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Faecal cortisol metabolites in Quarter Horses during initial training under field conditions

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Summary

The first month of training of a young horse is suspected to be stressful, but the endocrine responses to initial training are unknown. Therefore in our study a total of 40 Quarter Horses (QH), all at the age of almost 2 years, were followed during the first 30 days of their training. During this time faecal samples were collected twice daily and faecal cortisol metabolites (FCM) were measured.

Baseline values of FCM ranged between 1.3 and 20.1 (median: 6.7) ng/g faeces. No differences in FCM values between days of training were found. Mares showed the highest values. Significant diurnal variations were observed in mares (p=0.035) and stallions (p=0.003), but not in geldings (p=0.282). As in this study adrenocortical activity was not increased during initial training, horses seem to cope very well with this new situation.

The results of our large-scale study provide basic physiological data about initial training. This gives additional input in an emotional debate about animal welfare aspects of first time handling and training of horses.

Abbreviations: 11,17-DOA = 11,17-dioxoandrostanes; EIA = Enyzme Immunoassay; FCM = faecal cortisol metabolites; GC = glucocorticoids; HPA-axis = hypothalamic-pituitary-adrenocortical-axis; QH = Quarter Horses

Schlüsselwörter: Pferd, Stress, Nebennierenrindenaktivität, Westernreiten, nicht-invasiv.

Zusammenfassung

Messung der Kortisolmetaboliten im Pferdekot während der Grundausbildung von 2jährigen Quarter Horses

Das Einreiten eines jungen Pferdes steht unter Verdacht belastend zu sein. Bisher gibt es aber keine Veröffentlichungen über endokrine Vorgänge während dieser Phase. Mit der vorliegenden Studie wurde überprüft, ob Pferde aufgrund physischer und psychischer Belastungen während des Trainings höhere Konzentrationen an Kortisolmetaboliten im Kot (FCM) aufweisen. Es wurden dazu 40 Quarter Horses im Alter von 2 Jahren während der ersten 30 Tage der Grundausbildung des Westernreitens beobachtet und ihre FCM Werte gemessen. Während dieser Zeitspanne wurden täglich morgens und abends Kotproben der Pferde genommen. Die Basalwerte der FCM Konzentration variierten zwischen 1,3 und 20,1 (Median: 6,7) ng/g Kot, wobei Stuten die höchsten Werte hatten. Signifikante Unterschiede während der einzelnen Trainingstage konnten nicht festgestellt werden. In der Tagesrhythmik wurden signifikante Unterschiede bei Stuten (p=0,035) und bei Hengsten (p=0,003), jedoch nicht bei Wallachen (p=0,282) ermittelt.

In dieser Studie konnte keine erhöhte Aktivität der Nebennierenrinde im Verlauf der Grundausbildung eines Pferdes im Westernreitstil festgestellt werden. Das legt nahe, dass Pferde mit dieser neuen, zeitlich kurz andauernden Situationen gut zurechtkommen. Unsere Studie wurde an einer großen Anzahl von Tieren unter Feldbedingungen durchgeführt. Sie bietet daher eine gute Datenbasis über Belastungen während des Einreitens. Damit liefert sie einen zusätzlichen Beitrag zu einer mitunter emotional geführten Debatte über tierschutzrelevante Aspekte bei der Grundausbildung von Pferden.

Introduction

Almost all horses between 2-4 years of age experience some kind of initial training. The concept of training horses is well known and widespread. Untrained horses have to learn how to balance their own body mass as well as the weight of saddle and rider in the basic gaits of walking, trotting and cantering. Endocrine responses to exercise have been widely reported (CHURCH et al., 1987; NAGATA et al., 1999), but never under field conditions and with completely untrained and inexperienced horses. To our knowledge, no study has ever investigated to what extent horses are stressed from being unbroken to mastering the understanding of the principles of the basic gaits in the field. In most publications, hormonal changes through exercise were examined in the Standardbred and Thoroughbred horse (JIMENEZ et al., 1998; HADA et al., 2003). In this study, we used Quarter Horses. This breed is mainly used for Western riding, especially cow work. It is very common to start training Quarter Horses at the age of 2 years.

Besides other responses, stress increases glucocorticoid (GC) secretion by the adrenal cortex (MÖSTL and PALME, 2002; TOUMA and PALME, 2005). In the horse, cortisol, the main GC, is secreted in a circadian rhythm, with higher concentrations in the morning. In addition, concentrations are affected by episodic fluctuations and adaptation of the horse to its environment (IRVINE and ALEXANDER, 1994). Cortisol levels in blood are used as an indicator of stress (COVALESKY et al., 1992; TERLOUW et al., 1997; MÖSTL and PALME, 2002). However, blood sampling itself causes stress for the animals. In contrast, faecal samples can be collected easily. Even repeated sampling is possible without stressing the animals and will not disturb the endocrine status of the individuals. Therefore, non-invasive methods for the determination of GC metabolites in faeces were established in an increasing number of species (PALME et al., 1996; MÖSTL and PALME, 2002; TOUMA and PALME, 2005). In ponies, 41 % of infused radioactive cortisol is excreted via the faeces as metabolites (PALME et al., 1996). The authors describe a 24 h lag-time between peaks of radioactivity in blood and in faeces. This delay time in the faeces was also reflected by measured faecal cortisol metabolites (FCM) after ACTH injections (MÖSTL et al., 1999). An 11-oxoaetiocholanolone-EIA (PALME and MÖSTL, 1997) was successfully established to measure a group of FCMs. This non-invasive method has been physiologically validated in horses (MÖSTL et al., 1999; PALME, 2005). Several situations, such as painful events, transportation and gynaecological examinations in horses (MERL et al., 2000; BERGHOLD et al., 2007) or transportation, new surroundings and manipulations in cattle (MÖSTL et al., 1999; PALME et al., 2000; PESENHOFER et al., 2006) were reported to increase FCM.

The aim of this study was to evaluate exercise and psychologically induced endocrine responses, related to a new environment and the experience of the riding part itself, in young, untrained Quarter Horses under field conditions by measuring the concentrations of FCM. Our hypothesis that horses are stressed, while being trained for the first time, should be tested.

Materials and methods

Animals

A total of 40 Quarter horses (19 mares, 12 geldings and 9 stallions), all at the age of 19 to 22 months (mean: 20.6 months), were used for this study, which was performed in California, USA. One of the males was castrated on Day 16 and was not in training for 6 days. The training of the horses started on the same day (Day 1) and lasted for 30 days. All animals were clinically healthy at the beginning of the study. None of them had been trained before. Training was held in January 2005. Average temperature during the day was 9° C. The horses were transported to the 2 training facilities 2 weeks to 2 days before training started. Horses were kept in box stalls, which measured either 4 m by 8 m or 3 m by 6 m. The animals had visual contact with each other. They were fed a sufficient diet of alfalfa hay and commercially pelleted feed to maintain good body condition and had ad libitum access to water.

Facilities and equipment

All training sessions were held at 2 Western riding training facilities in Merced County, California. Training took place in an indoor arena, approximately 12 m in diameter, as well as in an outdoor arena that is 60 x 30 m. The flooring material throughout all the arenas was a mixture of sand and clay. Equipment used for initial training of the horses included a halter, saddle pad, bridle with a jointed snaffle bit and reins and western style saddles.

Training procedure

All horses were trained according to conventional cow work (Western riding style) procedures and completed the same amount of work at the same intensity. Training was held 6 days a week and for an individual horse was always performed at the same time of day. Training lasted 20 minutes on average. The horses had to learn to control their balance, their own body mass and the weight of saddle and rider while walking, trotting and cantering. In addition, they were tied up to the walker just to get used to being tied up and standing still.

On the first day the horses had to become confident in the trainer. They had to get used to the saddle pad, a saddle and a bridle. On the second day the horses had to learn to stand still and be mounted and dismounted by the trainer. After that, they were taken into the indoor arena to learn to balance with the weight of the saddle on their back in every gait. After about 15 minutes, when they felt comfortable with it, a trainer mounted the horse. For the next 3 to 5 days, the same procedure was repeated. From the second week onwards, horses were ridden in the bigger and open outside arena. There they were encouraged to move forward, stop and back up on command. Horses were walked, trotted and cantered. At the end of the third week they were ridden in the open field 2 to 3 times a week to get used to a novel environment. 5 trainers exercised all horses in the 2 participating training facilities. All were experienced, active western riding trainers.

Sampling

The first faecal samples were collected in the afternoon (Day 0) before training started. Each day during the first week, sampling was performed twice a day. Following the suggestion of TOUMA and PALME (2005) that sampling should be at the same time each day, collecting times were between 0700 h and 0900 h in the morning and between 1600 h and 1800 h in the afternoon. During the following 24 days, samples were picked up every third day twice a day at the same times as above. The sequence of the horses was the same for sampling and training. Within 30 minutes after defaecation, a handful of the inner part of the freshest faecal ball was taken directly from the bedding to standardize sample collections of FCM. Samples were thoroughly homogenized and put into Eppendorf tubes. Tubes were frozen immediately after collection at -24 °C. Samples were shipped by air on dry ice to the University of Veterinary Medicine Vienna for analysis.

Measurement of faecal cortisol metabolites (FCM)

To measure FCM in horses, we followed the protocol described earlier (MERL et al., 2000). Briefly, 0.5 g faeces were suspended in 4 ml methanol and 1 ml water (=80 % methanol) and shaken for 30 minutes. After centrifugation

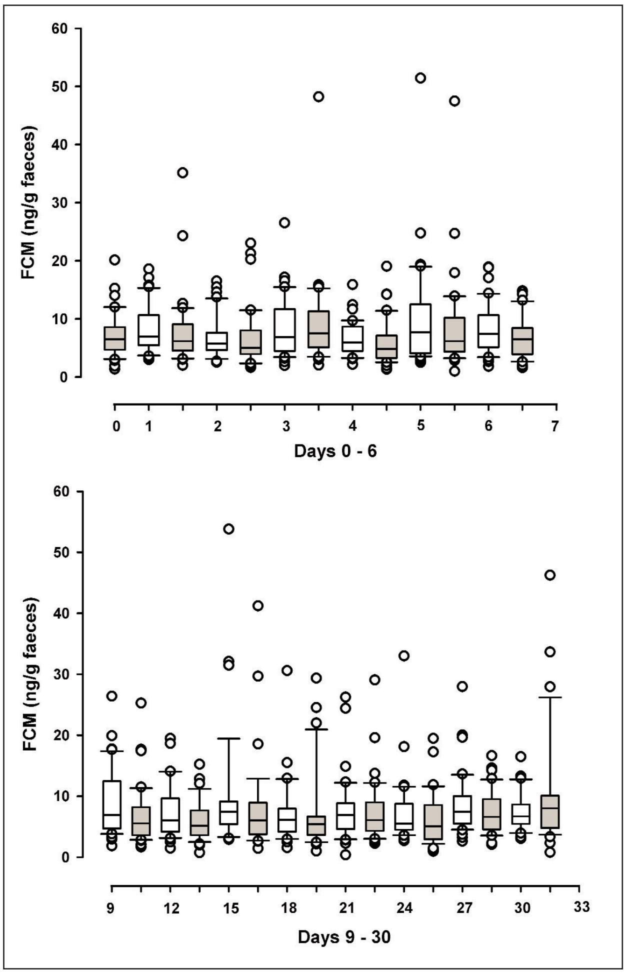

Fig. 1: Concentrations of faecal cortisol metabolites (FCM) in morning (white boxes) and evening (grey boxes) samples of Quarter Horses during the first 30 days of initial training; data are given as boxplot diagrams showing medians (lines in the boxes), 25 % and 75 % quartiles (boxes), 10 % and 90 % ranges (whiskers) and outliers (circles).

(2.500 g for 15 min), 1 ml of the supernatant was mixed with 4 ml diethylether and 0.25 ml 5 % $NaHCO_3$ in a new vial.

Samples were turned upside down 4 times and then vortexed for 10 seconds. The aqueous phase was frozen at -24 °C. Then the ether was decanted and dried down with a stream of nitrogen. The extract was redissolved in assay buffer (0.5 ml). Concentrations of FCM were measured with an 11-oxoaetiocholanolone EIA as described earlier (PALME and MÖSTL, 1997). This EIA, which has been physiologically and biologically validated in horses (MÖSTL et al., 1999; MERL et al., 2000), measures 11,17-dioxoandrostanes (11,17-DOA), a group of faecal cortisol metabolites.

Statistical analyses

The obtained data were statistically analyzed (software package SPSS, version 10.0, SPSS Inc., Chicago, USA) by analysis of variance (ANOVA) for repeated measures, followed by Dunnett's test at the 0.05 level, when the F value was significant. As the data were not normally distributed on 9 out of 30 sampling time points (Kolmogorov-Smirnov test), data are shown as boxplot diagrams (Fig. 1). Since the ANOVA for repeated measurements is robust against deviations from normal distribution, this will lead to small and tolerable deviations (see LINDMAN, 1974). To be on the safe side, data were also analysed with the Friedman's test, which however led to the same results. Median morning and evening FCM concentrations were calculated for each individual. They were used to compare morning and evening levels in mares, stallions and geldings with a t-test for paired samples using SIGMA-STAT© (Jandel GmbH, Erkrath, Germany). From Day 16 to Day 21 data from one male were excluded after it was castrated.

Results

Basal values of FCM before training started (on Day 0 in the evening and Day 1 in the morning) ranged between 1.3 and 20.1 ng/g (median: 6.7 ng/g) faeces. They showed high individual variations. No differences in FCM concentrations between days of training were present (Fig. 1), neither in morning nor in evening values. One male was castrated on Day 16. It was not in training for the following 6 days. In this individual increased values were found from Day 18 (30.6 ng/g in the morning and 29.4 ng/g in the evening) until the morning of Day 21 (12.3 ng/g). Afterwards values decreased again to pre-operative values (2.9 ng/g).

Significant diurnal variations of GC metabolites were observed in mares. Median values were 7.4 ± 0.6 ng/g (mean ± SE) in the mornings and 6.5 ± 0.4 ng/g in the evenings ($p = 0.035$). Stallions had also statistically significant ($p = 0.003$) higher median values (6.9 ± 0.8 ng/g) in the mornings as compared with concentrations (5.1 ± 0.6 ng/g) in the evenings. There were no significant diurnal differences ($p = 0.282$) found in geldings (6.5 ± 0.7 ng/g and 6.1 ± 0.7 ng/g, respectively). Besides, mares had a tendency toward higher values (although not statistically significant) as compared with stallions and geldings.

Discussion

This study examined changes in faecal cortisol metabolites (FCM), and thus adrenocortical activity, in horses in relation to the experience of initial training under field conditions with all its physiological and psychological components. Thereby we evaluated how young horses handle new situations such as basic training. Since we aimed to record adrenocortical activity over a longer period without disturbing the animal, we chose a non-invasive sampling method. FCM were measured with an 11-oxoaetiocholanolone EIA (PALME and MÖSTL, 1997). This method has been successfully validated and applied in horses (MÖSTL et al., 1999; MERL et al., 2000; TOUMA and PALME, 2005; BERGHOLD et al., 2007).

As transport causes stress for the animals, it was important that the horses in our study were brought to the training facilities at least 2 days in advance. Therefore evening values on Day 0 and morning concentrations of Day 1 reflected undisturbed basal levels. The measured basal values of FCM, ranging between 1.3 and 20.1 ng/g (median: 6.7 ng/g) faeces, were lower than those reported in previous studies (MÖSTL et al., 1999; MERL et al., 2000). That may indicate breed related differences in FCM values or reflect better housing conditions. Most box stalls are typically between 6 and 10 m² (RAMMERSTORFER et al., 1998; RIVERA et al., 2002) in size. The horses used in this study had the advantage of being in 18-32 m² sized stalls. Although there was no extra access to the pasture, the horses could move and jump around much better than in most of the commercial-sized box stalls. High individual variation in FCM concentrations, which were also reported earlier (MÖSTL et al., 1999), was similar to that described for cortisol concentrations in blood (CHURCH et al., 1987; JIMENEZ et al., 1998; MÖSTL et al., 1999).

Information about the influence of the diurnal rhythm and gender-specific effects could be of high relevance for studies investigating faecal hormone metabolites in horses as well as in other species (TOUMA and PALME, 2005). Well-defined circadian rhythms of plasma GCs have been described in horses (IRVINE and ALEXANDER, 1994). In our study higher concentrations of FCM were also found in the morning. Mares and stallions, but not geldings, showed significant differences between morning and evening values. This may indicate some sort of influence exerted by gonadal steroids on the hypothalamic-pituitary-adrenocortical (HPA)-axis. To our knowledge, there are not many other studies, which have investigated gender-specific differences in plasma cortisol concentrations in horses. COLBORN et al. (1991) reported that the cortisol response to acute exercise did not seem to be gonadally dependent, because it occurred similarly in stallions and geldings. That study did not examine the circadian rhythm of cortisol. Horses were only examined for 5 minutes with 2 days rest between the exercises. Mares showed a tendency toward higher FCM values, although it was not statistically significant. Higher FCM concentrations are in agreement with the data of PALME et al. (1996), who found a higher percentage of cortisol metabolites excreted in mares' faeces.

We assumed that the horses would have higher FCM concentrations in the beginning of the training, but then would adapt and show lower values afterwards. This hypothesis could not be confirmed. In the present study, elevated FCM were not observed at all during initial training in the 2 participating training facilities. Compared to others (FREESTONE et al., 1991; NAGATA et al., 1999; MARC et al., 2000), in our study a larger group of the same breed and same age was used. Therefore, our results should reflect the impact of training on these horses more reliably. There was no kind of pre-training period as in most studies, where exercise-induced changes in cortisol concentrations were observed (MARC et al., 2000). Our horses were not used to walking on a mechanical walker, or to any other kind of training. Even the beginning of the training, when the horses were saddled and mounted and wore a halter for the first time, did not elevate FCM values significantly. The horses probably adjusted to the new situation quickly. Therefore the HPA axis was not much stimulated. Also in weeks 3 and 4, no significantly increased values were measured.

Our results suggest that horses under the stimuli of a short training period, performed at the same intensity every day, are not stressed to such an extent that increased activity of the HPA axis is reflected by FCM measurements. Most studies investigating exercise-induced changes have been done with the help of a treadmill. Even though that is a good and exact method for measuring metabolic responses, there is no definitive conclusion as to how horses respond under field conditions. Cortisol responses to exercise depend on exercise duration (NAGATA et al., 1999). Peak plasma cortisol concentrations were found 30 minutes after completion of exercise (CHURCH et al., 1987). Other studies showed that maximum plasma cortisol concentrations were observed 5 min after the end of exercise. Cortisol values are almost back to resting levels 30 min after the end of exercise (NAGATA et al., 1999). This indicates that the adrenal cortex is working adequately, responding to higher energy demands. This is not a sign of stress but of a healthy responding metabolism. Therefore, the short-lasting training units of 20 minutes daily may be a reason why we did not find significant changes in FCM in our study. This specific amount of daily training does not seem to represent a stressful event for the horses. They were capable of coping with the new situation. There is a possibility that, if training had been longer or more strenuous, significant changes in FCM levels would have been found. Another reason for the unchanged FCM could be that horses participating in this study were no longer experiencing social or emotional stress. HADA et al. (2003) reported that plasma concentrations of ACTH and vasopressin increased significantly after horses were transported and ridden in a novel environment. It was suggested that emotional stress, in the form of novel stimuli, activates the HPA axis in horses.

We provide data showing that short training does not necessarily need to be stressful for horses when done properly. In our study, we were able to work with very professional and experienced trainers. Therefore, results may very well vary in different training facilities. A non-stressful initial training is dependent on many different factors such as handling, previous human-animal relationships and previous experiences. The experience of the trainer is also a very important factor to minimize stress for the horses while being trained.

The results of our large-scale study provide basic physiological data about initial training. We found no increased adrenocortical activity and therefore no indications of stress in relation to the initial training. This gives additional input in an emotional debate about animal welfare aspects of first time handling and training of western horses. However, caution is advised, as the conclusion drawn from our study is not a general statement that training is not stressful.

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References

BERGHOLD, P., MÖSTL, E., AURICH, C. (2007): Effects of reproductive status and management on cortisol secretion and fertility of oestrous horse mares. Anim. Reprod. Sci., in press.

CHURCH, D.B., EVANS, D.L., LEWIS, D.R., ROSE, R.J. (1987): The effect of exercise on plasma adrenocorticotropin, cortisol and insulin in the horse and adaptations with training. In: GILLESPIE, J.R., ROBINSON, N.E. (eds.): Equine exercise physiology 2. ICEEP Publications, Davis, California. p. 506-515.

COLBORN, D.R., THOMPSON, J.R., D.L., ROTH, T.L., CAPEHART, J.S., WHITE, K.L. (1991): Responses of cortisol and prolactin to sexual excitement and stress in stallions and geldings. J. Anim. Sci. **69**, 2556-2562.

COVALESKY, M., RUSSONIELLO, C., MALINOWSKI, K. (1992): Effects of show jumping performance on plasma cortisol and lactate concentrations and heart rate and behavior in horses. J. Equine Vet. Sci. **12,** 244-251.

FREESTONE, J. F., WOLFSHEIMER, K. J., KAMERLING, S. G., CHURCH, G., HAMRA, J., BAGWELL, C. (1991): Exercise induced hormonal and metabolic changes in Thoroughbred horses: effects of conditioning and acepromazine. Equine Vet. J. **23**, 219-223.

HADA, T., ONAKA, T., TAKAHASHI, T., HIRAGA, A., YAGI, K. (2003): Effects of novelty stress on neuroendocrine activities and running performance in Thoroughbred horses. J. Neuroendocrinol. **15**, 638-648.

IRVINE, C.H.G., ALEXANDER, S.L. (1994): Factors affecting the circadian rhythm in plasma cortisol concentrations in the horse. Dom. Anim. Endocrinol. **11,** 227-238.

JIMENEZ, M., HINCHCLIFF, K.W., FARRIS, J.W. (1998): Catecholamines and cortisol responses of horses to incremental exertion. Vet. Res. Commun. **22**, 107-118.

LINDMAN, H. R. (1974): Analysis of variance in complex experimental designs. Freeman & Co., San Francisco.

MARC, M., PARVIZI, N., ELLENDORFF, F., KALLWEIT, E., ELSAESSER, F. (2000): Plasma cortisol and ACTH concentrations in the warmblood horse in response to a standardized treadmill exercise test as physiological markers for evaluation of training status. J. Anim. Sci. **78,** 1936-1946.

MERL, S., SCHERZER, S., PALME, R., MÖSTL, E. (2000): Pain causes increased concentrations of glucocorticoid metabolites in horse feces. J. Equine Vet. Sci. **20**, 586-590.

MÖSTL, E., PALME, R. (2002): Hormones as indicators of stress. Dom. Anim. Endocrinol. **23**, 67-74.

MÖSTL, E., MESSMANN, S., BAGU, E., ROBIA, C., PALME, R. (1999): Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. J. Vet. Med. A **46**, 621-632.

NAGATA, S., TAKEDA, F., KUROSAWA, M., MIMA, A., HIRAGA, A., KAI, M., TAYA, K. (1999): Plasma adrenocorticotropin, cortisol and catecholamines response to various exercises. Equine Vet. J., Suppl. **30**, 570-574.

PALME, R. (2005): Measuring fecal steroids: guidelines for practical application. Ann. N. Y. Acad. Sci. **1046**, 75-80.

PALME, R., MÖSTL, E. (1997): Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Z. Säugetierkunde **62** (Suppl. II), 192-197.

PALME, R., FISCHER, P., SCHILDORFER, H., ISMAIL, M. N. (1996): Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. Anim. Reprod. Sci. **43**, 43-63.

PALME, R., ROBIA, C., BAUMGARTNER, W., MÖSTL, E. (2000): Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. Vet. Rec. **146**, 108-109.

PESENHOFER, G., PALME, R., PESENHOFER, RM., KOFLER, J. (2006): Comparison of two methods for fixation during functional claw trimming - walk-in crush versus tilt table - in dairy cows using faecal cortisol metabolite concentrations and daily milk yield as parameters. Vet. Med. Austria/Wien. Tierärztl. Mschr. **93**, 288-294.

RAMMERSTORFER, C., POTTER, G. D., CUDD, T. A., GIBBS, P. G., VERNER, D. D., HOUSEHOLDER, D. D. (1998): Physiological responses of mature Quarter Horses to reining training when fed conventional and fat-supplemented diets. J. Equine Vet. Sci. **18**, 175-183.

RIVERA, E., BENJAMIN, S., NIELSEN, B., SHELLE, J., ZANELLA, A. J. (2002): Behavioral and physiological responses of horses to initial training: the comparison between pastured versus stalled horses. Appl. Anim. Behav. Sci. **78**, 235-252.

TERLOUW, E.M., SCHOUTEN, W.G.P., LADEWIG, J. (1997): Physiology. In: APPLEBY, M.C., HUGHES, B.O. (eds.): Animal Welfare. CAB International, University Press, Cambridge, p. 143-158.

TOUMA, C., PALME, R. (2005): Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. Ann. N. Y. Acad. Sci. **1046**, 54-74.

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