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of LC-MS/MS measurements, considerations for sample extraction and examples of recent applications. We furthermore give estimates of the costs for the purchase, maintenance of LC-MS/MS systems and of sample measurements.

TMe3

Steroid extraction:

Get the best out of faecal samples

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Measurements of steroid hormone metabolites in faecal samples as non-invasive parameters for reproductive functions and stress hormone levels have become increasingly popular. The extraction of these steroids from the faecal matrix represents the initial step before quantification by different immunoassay techniques can be performed. The steroid metabolites present in the faecal matrix are of varying polarity and composition. Therefore the selection of a proper extraction procedure is essential. Furthermore, extraction should be kept as simple as possible, because additional steps increase the variation of determined concentrations. Some studies have already dealt with this complex but rather unnoticed matter. Radiolabelled steroids (e.g. cortisol or progesterone) were added to faecal samples to estimate the efficiency of used extraction procedures. However the added steroids are normally not present in the faeces and therefore results are artificial and do not accurately reflect the actual recoveries. In this respect recovery experiments based on faecal samples of radiometabolism studies are more helpful. Their metabolite content reflects the mixture of GC metabolites actually present in the given species very accurately. Consequently, in this study the evaluation of different extraction methods in faecal samples of sheep, horses, pigs, dogs (¹⁴C-steroids) and mice (³H-corticosterone) utilized samples containing the naturally metabolized, radiolabelled steroids. Based on our results, we recommend extracting faecal steroids by simply suspending the faeces in a high percentage of alcohol (for glucocorticoid metabolites 80% methanol

proved best suited for virtually all mammals tested so far). This significantly increased total radioactivity recovered, but also the relative portion of unconjugated metabolites, which are more likely to be recognized by the antibodies used in various immunoassays. Therefore the advantages of this extraction procedure are plain: it is easy to use (no evaporation step is needed), yields high recoveries and variation based on the extraction procedure is reduced to a minimum.

TMe4

A practical field extraction method for non-invasive monitoring of hormonal activity in the black rhinoceros, *Diceros bicornis*

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Non-invasive hormone analysis is a vital tool in assessing animal welfare and reproductive status, and is commonly utilized in zoos to improve breeding success and animal husbandry. However, it becomes harder to employ these techniques away from controlled laboratory conditions. Field researchers may find themselves without access to reliable electricity, which poses a problem when faecal samples need to be frozen to prevent sample degradation and stored for extended periods. The aim of this study was to develop a field-friendly technique for non-invasive monitoring of hormonal activity in the black rhinoceros, *Diceros bicornis*. Faecal samples were collected from male and female black rhinos, and a field extraction protocol developed which comprises two parts. The first stage allows the extraction of hormone metabolites from a faecal sample under field conditions; this extract is then loaded onto a C-8 Hypersep solid-phase extraction cartridge (Thermo Scientific) which can be easily stored in the field. The second stage of extraction can then continue back in the laboratory following the normal protocol for processing faecal extracts. This technique was validated on testosterone (R156/7, UC Davis), progesterone (CL425, UC Davis) and corticosterone (CJM006,

UC Davis) assays through parallelism and matrix interference assessment. Samples collected over a six-week period from one male (n=20) and one female (n=20) black rhinoceros were also used to demonstrate comparable results between the current laboratory method and the developed field extraction method. Results from all three hormones remained consistent, even after cartridges had been stored in either cool/dry or warm/humid conditions for 1, 2, 3 and 6 months before re-extraction of hormone metabolites for analysis (n=5 male and n=5 female for each condition). This technique for storing faecal extracts could be applied to a wide variety of species, and allows samples to be stored in the field for up to six months, without degradation of hormone metabolites.

TMe5

The confounding effect of GI activity on reliability of faecal glucocorticoids as biomarkers of stress

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Faecal corticosterone metabolites (FCM) have proven an invaluable tool in evaluating laboratory rodents' response to stressors; capable of estimating HPA axis activity when other measures come up inconclusive. However, some basic questions related to validation of this method remain unanswered. Whereas exogenously administered ACTH will produce a subsequent increase in FCM, will a stressful experience – of a certain duration and magnitude – under all circumstances do the same? And conversely, is an increase in FCM always preceded by elevated levels of circulating glucocorticoids? Falling between assay validations and applied experiments, these questions are often neglected.

When applying FCM as a straight-up metric we have found discrepancies with potentially worrisome consequences. When GI motility/loading is changed by surgery-associated temporary ileus or change in diet, mechanisms currently unaccounted for step in, compromising the reliability of FCM as measures of stress. Expressing FCM per weight of excreted dry mass is especially vulnerable to these effects as this approach inadvertently masks the effects. In recent

studies we demonstrated that the stress reaction, post-anaesthesia, in a mouse can be readily detected as an elevation in FCM. Combining the anaesthesia with a surgical procedure capable of inducing ileus will however abolish the elevation in FCM. In a less radical setting we further demonstrated that a change in diet could lead to a 30% reduction in excreted FCM which in turn, expressed per weight of dry mass, could be misinterpreted as more than a 50% increase.

In summary, the controversy over how to usefully express measures of FCM and the radical effect a change of diet can have will be discussed, as well as how surgical procedures may influence FCM, and disqualify these measures in relation to post-surgical pain and distress. Far from having all the answers, perhaps we can offer the right questions to ask.

TMe6

Is stress of the female bird reflected in her eggs?

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Quantification of corticosterone in bird eggs has been performed to non-invasively assess stress and to study effects of maternal stress on offspring. This study focused on validating applied analytical methods. Performing these validations, we found that in egg yolk and albumin of domestic chickens, our in-house corticosterone antibody cross-reacts with other steroids, mostly gestagens. Because of the high concentrations of yolk progesterone, pregnenolone and others, even low cross-reactivities seriously confound corticosterone measurements. These findings were consistent across all bird species examined to date (domestic chickens, jungle fowl, starlings, barn owls, Japanese quail, rockhopper penguins, imperial shags). In addition, we evaluated two commercial corticosterone immunoassays and compared them to the in-house assay. Although percentages of cross-reactivities with progesterone differed greatly between the antibodies, high-performance liquid-chromatographic (HPLC) immunograms of yolk