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Article type : Article

Editorial ref. code: **EVJ-TN-17-044.R2**

Glucocorticoid assessment in the domestic horse: the impact of time and climatic variables on sample integrity

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Keywords: horse; corticosterone; adrenal; faecal; welfare

Summary

Background: Assessment of faecal glucocorticoid metabolites (FGM) offers a non-invasive method of monitoring adrenal activity in domestic horses. Sample collection is on an opportunistic basis, and if samples are not fresh or have been exposed to the elements before they are identified, then they may not accurately reflect FGM concentration.

Objectives: To explore the impact of a range of environmental conditions upon the integrity of FGM levels in equine faeces.

Study design: In vitro experiment.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/evj.12726

Method: Equine faeces were exposed to six controlled environmental conditions meant to simulate a range of weather and seasonal patterns (temperate climate, high heat, high heat and rainfall, temperate climate and rainfall, high heat/temperate climate and freeze/thaw) over a period of five days. FGM were quantified using an enzyme linked immunoassay.

Results: Faecal samples exposed to room temperature and high heat demonstrated a significant increase in FGM over time. Changes in FGM were not observed in the remaining treatments.

Main limitations: The study should be repeated in field conditions and with known high and low levels of FGM to further inform sampling regimes.

Conclusions: Adrenal monitoring in the domestic horse should be performed with consideration of the impact of climate on integrity of faecal samples to further inform sampling schedules and improve reliability of results.

Introduction

Assessment of faecal glucocorticoid metabolites (FGM) offers a non-invasive method of measuring adrenal activity in domestic horses. Faecal sampling reflects an average glucocorticoid level over time rather than the point in time sampling offered by plasma or saliva and therefore is more suitable for long term monitoring [1]. FGM measurement has been utilised in horses to investigate the impact of veterinary treatment [2], and it continues to be a popular method of physiological stress assessment. The non-invasive nature of FGM assessment means that sample collection is on an opportunistic basis, and samples cannot always be collected immediately upon defecation. These samples may therefore not accurately reflect FGM concentration.

Studies in other species have reported artificially inflated faecal glucocorticoid levels in samples exposed to the environment [3], suggesting that microbes in the faeces may have metabolised the faecal glucocorticoids into metabolites, with an increased affinity for the corticosterone assay used. If researchers are unable to collect fresh samples, then they must have an understanding of the impact that time and sample condition has upon glucocorticoid metabolite concentrations. No standard protocol exists for faecal sample collection for glucocorticoid analysis in horses. It is therefore important to ascertain the impact that environmental conditions have on FGM levels in horse faeces and whether older samples have value or whether they should be discounted from any assessment. Optimum sampling would involve all faeces being collected upon defecation, but this requires constant observation of the study subject. If the integrity of samples over time and during exposure to various climatic variables is investigated further, then guidelines can be developed on appropriate sample collection in horses. This could potentially increase the amount of useful samples that can be collected or discount samples that are older or environmentally damaged and inform a more robust and specific collection protocol.

Materials and methods

Faecal sample collection

Faecal samples were collected from domestic horses (4 mares and 5 geldings) immediately upon defecation over a period of 90 minutes. At least two samples per individual were collected with 100 g of faecal matter retrieved each time faeces were produced. Horses were housed in individual stables and were fed ad libitum hay and water during the time of collection. Samples were stored in a cooler bag for transportation to the on-site laboratory within two hours of deposition.

Simulated environmental conditions

Faecal samples were pooled and thoroughly homogenised in two groups (1. male horse. 2. female horse). A sub sample of ~10 g was taken from each group and frozen at -20°C to provide our pre environmental treatment baseline (time 0). Immediately upon arrival at the laboratory (which was less than two hours post defecation), a second sample was taken (<2 h).

The remaining faeces in each of the two groups were separated into ~250 g sub samples, and each sub sample was randomly assigned to one of six environmental simulation treatments which were **1)** room temperature (samples kept at 15°C), **2)** high heat (samples kept in an oven at 36°C to simulate summer conditions), **3)** rainfall/room temperature (25 ml of rainwater every other day and kept at 15°C), **4)** rainfall/high heat (25 ml of rainwater every other day and kept in an oven at 36°C), **5)** high heat/room temperature cycle (samples kept at high heat in an oven at 36°C for 12 h and then at room temperature at 15°C for 12 hours over; simulating summer heat and night time cooling) and **6)** freeze/thaw cycle (samples frozen for 12 h at -20°C and then placed in an oven at 36°C simulating winter cold and daytime warming).

Sampling protocol

For treatments 1-4, a sub sample of 10 g was removed from both the male and female groups at <2 hours from baseline sample upon arrival at the lab and then at 4 h, 6 h, 8 h, 12 h, 24 h, 36 h and 48 h after the baseline sample. For treatments 5 and 6, a 10 g sub sample was removed from both the male and female groups at 12 h post baseline sample and then 24 h, 36 h, 48 h, 60 h and 72 h. All subsamples were placed into plastic sample bags and labelled according to their treatment group, time removed and gender and then frozen at -20°C until analysis.

Rainwater was collected in the week leading up to the study and stored in a refrigerator until it was applied to the appropriate samples. We introduced a total of 7 ml of rainwater to treatment 3 and 7 ml to treatment 4 split into four applications (every 12 h) over the 72 h treatment period (rainfall was calculated from average daily rainfall in UK, British meteorological office).

To account for moisture loss and standardise the weight of faecal sub samples, each sub sample was weighed and then divided by the number of remaining sub samples to be collected. Throughout the treatment, faecal samples in conditions 1, 2, 5 and 6 were kept in 13 cm by 13 cm petri dishes, and samples in the rainfall treatments 3 and 4 were kept in 15 cm diameter sieves to allow slow drainage of water.

Mean room temperature throughout the treatment period was 14.9 ± 2.1°C, oven temperature was 36.2 ± 0.75°C, and freezer temperature was -20°C ± 0.05°C (Thermosense, UK).

Faecal glucocorticoid analysis

Hormones were extracted from faecal samples following thawing and manual homogenisation using a wet-weight shaking extraction [4] as described elsewhere [5]. Faecal extracts were stored until analysis in duplicate using a previously described corticosterone antibody (CJM006 supplied by Coralie Munro; UC Davis) validated for horse faeces [6]; cross-reactivities for the assay are published elsewhere [7], and only data with intra-assay coefficient of variation of less than 10% and inter-assay CoV less than 15% were accepted and used for statistical analysis.

Data analysis

A mean was calculated from each subset of four samples for each sampling time point within a treatment. An independent T-test revealed no significant difference between samples of mares and geldings ($p<0.05$); therefore data were analysed together. Distribution of data was normal (Kolmogorow-Smirnov $p = 0.53$). Data were analysed using a repeated measures analysis of variance to compare pre-treatment (time 0) with post treatment samples (hours >2 to up to 48 hours). All data were analysed using IBM, SPSS V23 statistical software. All data are reported as mean ± s.d.

Results

During the room temperature treatment, there was a significant increase in mean FGM metabolite concentrations compared to time 0 (11.4 ± 5.7 ng/g) at 48 hours after baseline sample collection (25.5 ± 1.8 ng/g; $F (9, 30), = 7.7$, $p = 0.001$, multivariable partial eta squared = .472) (Fig 1).

Similarly, during the high heat treatment, there was a significant increase in mean FGM metabolite concentrations compared to time 0 (11.4 ± 5.7 ng/g) at 48 hours after baseline sample collection (22.2 ± 6.1 ng/g; $F (9, 30), = 2.7$, $p = 0.02$, multivariable partial eta squared = .100) (Fig 1).

There was no significant effect of time on FGM levels during the rain/room temperature treatment (mean of whole treatment 12.8 ± 4.4 ng/g), rain/high heat treatment (mean of whole treatment 15.6 ± 4.5 ng/g), room temperature/high heat treatment (mean of whole treatment 12.8 ± 2.4 ng/g), or the freeze thaw treatment (mean of whole treatment 15.02 ± 4.5 ng/g),

Discussion

These results help to identify an optimal window of less than 48 hours for sampling equine faeces, increasing accuracy of the interpretation of the results as being a true reflection of adrenal activity in the domestic horse.

The simulated environmental treatment that included high heat had an impact upon FGM levels. It has been suggested that biochemical processes, such as cleavage of conjugate side groups from hormone metabolites or release of faecal glucocorticoids from lipid micelles, may increase faecal glucocorticoid metabolite levels [3]. This may also be the case in this study. The presence of high heat may have provided a favourable growth environment for microbes causing the increase in FGM.

These findings have implications for field based sampling in similar climatic conditions. Studies taking place during seasons of hot weather with limited rainfall may result in degradation of samples. In the current study, the room temperature treatment also demonstrated a significant increase in FGM over time, and the authors suggest faecal samples older than two days post defecation be discounted from any adrenal assessment protocol.

The simulated rain conditions were conducted at both room temperature and with heat. Given that both heat and room temperature treatments on their own caused an increase in FGM, it was unexpected to see no change in the rainfall treatments. It may be that the presence of moisture inhibits degradation of the sample.

Freezing and thawing faecal samples had no impact on FGM levels, and concentrations remained stable (mean 15.02 ± 4.5 ng/g) until sampling ceased. This supports previous work, as a part of the current recommended sampling protocol is to freeze samples until analysis. In

this case, the 12 hours at room temperature before samples were reintroduced into frozen storage did not affect FGM levels.

Time post defecation of faecal samples needs to be closely monitored, and climate at time of collection must also be carefully considered. Based on the findings of the current study, we suggest that any planned adrenal monitoring needs to consider the impact of climate on integrity of faecal samples and would encourage similar work to be carried out in addition to assay validation for species and gender. Findings can then inform a more robust methodology and improve reliability of results.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The study was approved by the Nottingham Trent University ethics committee. Faeces were collected from horses stabled at the NTU equine unit which was a non-invasive procedure and did not alter the usual routine of the horses.

Source of funding

The study was funded by Nottingham Trent University.

Authorship

K. Yarnell contributed to the study design, study execution, data analysis and interpretation, and preparation of the manuscript. S. Walker contributed to the study design, data analysis and interpretation, and preparation of the manuscript. Both authors gave their final approval of the manuscript.

Figure legend

Fig 1: Mean faecal glucocorticoid metabolite levels over time for the room temperature and high heat treatments. The box identifies the quartile range with the median marked as a line within the box. The two lines outside the box extend to the highest and lowest observations. An * identifies the significant rise in FGM concentration between time point 0 and 48 hours during both treatments.

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