Video Article

Fecal Glucocorticoid Analysis: Non-invasive Adrenal Monitoring in Equids

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Abstract

Adrenal activity can be assessed in the equine species by analysis of feces for corticosterone metabolites. During a potentially aversive situation, corticotrophin releasing hormone (CRH) is released from the hypothalamus in the brain. This stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland, which in turn stimulates release of glucocorticoids from the adrenal gland. In horses the glucocorticoid corticosterone is responsible for several adaptations needed to support equine flight behaviour and subsequent removal from the aversive situation. Corticosterone metabolites can be detected in the feces of horses and assessment offers a non-invasive option to evaluate long term patterns of adrenal activity. Fecal assessment offers advantages over other techniques that monitor adrenal activity including blood plasma and saliva analysis. The non-invasive nature of the method avoids sampling stress which can confound results. It also allows the opportunity for repeated sampling over time and is ideal for studies in free ranging horses. This protocol describes the enzyme linked immunoassay (EIA) used to assess feces for corticosterone, in addition to the associated biochemical validation.

Video Link

The video component of this article can be found at https://www.jove.com/video/53479/

Introduction

The method described aims to analyse corticosterone concentrations in equine feces in order to provide a non-invasive evaluation of adrenal activity. Measuring hypothalamic-pituitary-adrenal (HPA) axis activity is an accepted approach to study the response to potentially aversive situations in both captive and domestic species. The reference technique and the most widely used method is the use of blood plasma[1] however, alternate methods such as fecal analysis have been developed in order to overcome the stress induced by blood sampling itself and allow the ability to monitor free ranging species.

During an aversive situation, physiological homeostasis is disrupted. The hypothalamus in the brain releases corticotrophin releasing hormone (CRH) which acts on the anterior pituitary gland and stimulates release of adrenocorticotrophic hormone (ACTH). ACTH enters the bloodstream and stimulates the adrenal cortex to secrete species specific glucocorticoids (GC). Glucocorticoids are closely linked to stressful events rather than being consistently produced in all energy heightened states therefore they are often measured in preference over other stress linked hormones[2]. Glucocorticoids are responsible for several adaptive effects in horses. Energy is rapidly mobilised from storage sites in the body in the form of fatty acids and glucose, oxygen intake is increased, sensory function is enhanced[3] and blood flow is decreased to areas not necessary for movement[4]. As well as acting as a coping mechanism, the stress induced rise in Glucocorticoids may also help to prepare the animal for the next stressor[5].

Assessing hormone levels in the plasma and saliva involves measuring the actual circulating hormone however, measuring the metabolites in the faeces measures the metabolic end product of the hormone. Circulating steroids are catabolized in the liver before excretion in to the bile where they undergo further changes facilitated by the enzymatic activities of bacterial flora within the intestinal track[6]. Therefore, immunoassays directed towards blood glucocorticoids may not be suitable for analysis of fecal glucocorticoid metabolites[7].

As fecal collection can be carried out with no disturbance to the horse, analysis of feces for corticosterone, has been used extensively to monitor HPA activity in a number of circumstances. Elevated corticosterone in the feces of horses has been reported in response to potentially aversive situations including during post-operative veterinary treatment[8] and in restrictive housing[9]. Fecal sampling reflects a pooled glucocorticoid level over time rather than the point in time sampling offered by plasma and saliva making it appropriate for monitoring long term, chronic or seasonal patterns[10]. Due to the non-invasive nature of the method, samples can be collected repeatedly for an individual without the need for capture or restraint[11]. However, species specific gut transit time must be taken into account when planning a sampling protocol. In horses, gut transit time is around 18 hr[12] therefore, adrenal response and subsequent corticosterone metabolites can be detected in the feces one day after initial activation of the HPA axis.

When utilising non-invasive immunoassay techniques a careful validation for the species being investigated is essential[13]. In addition, sex differences in hormone metabolite excretion have been reported probably due to differences in metabolic rate and type of corticosterone

metabolite excreted in various species including mice[14], and chickens[15]. It was therefore important as part of this method that the assay was validated for use in both male and female domestic horses as is detailed in the protocol. This difference in hormone metabolism between genders has consequences for data quality yet it is rarely addressed and included as part of assay validation.

This non-invasive method allows long term assessment of adrenal activity in domestic horses. The protocol details both the validation of the assay and the assay technique itself.

Protocol

Ethics statement: procedures involving field sampling and animal subjects have been approved by the School of Animal, Rural and Environmental Science (ARES) at Nottingham Trent University.

1. Collection of Fecal Samples

NOTE: Gloves should be worn when handling fecal samples and methanol. If there is a strong suspicion that an animal could be suffering from a zoonotic disease, protective clothing such as a lab coat should also be worn.

1. Collect the fecal samples as soon as possible (within min to a few hr) following defecation and place them into a sample bag. Take three to five subsamples of any defecated sample with a total weight of approximately 10 g. Label the bag with the time and date of collection, individual ID, and freeze the sample at -20 °C until analysis.
2. To minimize error, freeze all samples at -20 °C until the study is complete then gather the samples together for analysis as a single batch.

2. Fecal Hormone Extraction

1. Leave the fecal samples to thaw at RT.
2. Ensure that the samples are homogenized by manually mixing each sample in its own sample bag.
3. For each sample weigh 0.50 (± 0.003 g) of fecal material on a microbalance in a weigh boat then transfer the feces to a 8 ml glass vial. Use a new weigh boat for each sample and clean the tweezers with 30% methanol between each sample.
4. Combine the 0.5 g of fecal material with 5 ml of 90% methanol: water, and shake O/N on an orbital shaker at RT.
5. The following morning, vortex the sample and then centrifuge for 20 min at 600 g.
6. Decant the methanol fraction into a 16 x 125 mm^2 glass tube and place in a rack in a water bath set to a temperature of 37 °C. Evaporate to dryness using air in a fume cupboard. Rinse the extraction vial and place the remaining fecal pellet into a clinical waste bag for disposal.
7. Re-suspend the fecal extracts in 100%, 1 ml methanol and transfer to a 12 x 75 mm^2 polypropylene tube. Immediately cap and store at -20 °C until analysis.

3. Hormone Analysis

1. Carry out the analysis with an enzyme linked-immunoassay (EIA) using polyclonal corticosterone CJM006 antiserum.
   NOTE: A second EIA was tested (Cortisol R4866 antiserum, but this failed to achieve the biochemical validation step (See Section 4.1). The Polyclonal corticosterone CJM006 antiserum and Cortisol R4866 antiserum and the respective HRP's are supplied by CJ Munro, University of California, Davis, CA.
2. Dilute the antiserum at 1:15,000 in coating buffer (0.05 M NaHCO3, pH 9.6) using a repetitive pipette and a 50 µl pipette tip, load a 96-well microtitre plate with 50 µl/well. Cover the plate with a plate sealer and incubate O/N at 4 °C. Leave two wells uncoated to represent the non-specific binding wells (NSB).
3. Immediately before use wash the microtitre plates five times using an automated microtitre plate washer (wash solution; 0.15 M NaCl, 0.05% Tween 20).
4. Load the plates with 50 µl per well for the NSB's and 'zero' wells [EIA buffer (0.1 M $NaPO_4$, 0.149 M NaCl, 0.1% bovine serum albumin, pH 7.0) only], standards (corticosterone, 3.9 - 1,000 pg/well) or fecal extract samples (appropriately diluted at 1:20 EIA buffer, see section 4.1). Standards and zeros should be run in triplicate and quadruplicate, respectively. Diluted fecal extract samples and NSB's should be run in duplicate.
5. Follow this immediately with addition of 50 µl/well of the label horseradish peroxidase conjugate[I] diluted in EIA buffer to 1:70,000.
6. Incubate the microtitre plates in the dark for 2 hr at RT.
7. Wash the microtitre plates with wash solution 5 times and incubate the plates at RT in the dark with 100 µl/well of RT substrate [0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6 mM H_2O_2, 0.05 M citrate, pH 4.0]. Prepare the substrate immediately prior to use.
8. Use a spectrophometer at a wavelength of 405 nm to read the microtitre plates. Incubation of plates is considered complete when the optical density of the zero wells reaches 0.8 to 1.0.
9. To insure repeatability, use controls [EIA standards or a biological sample diluted in EIA buffer to bind at approximately 30% (EIA standard), 50% (a biological sample *e.g.,* pool of domestic horse fecal extract) and 70% (EIA standard)] to demonstrate an intra- and inter-assay coefficients of variation of < 10% and < 15% respectively.

4. Enzyme Linked-immunoassay: Validation for the Study Species and Sex

1. Biochemical Validation (Parallelism)
   1. Using EIA buffer, perform a serial dilution on pooled fecal extract, ideally taken from suspected high and low hormone concentration samples from several individuals (range: neat, 1:2 to 1:8192) and run on the EIA as described in section 3.

NOTE: A parallelism is considered achieved when the serial dilutions of fecal extracts yield a displacement curve parallel to the standard curve and linear regression results demonstrate R2 > 0.90, a slope > 0.50 and p < 0.05. The serial diluted fecal extract that binds around 50% on the standard curve should be considered the ideal dilution to run all fecal extracts (*e.g.,* 1:20). There is no interference considered on the assay when the linear regression results demonstrate R2 > 0.90 and p < 0.05.

2. Biochemical Validation (Interference)
    1. Spike 200 µl of serially diluted standards (zero, 3.9 to 1000 ng/well corticosterone) with 200 µl of the appropriately diluted pooled fecal extract [diluted at the 50% binding determined by parallelism (1:20)] and run on the EIA as in section 3. Following EIA analysis plot the observed concentration minus the background (concentration of the zero sample) versus the expected concentration.
    NOTE: If the addition of diluted pooled fecal extract to standards does not significantly alter the amount expected and linear regression results yields R^2 > 0.90, a slope close to 1.0 and p < 0.05. No interference with the EIA has been achieved.

3. Biological Validation:
NOTE: A demonstration that the chosen EIA has the ability to measure biologically relevant changes of the hormone of interest [*e.g.,* the rise in glucocorticoid concentrations following a pharmacological (ACTH is often a licenced procedure) or biological challenge (*e.g.,* psychological and/or environmental; more likely to be an opportunistic unregulated event)].
    1. Select, extract and analyse fecal samples for glucocorticoid metabolite concentrations using the process detailed in sections 1 - 3 before and after a challenging event.
    NOTE: A challenging event will be species specific. An example method is provided below for the domestic horse. Full examples of challenging events for horses have been published previously including the use of management[9] and husbandry procedures[16].
    A significant rise in glucocorticoid metabolite concentrations following the challenging event must be observed and confirmed using statistical analysis. This must incorporate where appropriate, the effect of repeated measures, sample date and individual. Biological validation is then considered achieved.

Representative Results

Domestic horses (n = 16, 8 mares, 8 geldings) with a mean age of 15 years (± 3) were grouped according to gender and subjected to four housing designs with increasing levels of social isolation (n = 4 horse/treatment). Housing 1 involved horses living in a herd environment, closely simulating their natural habitat. Housing 2 involved horses living in pairs in an indoor barn. Housing 3 involved horses housed alone in stables but with visual contact to other horses and housing 4 involved total isolation of the horses.

Exposure to each treatment was in a randomized block design for a period of five days. Following this the horses were turned out into grass paddocks in their experimental groups for two days before exposure to the next housing treatment. Fecal samples were collected once per day on days 1, 2, and 3 spent within each housing treatment from n = 8 horses (n = 2 horses within each treatment). Fecal samples were collected as soon as possible and within 1 hr after defecation. All samples were collected after 1,200 hr on the first full day of stabling meaning that the first sample on day one was collected at least 20 hr after the horse was introduced to the housing treatment. Samples from day one, two and three (total of 24 samples per housing design) were assessed for fecal corticosterone levels reflective of the past 18 hr due to rate of passage of digesta[12]. The same individual horses were used for fecal analysis throughout the study. For full details of this biological challenge, including additional measured parameters please refer to the published work[9] Alternate examples of challenging events for domestic horses can also be found in the literature[16].

This section provides example results of a biochemical validation (parallelism) for both corticosterone and cortisol in male and female domestic horse feces. This section also provides representative data obtained from fecal extraction and EIA analysis. Results were obtained as part of a larger study that investigated the impact of social isolation caused by housing design, upon equine behaviour and physiology[9].

The results of the biochemical validation revealed that the corticosterone EIA was appropriate to measure adrenal activity [**Figure 1A** male sample % binding = 25.349 + 0.729 (standard % binding), R2 = 0.9545, F (1, 7) = 146.710, p less than 0.001); **Figure 1B** female sample % binding = 29.989 + 0.7198 (standard % binding), R2 = 0.95594, F (1, 7) = 151.865, p less than 0.001] but not the cortisol EIA [**Figure 1C** male sample %binding = 79.089 + 0.0629 (standard % binding), R2 = 0.175, F (1, 7) = 1.485, p =0.262); **Figure 1D** female sample %binding = 81.652 + 0.0772 (standard % binding), R2 = 0.257, F (1, 7) = 2.422, p = 0.164]. As the corticosterone EIA demonstrated parallelism unlike the cortisol EIA and interference test on the corticosterone EIA revealed no evidence of matrix interference, as addition of diluted faecal extract to corticosterone standards did not alter the amount expected (male: Observed = 1.302 + 1.017 (Expected), R2 = 0.9972, F1, 7 = 1287.128, p <0.001; female: Observed = 0.3169 + 1.0931(Expected), R2 = 0.9972, F1, 7 = 2432.65, p <0.001;).

The results of the EIA revealed that there was no significant difference in corticosterone levels between male horses (M = 37.7, SD = 14.1) and female horses (M = 33.8, SD = 12.7; t (70) = 1.23, p = 0.22). The level of fecal corticosterone increased as the level of isolation increased. Isolated horses (housing design 4) had significantly higher levels of fecal corticosterone compared to all other housing designs (Wilks Lambda = 0.58, F (3, 18) = 4.29, p = 0.01, multivariate partial eta squared = 0.42; p ≤0.02). The level of fecal corticosterone was higher for all horses on all three sample days during the most isolated housing when compared to the other housing treatments. The concentrations of mean fecal corticosterone for each housing design for each day are presented in **Table 1**.

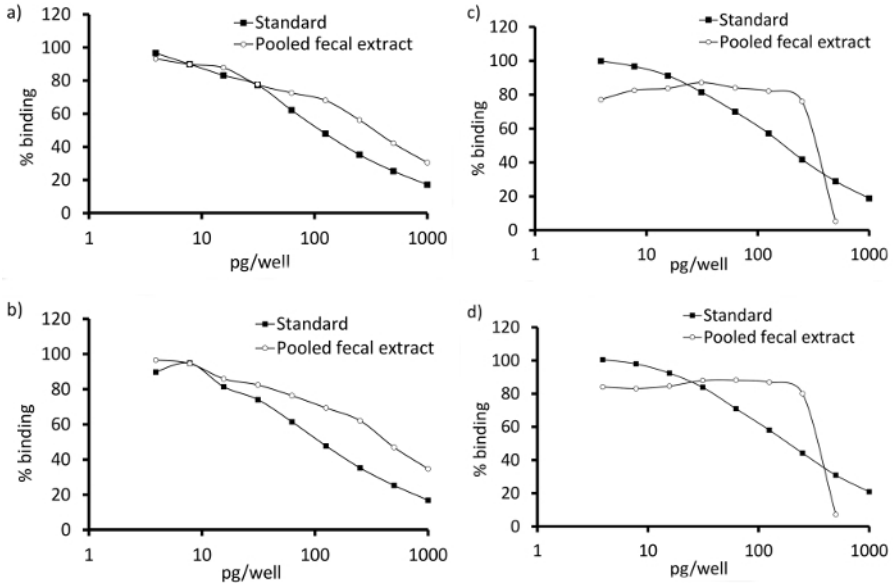

Figure 1. Biochemical Validation (Parallelism) of a Corticosterone and Cortisol Enzyme Linked-Immunoassay for Male and Female Horses. This figure details the percent binding of serial diluted pools of male and female domestic horse fecal extracts against increasing corticosterone and cortisol standard curve concentrations on an EIA. The results demonstrate that the corticosterone (**A,** male; **B**, female) and not the cortisol EIA (**C**, male; **D**, female) yielded a successful displacement curve parallel with the corticosterone EIA standard curve. Please click here to view a larger version of this figure.

	MEAN (±SD) FECAL CORTICOSTERONE (ng/g) IN EACH HOUSING TREATMENT			
HOUSING TREATMENT	**Day 1**	**Day 2**	**Day 3**	**OVERALL**
1	31.73 ± 10.2	32.18 ± 8.0	29.22 ± 5.9	31.05 ± 7.8
2	32.75 ± 10.0	33.66 ± 12.9	34.67 ± 9.3	33.69 ± 10.3
3	35.06 ± 14.6	35.14 ± 15.9	33.13 ± 12.5	34.44 ± 13.6
				*
4	38.16 ± 17.8	42.00 ± 16.7	41.52 ± 17.6	40.56 ± 16.5

Table 1. Mean (± SD) Fecal Corticosterone (ng/g) for each Housing Treatment for Days One, Two and Three and Mean Fecal Corticosterone (ng/g) for all Three Days (Overall) in each Housing Treatment. This table shows the mean values for fecal corticosterone (ng/g) ± standard deviation for each of the housing treatments during day one, two and three. Samples were collected at least 20 hr after horses entered the housing. It also shows mean fecal corticosterone (ng/g) ± standard deviation for all three days (overall) in each housing treatment. The fecal corticosterone concentration was significantly higher (*) during the isolated housing design. The lowest concentration of fecal corticosterone for all days was found in the group housed treatment (treatment 1). Adapted from Yarnell *et al.* (2015), with permission from the Journal of Physiology and Behaviour.

Discussion

Fecal corticosterone analysis provides a means of assessing long term patterns of adrenal activity in horses. The non-invasive nature of the method overcomes the confounding effects of other sampling methods used to assess adrenal activity including saliva and plasma analysis[9]. In addition the technique has a clear non-invasive advantage if studying free ranging horses.

There are several key points to discuss regarding this method and its appropriate use. A critical step in the protocol is the validation of the assay for the species in question and appropriate antibody choice. If the antibody used cross-reacts with metabolites of structurally similar but functionally different glucocorticoids this could confound results[17]. Therefore, assays to measure hormone metabolites require a careful physiological or biological validation, details of which exist in the literature[18,19] and within the protocol described.

The condition of the fecal sample needs to be considered including freshness of the sample and environmental exposure which have both been shown to affect hormonal metabolite levels[20]. Excessive rain or sunshine exposure are known to cause increases or decreases in metabolite concentrations due to bacterial metabolization[21]. Researchers who wish to collect samples of varying degrees of freshness must investigate the

effects of time on sample integrity, in addition to environmental impact. This can be done prior to the study by setting up a small experiment and periodically sampling faeces from male and female individuals, with varying degrees of freshness and exposure to climatic variables.

In most research scenarios, samples from known individuals will be helpful or necessary[10]. In domestic horses this can be achieved by monitoring a group for defaecation or using ingested food markers to identify feces from individuals in larger groups[12]. In free ranging species this may prove time consuming as individual tracking will be required.

When selecting the method of glucocorticoid analysis it must be considered that the results of fecal corticosterone assay represent a pooled sample over time rather than a point in time measure; therefore, there will be less fluctuation in the data. If the researcher is interested in long term effects of a situation, husbandry technique or management practice then fecal analysis offers an appropriate technique. If assessment of short term patterns in hormone concentration is required then plasma or salivary analysis would be the preferred method.

Disclosures

The authors have nothing to disclose.

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