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Fecal cortisol metabolites indicate increased stress levels in horses during breaking in

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Abstract

Sport horses are frequently exposed to situations that were identified as stressors, indicated by an increased cortisol release, that might impair animal welfare. However, while many studies deal with the impact of exercise, transport and competition on stress in horses, there is only rare knowledge about the early phase of a horse's sports career and studies investigating the stress level of young horses during breaking in are limited. To compare stress levels in unriden horses, horses during breaking in, and horses in training, we collected fecal samples of young, unriden horses (n=28) and of horses in different training stages (n=13) and measured fecal cortisol metabolite (FCM) concentrations. Our results showed that FCM concentrations of unriden horses were significantly lower than those of horses in training (Mann-Whitney Rank Sum Test, $p < 0.001$). Particularly in the first year under the saddle FCMs were significantly higher than in unriden horses (One Way ANOVA + posthoc Holm-Sidak-Test, $p < 0.05$), with a tendency of decreasing FCM levels with time in training. Furthermore, we observed that within the group of ridden horses, there was a larger range of variability in FCM levels, suggesting individual variations regarding their ability to deal with (training-induced) stress. These results indicate that breaking in is a stressful time for young horses, underlining the importance of carrying out the initial training as carefully as possible.

Zusammenfassung

Sportpferde werden regelmäßig Situationen ausgesetzt, welche zu einer erhöhten Cortisolausschüttung führen, dadurch als Stressoren für sie identifiziert werden konnten und folglich ihr Wohlbefinden beeinträchtigen können. Obwohl bereits viele Studien durchgeführt wurden, die sich mit dem Einfluss von Training, Transport und Wettkämpfen auf das Stresslevel von Pferden beschäftigen, ist der Wissensstand über die frühe Phase der Karriere eines Sportpferdes noch gering und es gibt wenige Studien, die das Maß an Stress, dem junge Pferde während des Einreitens ausgesetzt sind, erforschen. Um die Stresslevel ungerittener Pferde mit denen von Pferden während des Einreitens und Pferden, die im Training sind, zu vergleichen, haben wir Kotproben ungerittener Jungpferde (n=28) und von Pferden unterschiedlicher Trainingsstadien (n=13) gesammelt und darin die fäkalen Cortisolmetabolitkonzentrationen (FCM) gemessen. Unsere Ergebnisse zeigen, dass die FCM-Konzentrationen der ungerittenen Pferde signifikant niedriger waren, als die der Pferde im Training (Mann-Whitney Rank Sum Test, $p < 0.001$). Besonders im ersten Jahr unter dem Sattel waren die FCMs signifikant höher als bei ungerittenen Pferden (One Way ANOVA + posthoc Holm-Sidak-Test, $p < 0.05$), mit einer Tendenz zu sinken, je länger die Pferde im Training waren. Darüber hinaus konnten wir beobachten, dass innerhalb der Gruppe der Reitpferde die FCM-Levels stärker variierten, was darauf schließen lassen könnte, dass die Fähigkeiten der Pferde mit dem (trainingsinduzierten) Stress umzugehen sich individuell unterscheiden. Zusammenfassend konnten wir mit unseren Ergebnissen bestätigen, dass das Anreiten junger Pferde eine stressreiche Zeit für sie ist und unterstreichen wie wichtig es folglich ist die Grundausbildung so vorsichtig wie möglich durchzuführen.

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1. Introduction

Horses have been a means of transportation, warfare and companions for humans for centuries and the popularity of horseback riding as a leisure activity continues. In the past decades, horses have gained importance as partners and their welfare is increasingly being considered. Nonetheless, stress is a daily companion in the life of many horses, especially when they are in training, taking part in competitions, being transported [1–4], but also common diseases such as lameness and external influences, e.g., changes in the weather, are important stressors for horses [5]. “Stress” is a vague term, and a large number of researchers have already tried to find the ideal definition. According to Ramos and Mormède, “stress is considered as the response of an organism to environmental stimuli (stressors) which threaten its internal equilibrium, also called homeostasis” [6]. Fraser et al. propose that “an animal is said to be in a state of stress if it is required to make abnormal or extreme adjustments in its physiology or behavior in order to cope with adverse aspects of its environment and management” [7]. Both definitions lead to the conclusion that stress is an uncomfortable condition and that it is essential, for the physical and mental wellbeing of horses, to avoid stressful situations. Besides the adaptations in the physiology and behavior of the animals that are necessary to deal with stress, it could be shown that particularly transport and lameness can cause reduced pulmonary defense mechanisms and also have negative effects on other immunological functions [5], and thus impair the horses’ health. Therefore, in the interest of animal welfare and health, it is important to identify triggers potentially activating the stress response in horses.

So far, stress is usually measured by assessing clinical (heart rate, body weight and activity level) and endocrinological (plasma cortisol, catecholamine and beta-endorphin concentrations) parameters [5]. Due to the fact that stress leads to an increased glucocorticoid secretion by increasing the activity of the hypothalamo-pituitary-adrenal axis [8], there are already numerous studies that identified assumingly stressful situations for horses by means of increases in cortisol release. Besides typical situations like training [9, 1], transport [10–12, 3] and competitions [13, 14, 2], but also veterinary examinations [15, 16] and social instability [17] that have been shown to increase plasma cortisol levels, breaking in, i.e., the transition from a young, unriden horse to a riding horse and sport partner for humans can also be seen as stressful situation for the animal. Animal welfare organizations are increasingly criticizing equestrian sports for the potentially negative impact it may have on horses, particularly equine athletes at a high-performance level and young horses during breaking in. However, there are

only few studies that investigate the actual stress level of horses during initial training. Schmidt et al. identified particularly the mounting of a rider as a stressor for 3-year-old sport horses [4] and demonstrated a quick adaptation to the new situation, while Pilger et al. also reported that the pretraining (e.g. free movement, lunging, training on an automatic horse walker) of young stallions leads to an initial stress response [18]. Both studies determined changes in salivary cortisol concentrations, heart rate and heart rate variability and thus had to manipulate the animals repeatedly, which could additionally have stressed the young horses. In contrast, Gorgasser et al. did not find any increase in adrenocortical activity in young Quarter horses within the first month of training using fecal cortisol metabolites (FCMs) as indicator and thus concluded that the animals were able to deal well with their new tasks [19]. It cannot be excluded that the diverging results may be due to the breed of the horses and/or the Western training style used in the latter study. Furthermore, all of these studies investigated only stress levels in the horses during the training period, neglecting the possibility of generally increased baseline values due to the onset of training, and a comparison over a longer period of time, e.g., between horses that are not trained at all, more experienced riding horses and horses during initial training is lacking. Therefore, from the current state of the literature, it cannot be concluded whether the transition phase is particularly stressful for horses or not. In this study, we aimed to examine if the stress levels in horses during breaking in differ from those in horses that are not ridden or more experienced horses in training.

While many studies utilized plasma or saliva cortisol concentrations [20–22] as a marker, we chose to determine FCMs in order to avoid additionally stressing the animals by venipuncture or taking saliva samples. Non-invasive techniques to measure glucocorticoid metabolites in feces have been developed for several species [8, 23, 24]. Also in horses an 11-oxoaetiocholanolone enzyme immunoassay [25] was proven to reliably quantify a group of FCMs without the need of manipulating the animal [26–28]. Several studies already demonstrated that increased plasma cortisol concentrations aligned with increased FCM levels in horses exposed to potential stressors like transport and veterinary examinations [12, 13, 2].

2. Materials & Methods

2.1. Horses

We included a total of 41 horses from one stud farm, aged between 1 and 20 years, in this study. According to their level of training, they were categorized into two groups: “unridden” and “in training”.

The group of unridden horses (n=28) was composed of female, male intact and castrated warmblood horses, Haflingers and a Noriker. They were housed in open stables with seasonal paddocks. Their barns were strewn with straw, equipped with a deep litter system and were usually cleaned every two weeks, depending on the amount of manure. Water was provided ad libitum and the horses were fed with hay, haylage and grass. The animals had contact with humans from birth and were halter trained, but were not trained otherwise before breaking in. This group was further subdivided in three different age groups: “1-2 years old” (n=6), “2-3 years old” (n=10) and “3-4 years old” (n=12).

The group of horses in training contained n=13 warmblood geldings, which were kept individually in boxes (3x3m) with straw bedding and daily mucking out. They were fed with hay and oats and had ad libitum access to water. Their training involved two to three sessions of approximately 30 minutes of riding or free jumping per week, with few small individual modifications. This group was further classified, based on the time the horses had been under the saddle: “ridden for 3-6 months” (n=6, all 4 years old), “ridden for 1 year ” (n=3, 4-5 years old) and “ridden for 3 years or longer” (n=4, aged 8-20 years).

2.2. Sample collection and processing

A fresh fecal sample of every horse was taken at the beginning of May 2023. The samples of the ridden horses were collected during the day after training, and all samples were immediately stored at -20 °C.

Extraction of FCMs was performed as described by Merl et al. [29]. In brief, 0.5 g of feces were mixed with 5 ml 80% methanol, vortexed for 30 minutes and centrifuged at 2,500 x g for 15 min. 1 ml of the supernatant was transferred into new vials and mixed with 0.25 ml 5% NaHCO₃ and 5 ml diethylether before spinning down again and freezing at -20 °C overnight. The next day, the supernatant was transferred into new vials and dried down before being redissolved in 0.5 ml assay buffer. Concentrations of 11,17-dioxoandrostanes were measured via an 11-

oxoetiocholanolone enzyme immunoassay as described before [25]. This method has been successfully validated for use in horses [26]. Each sample was measured in duplicate and the mean value of all technical replicates was used for statistical analysis.

2.3. Statistical analysis

For statistical analysis, FCM values were compared using Sigma Plot 14.5 (Systat Software Inc., Germany) with either a Mann-Whitney Rank Sum Test for comparison of two groups or a One Way ANOVA with a post-hoc Holm-Sidak-Test for comparison of more than two groups. The data were tested for normality using a Shapiro-Wilk test and for equal variance with a Brown-Forsythe test. Correlation analysis was performed using a Pearson Product Moment correlation. Significance was assumed at $p < 0.05$.

3. Results

3.1. FCM concentrations are higher in horses in training

FCM concentrations among the unriden horses ranged from 4.5 to 14.0 ng/g feces (median 7.3 ng/g), while FCM levels of the horses in training were between 7.5 and 18.1 ng/g feces (median 11.6 ng/g). There was a statistically significant difference ($p < 0.001$, Mann-Whitney Rank Sum Test) between the two groups (Fig. 1).

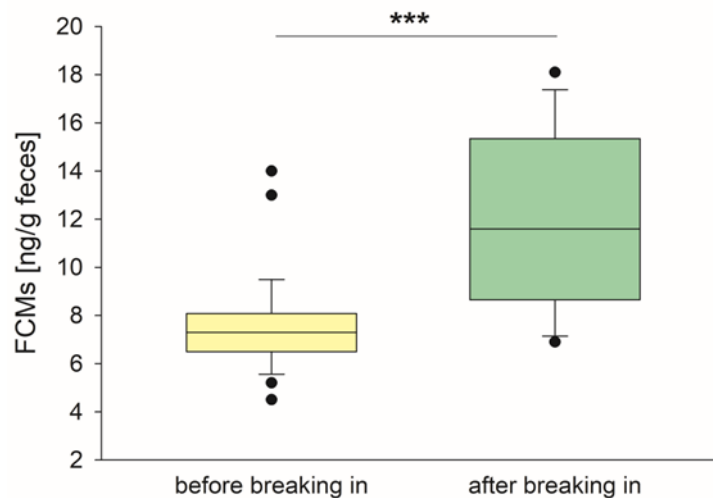


Figure 1. FCM concentrations in unriden horses (yellow box, $n=13$) were significantly lower than in horses in training (green box, $n=28$). Boxplots represent median FCM concentrations (line) in the different age groups $\pm$ 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers), outliers are marked by dots. *** $p < 0.001$, Mann-Whitney Rank Sum Test.

3.2. FCM concentrations are higher directly after breaking in

There was no difference regarding the FCM concentrations between the different age groups of unriden horses (Fig. 2). Within the group of “1-2 years old” horses, a range from 5.6 to 13.0 ng/g feces (median 6.6 ng/g) was measured. The values of the “2-3 years old” horses were between 5.2 and 9.1 ng/g feces (median 7.3 ng/g) and the “3-4 years old” horses had levels from 4.5 to 14.0 ng/g feces (median 7.7 ng/g).

In contrast, horses, which had been in training for less than one year had significantly higher FCM concentrations ranging from 7.5 to 18.1 ng/g feces (median 13.1 ng/g) compared to unriden horses ($p < 0.05$, One Way ANOVA + post-hoc Holm-Sidak-Test). FCMs in horses

that had been in training for at least one year no longer differed significantly from those in the group of unriden horses and showed a tendency to have lower FCMs in relation to the time under the saddle. FCM values in horses, which have been ridden for one year, ranged from 6.9 to 12.1 ng/g feces (median 13.2 ng/g). Among the horses which have been under the saddle for the longest period ("ridden for 3 years or longer"), FCM values between 8.6 and 16.2 ng/g feces (median 8.8 ng/g) were measured (Fig. 2 and Tab. 1).

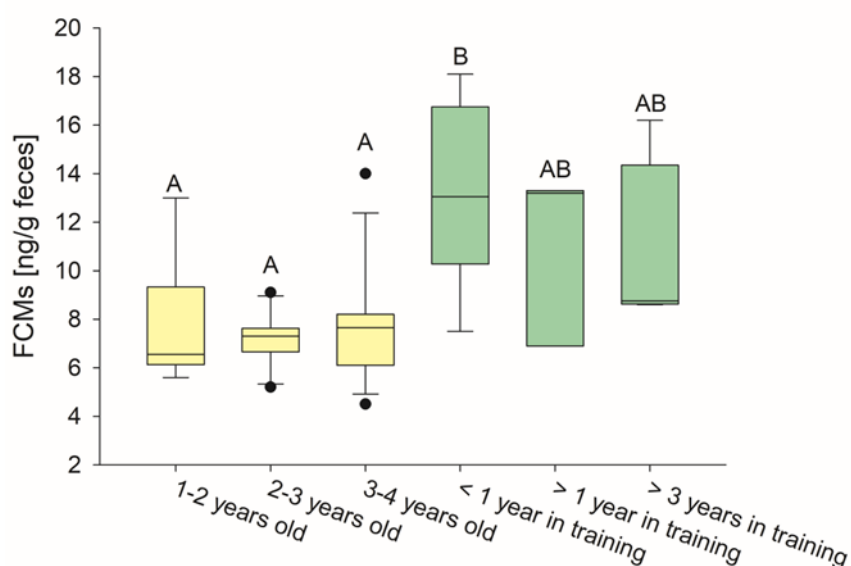


Figure 2. FCM concentrations were higher when the horses were newly broken in compared to unriden horses. Horses that have been ridden for less than one year had significantly higher FCM levels than horses that were not yet broken in. After more than one year in training, there was no statistical difference between FCM levels in horses in training (green boxes) and unriden horses (yellow boxes). Boxplots represent median FCM concentrations (line) in the different age groups $\pm$ 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers), outliers are indicated by dots. Different letters indicate significant differences between groups, $p < 0.05$, One Way ANOVA + posthoc Holm-Sidak-Test.

Table 1. Median and quartiles of FCM concentrations in ng/g feces in the different groups of horses.

75%	25%	median	group
9.3	6.1	6.6	unriden, 1-2 years old
7.6	6.7	7.3	unriden, 2-3 years old
8.2	6.1	7.7	unriden, 3-4 years old
16.8	10.3	13.1	in training for 3-6 months
13.3	6.9	13.2	in training for 1 year
14.4	8.6	8.8	in training for > 3 years

3.3. FCM concentrations show a weak correlation to training status

Pearson Product Moment correlation showed a weak positive correlation ($r=0.440$, $p<0.01$) of FCM levels with the time the horses had been under the saddle at the time point of measurement (Fig. 3).

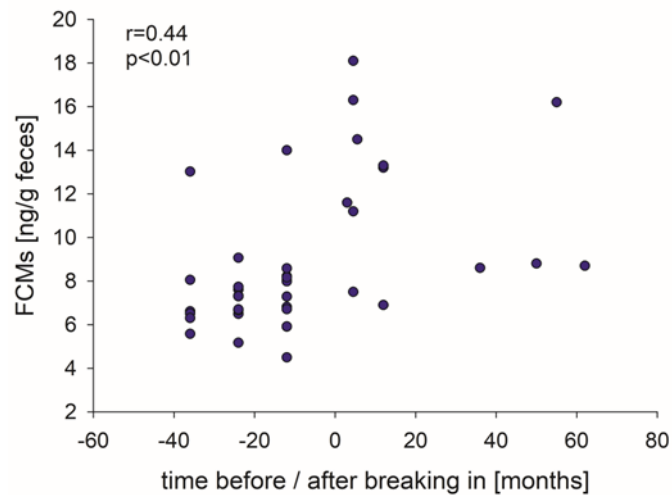


Figure 3. Relation between FCM levels of individual horses and the time (months) before and after breaking in, respectively.

4. Discussion

Animal welfare in equestrian sport is increasingly under debate. A common point of criticism concerns the breaking in of the horses, i.e., getting them used to the saddle, bridle and rider. However, it is hard to objectively judge or even quantify the strain that is actually induced in the animals by this procedure. The aim of this study was to investigate whether horses in training, and particularly those that have recently been broken in, have higher stress levels indicated by elevated FCM concentrations compared to unriden horses. Fecal samples of young and unriden horses of different age and of horses with various training status were compared. It is important to highlight that we did not only measure FCMs during breaking in, but also months and years before and after the initial training started, providing a comprehensive investigation of FCM levels in horses of different training stages. Additionally, in our study the horses were all from the same farm and not transported to another facility, but the measurements took place in their familiar environment, thus avoiding a bias that is present in many other studies.

The FCM values we observed, particularly in the unriden horses, align well with baseline values measured with the same method in another study using a larger horse population [30], supporting the validity of the measurements and indicating the baseline character of these measurements. We showed that the median FCM concentrations of unriden horses were lower than those of horses in training. Furthermore, our results suggest that after reaching a peak within the first year under the saddle FCM levels are lower in more experienced horses. Interesting findings were not only the higher median concentration of FCMs in horses in training, but also a larger range of variation in these horses, although the group of ridden horses was more homogenous regarding sex and breed than that of the unriden horses. This might be interpreted as a higher stress level in horses that are being ridden [27] with an individual variability of their ability to deal with that stress. This should not be disregarded, as individual horses have different coping mechanisms in order to deal with excessive strain that can lead to unwanted behavior [31]. The variability could also be caused by different riders, whose level of experience as well as the arousal experienced by the rider can influence the horse's behavior and trigger the animal's flight response [31]. It is therefore important to ensure that training is carried out by an experienced, confident and calm person, especially in the early stages of training, when the horse is already exposed to several new stimuli. Unfortunately, detailed information on the riders was not available for this study, but all of them were experienced in training (young) horses.

Our findings add to previous studies showing that the measurement of FCMs is a reliable and sensitive method to assess stress levels in horses [27, 26] and that breaking in is a stressful period of time for young horses. Schmidt et al. demonstrated that equestrian training is challenging for the horses, especially in the beginning, by measuring salivary cortisol concentrations, heart rate and heart rate variability [4]. On the contrary, a study performed in young Quarter horses did not show any increase in FCMs during the first month of breaking in [19]. These conflicting results may be caused by different breeds, housing conditions and/or training style. Although training horses without a rider through activities like free movement, jumping obstacles, lunging, or using a horse walker, can already elevate the stress response [18], it is important to highlight that the mounting of the rider is a very sensitive step, when it comes to the process of breaking in [4]. In wildlife nothing comparable to a human riding on the back of a horse can be found, except the situation of a predator catching its prey. Therefore, to make equitation even possible, a high tolerance from the young horses is needed towards their rider [32], who is not only on their back, but also out of their field of vision [33]. Observing and understanding the individual reaction of horses to new and potentially stressful situations (e.g., the first mounting of the rider) could not only be helpful to select them for different intended purposes, according to their ability to cope with this kind of stress [34], but is also relevant to avoid undesirable, “negative” behavior and resulting accidents [35]. However, it must also be mentioned, that the highest stress levels observed both in our and other studies during initial training do not reach those induced by transport, pain or excitement due to unusual events [30, 29], indicating that the training process might cause some distress in the animals but no serious impairment of animal welfare.

It is important to highlight that as soon as the young horses are used to their rider, initial training is rather a physical exercise than a mental task [4]. However, it should be borne in mind, that physical exercise can also lead to an increased stress response [36]. Yet, in our study, it is worth noting that horses which had been in training for longer exhibited lower FCM levels. This could either indicate that physical activity may not be the primary factor for elevated FCM values or that the initial training is more demanding for the young horses until they get used to it and this causes the observed increase in FCMs rather than mental distress. To maintain the horses' will to cooperate with humans, it is essential to know that handling and training can influence them either in a positive or a negative way [37] and that negative experiences are able to delete positive ones from their memory [38]. For these reasons, it is even more relevant to be careful during initial training of young horses.

It has been reported that horses kept on pastures and in groups are able to deal better with new training situations [39] and show less stereotypies and abnormal stress-related behavior than horses kept individually in boxes [40]. Our findings also suggest that keeping young horses in open stables in groups has a positive impact, as they were already being accustomed to human contact and halter training without showing a corresponding increase in FCM values. This implies that exposure to human interaction and basic handling does not induce stress per se, further underlining that the primary triggers occur during the process of breaking in. However, since this was also the time point when the horses' environment changed from being kept in an open stable in groups to being stabled in individual boxes for the first time, it is not possible to differentiate precisely which experience is the main reason for the rise of FCM concentrations in our study and whether keeping them in groups during the process of breaking in would have attenuated the stress response. However, other studies suggest no or rather an inverse impact of group housing on the horses' resilience during breaking in. Schmidt et al. distinguished between two groups, one of which included only mares housed in group stables and one including stallions that were kept in individual boxes. In both groups salivary cortisol levels increased during breaking in and even more in the group of mares than in the stallions, emphasizing that this probably has a greater influence on the animals than the housing conditions [4]. Similarly, Pilger et al. reported that group housing had no stress-reducing effects during pretraining [18].

The main limitation of our study is that we could only measure one sample per animal and thus we could not perform a longitudinal observation of FCMs in individual horses before, during and after breaking in to identify different factors influencing FCM levels. Further studies, including long-term observations of the same pasture-kept horses without any changes in housing and social groups while breaking in, would be interesting and should be performed in the future. In addition, it would be helpful to combine different methods to detect and quantify stressful events in horses, for example salivary cortisol concentrations, heart rate measurement and behavioral analysis. Determining salivary cortisol may be a better method to detect increases of cortisol levels induced by specific events throughout the day, whereas FCMs appear with a delay of 24 hours after a stressful event in the feces, because of the intestinal passage of the horse [23]. FCMs may thus be more representative for the general wellbeing of a horse within the last 24 h, while it is easier to identify a certain stressor in salivary or plasma samples. However, when measuring plasma or salivary cortisol, the sampling process itself is stressful for the horses, when it comes to repeated venipuncture or fixation of

the head, particularly when the horses did not have a lot of contact with humans before. Therefore, we chose a non-invasive method using fecal samples in this study.

5. Conclusions

In conclusion, we show that the FCM concentrations of unriden horses were significantly lower than those of horses in training. The larger range of variability in the FCM levels of ridden horses suggests individual variations regarding their ability to deal with stress. Another interesting finding was that FCM level reach a peak within the first year of being ridden, confirming that breaking in is a critical time point for the young horses.

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