Measuring Fecal Steroids

Guidelines for Practical Application

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ABSTRACT: During the past 20 years, measuring steroid hormone metabolites in fecal samples has become a widely appreciated technique, because it has proved to be a powerful, noninvasive tool that provides important information about an animal's endocrine status (adrenocortical activity and reproductive status). However, although sampling is relatively easy to perform and free of feedback, a careful consideration of various factors is necessary to achieve proper results that lead to sound conclusions. This article aims to provide guidelines for an adequate application of these techniques. It is meant as a checklist that addresses the main topics of concern, such as sample collection and storage, time delay extraction procedures, assay selection and validation, biological relevance, and some confounding factors. These issues are discussed briefly here and in more detail in other recent articles.

KEYWORDS: steroid hormones; estrogens; gestagens; androgens; glucocorticoids; noninvasive monitoring; feces/faeces; validation; sex differences; stress

INTRODUCTION

Noninvasive methods of measuring fecal steroid metabolites to assess an animal's endocrine status were pioneered in the late 1970s (birds[1]) and early 1980s (mammals[2,3]) and have been established during the past two decades in an increasing number of species. These methods are now widely used to investigate hormone–behavior relationships, as well as questions in the fields of reproduction, animal welfare, ecology, conservation biology, and biomedicine (for a review, see Refs. 4–9). Because metabolism and excretion of steroids differ significantly between species, and sometimes even between sexes, these noninvasive methods must be rigorously validated for each species before application. Researchers who are not familiar with these endocrine techniques and who want to use them as a noninvasive tool in their field of research need to be especially aware of this caveat. Therefore, the following guidelines highlight the main points of concern and serve as a kind of checklist that briefly addresses these topics.

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MAIN POINTS OF CONCERN

Sample Collection and Storage

Undoubtedly, the best option is to collect a sample shortly after defecation and to freeze it immediately.[7–9] However, this is not always possible, especially in field studies. Because several authors have highlighted that fecal steroids are not stable and undergo metabolism,[6–8,10] different preservatives were evaluated to find one that does not compromise results. Bacterial enzymes are the main source for the observed metabolism; therefore, water has to be removed (by drying or lyophilization) or heat or chemical substances, such as alcohol or acids (the latter is necessary when import regulations demand disinfection7), have to be applied.[9,10] However, it is important to notice that, for example, the addition of alcohol to the sample will initiate steroid extraction, and its loss will bias the results. It is therefore advisable to standardize the amounts of both the feces and alcohol, and to use tightly sealed vials to avoid any loss by leakage. Because increased levels of fecal steroid metabolites have been reported in mammals,[7,11] caution is advised when samples are stored in ethanol for long periods of time. However, storage experiments also should be performed on bird feces.

Because steroids are often not evenly distributed within fecal samples,[7,12] homogenization of samples is recommended. In birds, feces and urine are excreted together in a species-dependent way[8–9,13–15] in the form of droppings; the relative proportion of both may vary between samples. This may increase sample-to-sample variation; therefore, a standardized protocol (e.g., homogenization or fecal portion alone) for the analysis of droppings is important.

Wet or dry feces are used for analysis, and both measures usually correlate quite well.[5,16] Wet samples are easier to handle and are therefore favored by many researchers.[6,8,10] However, when fresh samples are not available or when undigested materials need to be sorted out, dry feces are used.[7] Furthermore, it is advisable to test the stability of fecal steroids under the same environmental conditions expected during the experiment.[13,14] Fecal bacteria may differ from animal to animal, both qualitatively and quantitatively, which will result in individual differences concerning stability of fecal steroids.[10] Therefore, it is necessary to perform storage experiments with samples from several animals (and both sexes) of a species under investigation, if samples cannot be frozen right away. In addition, attention must be paid to the fact that contamination with water (rain[7]) or urine in mammals may affect concentrations of measured steroid metabolites.

Time Delay of Fecal Excretion

Steroids are metabolized in the liver and excreted via the bile into the gut. Therefore, measured amounts of fecal steroids reflect an event a certain time ago, which allows separation of the experimental and sampling phase. As postulated earlier, on the basis of radiometabolism studies,[12] the gut passage time (confluence of the bile fluid to the rectum) reflects that time delay quite well and therefore provides a rough estimate of the expected delay. This lag time may range from less than 30 min to more than one day, depending on species, sometimes even within species, depending on the activity rhythms of animals.[8,9,13,14,16,17] Knowledge of those delay times of

fecal excretion is crucial for the experimental setup, because these times will determine sampling intervals, depending on the events that should be monitored (e.g., basal values or acute vs. chronic stress). In addition, the number of samples necessary will differ from only a few (e.g., in the case of a pregnancy diagnosis) to many samples per individual (e.g., in behavioral studies to evaluate stress).[6,18]

Extraction Procedures

Before analysis, steroid metabolites must be extracted from the feces. Selection of extraction procedures is a significant concern, because fecal steroid metabolites are a mixture of several metabolites with different polarities.[8–10,15] Nevertheless, extraction should be kept as simple as possible. Additional steps increase the variation of determined concentrations; however, low amounts of fecal metabolites demand more sophisticated extraction procedures. To measure fecal glucocorticoid (GC) metabolites in mammals (and progesterone and androgen metabolites), the recommended procedure is to shake a portion of the wet feces (e.g., 0.5 g) suspended in 5 mL of 80% methanol. This percentage of methanol yielded the highest recovery of naturally occurring metabolites in all species tested so far (for a review, see Refs. 6, 8, 10, and 19). This method is practicable, because no evaporation step is needed; at the same time, it yields high recoveries. So far, similar experiments in birds are lacking, but because of the high portion of polar metabolites, 60% methanol is used for extraction of GC metabolites by some authors.[10,13,14] In most studies reported, no additional hydrolysis step is performed in the course of the extraction (for details, see Ref. 10). Although some authors have described extraction procedures with boiling ethanol, most by now have shifted to protocols using a high percentage of methanol for extraction of the fecal samples.[5,7–10] In some studies, radiolabeled steroids (e.g., cortisol or progesterone) were added to estimate the efficiency of extraction procedures, but their results do not reflect the actual recoveries, because these steroids are normally not present in the feces (for a detailed discussion, see Refs. 9, 10, 15, and 20).

Sample volumes of less than 0.05 g were reported to bias the results.[7] However, this may be caused mainly by spurious correlations, and thus it is unlikely to be meaningful.[15] Nevertheless, measurement error (depending on the sensitivity of the balance used to weigh the samples) may increase with small samples and can be avoided by pooling samples over a longer period of time.[7,9,16]

Selection of an Appropriate Immunoassay

Most steroids, particularly GCs, are heavily metabolized by the liver and in the gut.[6,10] Therefore, cortisol or corticosterone itself is virtually absent in the feces. The same is true for other steroids such as progesterone and testosterone. This is demonstrated by almost all radiometabolism studies conducted so far (for a review, see Refs. 8 and 20), which report only very small amounts, if any at all, of radioactive substances with chromatographic properties similar to the steroids present in the blood. Although the terms “fecal cortisol/corticosterone” and “fecal GCs” (and in analogy, the same for gonadal steroids) are often used in the literature, they are incorrect and should therefore be avoided. Instead, the group of metabolites recognized by the respective immunoassay should be mentioned (e.g., 11,17-

dioxoandrostanes[6,10,19]). Alternatively, the measured metabolites should be called "cortisol/corticosterone (glucocorticoid) metabolites" or simply "immunoreactive cortisol or corticosterone.

On the basis of the diverse array of metabolites present in the feces, it is advisable to apply group-specific immunoassays for their measurement.[4,8,10,19] Radioimmunoassays or enzyme immunoassays are most commonly used. Because of several advantages, the latter are becoming more popular.[10] However, all immunoassays must be validated analytically with regard to sensitivity, accuracy, precision, and cross-reactions with the 5α5β-reduced metabolites present in the feces, as described in detail by Möstl *et al.*[10] To characterize fecal metabolites, high-performance liquid chromatography immunograms should be performed. After chromatographic separation, the presence of immunoreactive metabolites in collected fractions is determined with different assays and, in the case of radiometabolism studies, the radiolabeled metabolites present are measured. This procedure helps to clarify whether and which metabolites derived from the plasma hormones of interest are measured by the applied immunoassays.[8,10,15]

Biological Relevance

After the analytical validation of an immunoassay, a physiological validation must be conducted to demonstrate that an assay technique is capable of detecting changes in the levels of fecal steroid metabolites compared to respective changes of steroid concentrations in the blood. In the case of GCs, a widely used method is an adrenocorticotrophic hormone (ACTH) challenge test.[5,9,15] Many authors who have performed such a stimulation of the adrenocortex in a larger number of animals described a considerable variation between individuals (for a review, see Ref. 9). Therefore, a sufficient number of animals of both sexes should be used. A suppression of the adrenocortical activity (e.g., by dexamethasone) also might be performed for a more profound validation.[9] Different assays are available and should be tested to select the one that shows the most expressed differences between basal and peak concentrations (e.g., after an ACTH test), and thus yields the highest signal-to-noise ratio.[10,15]

However, rigorous physiological validations (and radiometabolism studies) sometimes are not possible (e.g., in endangered species). Even under these constraints, a biological validation should be performed.[9,15] In the case of GC metabolites, serial samples before and after some known stressful events such as immobilization or transportation and for gonadal steroids samples from different reproductive stages can be used to evaluate the biological relevance of such an established noninvasive method.

Confounding Factors

Many factors influencing blood GC levels (and those of other steroids' levels) are expected to be reflected in the concentrations of the metabolites in the feces. These include individual and species differences; daily rhythms; seasonal variations; effects of weather, sex, and age; life-history stages (such as molt or reproductive status); sensitization; and habituation.[7,9,15,16,21] These factors should be kept in mind when designing an experiment to produce meaningful, biologically relevant results.

In general, episodic fluctuations reported in the blood plasma (e.g., androgens, GCs) are smoothed in the feces.[9]

In addition, sex was reported to play a role in the metabolism and excretion of steroids. This is probably the reason why some assays for measuring fecal steroid metabolites yielded good results in one sex, but not in the other.[9,15] The influence of changing diets or metabolic rates (e.g., because of season), especially in birds, has been little evaluated so far,[15] but must be taken into consideration.

CLOSING REMARKS

Even though the highest standards need to remain, compromises cannot always be avoided (especially in field studies). In this case, these compromises should be addressed frankly. Last but not least, it is advisable to search and read the literature carefully. Currently, approximately 140 articles that deal with fecal GC metabolites (for a review, see Refs. 6–9) and an even larger number dealing with fecal metabolites of gonadal steroids[1,14,16] have been published in peer-reviewed journals. The quality of analytical procedures differs significantly among those articles. Analyzing hormone metabolites in the feces may appear to be a quick and easy solution for many problems. In fact, it is not, and there is a strong need to carefully validate assays analytically, physiologically, and biologically in each new species under investigation. Hence, to save time and money and to avoid some of the many pitfalls, it is advisable to contact an experienced laboratory before the start of the experiments.

Applying the recommendations addressed by these guidelines and considering the conclusions of the respective articles in this volume will help to keep the standards of the methods high to get the best out of these noninvasive methods. This will help to establish, confirm, and spread these helpful tools to answer questions related to the endocrine status of an animal (or even a population). In addition, more research is needed to clarify unresolved problems (e.g., standardization among laboratories[10]) or to address open questions (e.g., the influence of diet or plasma binding proteins[9,10,15]).

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