjiang Province for their help and support. This work was financed by the National Natural Science Foundation (No.30970545) and Special Fund for Forestry Scientific Research in the Public Interest, China's Ministry of Science and Technology (No. 201004054).

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