Measuring Fecal Glucocorticoid Metabolites in Mammals and Birds: The Importance of Validation

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ABSTRACT: **In recent years, the noninvasive monitoring of steroid hormone metabolites in feces of mammals and droppings of birds has become an increasingly popular technique. It offers several advantages and has been applied to a variety of species under various settings. However, using this technique to reliably assess an animal's adrenocortical activity is not that simple and straightforward to apply. Because clear differences regarding the metabolism and excretion of glucocorticoid metabolites (GCMs) exist, a careful validation for each species and sex investigated is obligatory. In this review, general analytical issues regarding sample storage, extraction procedures, and immunoassays are briefly discussed, but the main focus lies on experiments and recommendations addressing the validation of fecal GCM measurements in mammals and birds. The crucial importance of scrutinizing the physiological and biological validity of fecal GCM analyses in a given species is stressed. In particular, the relevance of the technique to detect biologically meaningful alterations in adrenocortical activity must be shown. Furthermore, significant effects of the animals' sex, the time of day, season, and different life history stages are discussed, bringing about the necessity to seriously consider possible sex differences as well as diurnal and seasonal variations. Thus, comprehensive information on the animals' biology and stress physiology should be carefully taken into account. Together with an extensive physiological and biological validation, this will ensure that the measurement of fecal GCMs can be used as a powerful tool to assess adrenocortical activity in diverse investigations on laboratory, companion, farm, zoo, and wild animals.**

KEYWORDS: **stress hormones; glucocorticoids; cortisol; corticosterone; HPA axis; noninvasive monitoring; feces/faeces; validation; ACTH challenge test; dexamethasone suppression test; sex differences; diurnal variation; seasonal variation; life history stages; review**

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INTRODUCTION

Hormones, Stress, and the Hypothalamic–Pituitary–Adrenal Axis

Hormones are of great interest to scientists from various fields because they are largely involved in virtually all bodily functions in health and disease, including the regulation of reproduction, development, and the expression of behavior. A wide variety of endocrine factors has been linked to genetic, environmental, and social variation, including gonadal and adrenal steroids, pituitary peptides, growth factors, and biogenic amines. In particular, the so-called stress hormones and the concepts of stress have a very long history of research (going back to the ancient Greeks), as they deal with the daily social and nonsocial stimuli that are challenging or threatening to the survival, health, and reproductive success of animals (for reviews, see Refs. 1–9).

Stress in its broadest sense is well known to have a substantial impact on a variety of bodily functions. Its disruptive effects, for example, on the immune system, reproduction, cognition, and behavior of vertebrates have been broadly demonstrated (for reviews, see Refs. 2, 4, 6, and 8–12). Furthermore, stress hormones have been implicated in a wide range of human disorders, including depression, anxiety, cancer, asthma, cardiovascular diseases, diabetes, and dementia.[2–7,9,11,13,14] Assessing physiological parameters related to stress is therefore essential for the understanding and improvement of animal welfare, health, and reproduction.

When confronted with a stressor (environmental, physiological, or psychological), an individual typically displays a stress response consisting of a suite of physiological and behavioral alterations to cope with the challenge. One of the main mediators of this response is the hypothalamic–pituitary–adrenal (HPA) axis, which is responsive not only to stressors but also to other types of activity that are associated with emotional arousal (e.g., courtship or sexual behaviors).[4,5,8,9] Within minutes of the onset of a perceived stressor, the adrenal cortex begins to secrete glucocorticoids (GCs), mainly cortisol or corticosterone. Which GC is predominantly produced depends largely on the species and should be considered when choosing an appropriate assay system.[15–17] The major GC in most primates, carnivores, and ungulates, for example, is cortisol, whereas in most rodents, birds, and reptiles it is corticosterone. These GCs orchestrate the organism's response to challenges, acting on many organ systems, including the brain, to modulate physiology and behavior.[2,4–9,12,18] The secretion of GCs from the adrenal cortex is regulated by the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which in turn is stimulated by corticotropin-releasing hormone (CRH) and vasopressin (AVP) derived from neurons of the paraventricular nucleus (PVN) of the hypothalamus.[1,2,5–7,9,18] Plasma GC concentrations are therefore widely used to assess stress responses in various species.[2,4,5,8,9,12,19,20,26]

However, constraints of the blood sampling procedure pose some limitations to this approach, particularly for small animals, such as most rodents and birds, or for free-ranging animals. A further limitation of invasive sampling techniques is that circulating hormone levels are affected rapidly in response to the stress of handling, physical restraint, and the blood sampling procedure itself, which can substantially alter physiological and behavioral parameters investigated in the experiment.[4,8,21] An additional drawback of measurements in the plasma is that blood samples repre-

sent concentrations at only a single point. Because steroid hormones and especially GCs may exhibit regular as well as episodic changes over time (i.e., circadian variations and pulsatile secretion patterns),[1,4,5,8] hormone levels representing a very narrow time frame might be biased. However, alternative techniques of measuring steroid hormone metabolites in excreta like feces of mammals or droppings of birds offer a possible solution to overcome some of these problems.

NONINVASIVE MONITORING OF HORMONES

In general, circulating steroid hormones are metabolized by the liver and excreted as conjugates via the kidneys into the urine or via the bile into the gut.[22–26] Although steroids in the gut are subjected to some extent to an enterohepatic circulation (i.e., reabsorption into the blood stream) and are intensively metabolized by the microbial flora, the sterane skeletal structure is not degraded.[22,27,28] Therefore, specific steroid metabolites can be detected in the feces of mammals and in droppings of birds.[16,25,26,29,30] When a lag time between hormonal events in the plasma and the appearance of the respective signal in the feces is considered, a similar pattern to that found in the plasma is reflected in the feces.[16,22,24,26,30] This lag time, which depends mainly on the intestinal transit time from the duodenum to the rectum, is largely species-specific and must be taken into account when comparing endocrine patterns found in plasma and feces.[16,23,26,31] Considering these points, it is possible to use fecal hormone metabolite analyses as a noninvasive tool to assess various endocrine functions in mammals and birds.

In recent years, this completely noninvasive technique has been established in an increasing number of species, ranging from laboratory animals, companion and farm animals, to wild animals (in zoos and in the field). It is now widely used to investigate hormone–behavior relationships as well as various questions in the realms of stress and animal welfare, reproductive physiology, behavioral ecology, conservation biology, and biomedical research (see TABLE 1 and studies discussed in the text).

Using fecal samples offers several advantages. Feces can be collected very easily, and the sampling is feedback free, because there is no need to capture and handle the animal. Therefore, repeated sampling of the same individual is possible without affecting the animal's behavior or its endocrine status. This method allows the monitoring of short-term hormonal changes in reaction to specific situations, social encounters, or treatments, as well as assessing day-to-day changes or even long-term endocrine profiles. In addition, circulating hormone levels in the feces are integrated over a certain period. Hence, rather than the actual steroid concentration, fecal hormone metabolite levels reflect the production rate, that is, the cumulative secretion and elimination of hormones, over several hours.[16,23,26,30] Therefore, unlike blood samples, fecal samples are less affected by episodic fluctuations or the pulsatility of hormone secretion. Consequently, depending on the research question, steroid metabolite concentrations measured in feces might represent the hormonal status of an animal more accurately than a single plasma sample. However, dampening of short peaks of hormone secretion can also be a disadvantage if fecal samples are used to monitor these short-term alterations.

TABLE 1. Selected studies describing experiments to physiologically and/or biologically validate techniques to measure fecal glucocorticoid metabolites as a noninvasive tool to assess adrenocortical activity in different species of mammals and birds

Species		Sample size				
Scientific name	Common name	Males (M)	Females (F)	Physiological validation (pharmacological treatment)	Biological validation (challenging procedure/situation/condition)	Ref.
Mammalia						
Primates						
Macaca fascicularis	long-tailed macaque		10	ACTH (data for 6 shown)		32
Papio cynocephalus	yellow baboon		2	ACTH		32
Pan troglodytes	chimpanzee	1	3		anesthesia	33
Lemur catta	ring-tailed lemur	12			capture+blood sampling, correlation plasma–feces	34
Rodentia						
Mus musculus f. *domesticus*	laboratory mouse	36	36	ACTH, Dex (2 doses each, 12M/12F)	saline injection, diurnal variation (6M/6F, each)	35
Rattus norvegicus f. *domesticus*	laboratory rat	24		ACTH, Dex (6M, each)	saline injection, handling (6M, each, no effects)	36
		10	9		effects of the estrous cycle, diurnal variation	37
Clethrionomys gapperi	red-backed vole	10 (5)			novelty, cold stress	38
		5			confinement in a trap (4/12 h)	39
Peromyscus maniculatus	deer mouse	18/17/16			confinement in a trap (<4/4–8/>8 h)	39
Lagomorpha						
Lepus europaeus	European hare	10	10		effects of rousing	40
Carnivora						
Crocuta crocuta	spotted hyena	4	2	ACTH	translocation (1M) agonistic interactions (1M/1F)	41
Ursus acrtos horribilis	grizzly bear	1	1	ACTH		42

TABLE 1. (*continued*) Selected studies describing experiments to physiologically and/or biologically validate techniques to measure fecal glucocorticoid metabolites as a noninvasive tool to assess adrenocortical activity in different species of mammals and birds

Species		Sample size				
Scientific name	Common name	Males (M)	Females (F)	Physiological validation (pharmacological treatment)	Biological validation (challenging procedure/situation/condition)	Ref.
Carnivora (*continued*)						
Helarctos malayanus	Malayan sun bear		1	ACTH		32
Ursus thibetanus	Himalayan black bear	1		ACTH	aggression (1F), no consistent effects	43
Canis lupus f. *familiaris*	domestic dog	5	5	ACTH, Dex		44
Canis lupus	wolf	2		ACTH (data not shown)		45
		>40 (3 packs)			effects of rank, aggression	45
Canis rufus	red wolf	1		ACTH	various stressors (1M/1F), inconsistent effects	43
Lycaon pictus	African wild dog	2	3	ACTH		46
		34	22		effects of social status	47
Acinonyx jubatus	cheetah	2	2	ACTH		48
			7		immobilization, translocation, mate introduction	48
		2	2	ACTH (data for 1 shown)		32
			1	ACTH	anesthesia	43
Neofelis nebulosa	clouded leopard	2	2	ACTH		49
		36	36		effects of housing condition	49
		2	1	ACTH	anesthesia (1F)	43
		3	2	ACTH (data for 1 shown)		32
Felis silvestris f. *catus*	domestic cat	5	5	ACTH, Dex		44
		1	1	ACTH	anesthesia (1F), no clear effect	43
Suricata suricatta	Slender-tailed meerkat	2		ACTH		43
Enhydra lutris kenyoni	Alaskan sea otter	1		ACTH		32
Mustela nigripes	black-footed ferret	4	6	ACTH (partly inconsistent)	restraint, saline injection (inconsistent effects)	50
Eumetopias jubatus	Steller sea lion	1	2	ACTH (feces + plasma)		51

TABLE 1. (*continued*) Selected studies describing experiments to physiologically and/or biologically validate techniques to measure fecal glucocorticoid metabolites as a noninvasive tool to assess adrenocortical activity in different species of mammals and birds

Species		Sample size				
Scientific name	Common name	Males (M)	Females (F)	Physiological validation (pharmacological treatment)	Biological validation (challenging procedure/situation/condition)	Ref.
Proboscidea						
Loxodonta africana	African elephant	1		ACTH		52
			4 (juv)	ACTH (feces + plasma)	effects of enclosure size (in total, 23F)	53
			2	ACTH (data for 1 shown)		32
Perissodactyla						
Equus caballus	domestic horse	3	3	ACTH, Dex (feces + plasma)		54
		10			anesthesia + surgery (castration)	55
Diceros bicornis	black rhinoceros	4		ACTH		56
		1	1	ACTH (data for 1 shown)		32
Artiodactyla						
Bos taurus	cattle	3	3	ACTH (3 doses), Dex (feces + plasma)		57
			8 + 4		loading into cattle carrier, transportation	58
			5	ACTH (feces + plasma)		59
			10 + 8		novel environment, transportation	59
Ovis ammon f. *aries*	domestic sheep	3	3	ACTH, Dex (feces + plasma)		57
Cervus elaphus	elk/red deer		6	ACTH	saline injection, disturbance	60
Cervus elaphus roosevelti	Roosevelt elk	1	1	ACTH (data for 1 shown)		32
Capreolus capreolus	roe deer	5		ACTH, Dex	capture, injection, transportation (4M)	61
Litocranius walleri	gerenuk	2		ACTH (data for 1 shown)		32
Oryx dammah	Scimitar-horned oryx	2		ACTH (data for 1 shown)		32
Sus scrofa f. *domestica*	domestic pig	3	3	ACTH, Dex (partly inconsistent)		54

TABLE 1. (*continued*) Selected studies describing experiments to physiologically and/or biologically validate techniques to measure fecal glucocorticoid metabolites as a noninvasive tool to assess adrenocortical activity in different species of mammals and birds

Species		Sample size				
Scientific name	Common name	Males (M)	Females (F)	Physiological validation (pharmacological treatment)	Biological validation (challenging procedure/situation/condition)	Ref.
Aves						
Anseriformes						
Anser domesticus	domestic goose	24		ACTH (8M)	social stimulation/confrontation (8M per group)	62
Anser anser	greylag goose	43			effects of higher competition (mating season)	63
		37			correlation with agonistic/courtship behavior	64
Galliformes						
Gallus domesticus	domestic chicken	10	10	ACTH, Dex (no Dex response)		65
			15	ACTH, Dex (feces + plasma, 5F, each)	saline injection (5F, Dex + saline, no clear effects)	66
Tetrao urogallus	capercaillie	3	2	ACTH		67
Tetrao tetrix	black grouse	2	2	ACTH		68
Stringiformes						
Strix occidentalis caurina	northern spotted owl		1	ACTH	translocation (feces + plasma)	69, 32
Passeriformes						
Parus major	great tit	10 + 6			social challenge, clear effects in one line (6M)	70
Saxicola torquata rubicola	European stonechat	7	1	ACTH		71
Spiza americana	dickcissel	10			capture, radio-tagging (partly inconsistent)	72
Columbiformes						
Zenaida macroura	mourning dove	9	7	ACTH (2 doses, 2M/2F each)	saline injection (saline + low dose ACTH, no effect)	73
Sphenisciformes						
Pygoscelis adeliae	Adelie penguin	1	1	ACTH		74

Measuring Fecal Glucocorticoid Metabolites

Prior to the analysis, fecal glucocorticoid metabolites (GCMs) must be extracted from the fecal matrix. Because fecal steroid metabolites are a mixture of several different metabolites with a wide range of polarities, the selection of an appropriate extraction procedure is a serious issue.[15,16,25,30] We recommend extracting fecal steroid metabolites simply by suspending (and shaking) a certain amount of homogenized feces (e.g., a portion of 0.5 g of fresh or dry weight) in a fixed volume (e.g., 5 mL) of methanol (80% in mammals and 60% in birds proved to work best).[15,17,26] This very simple extraction technique is highly practical (no complex apparatuses or evaporation steps are needed) and yielded good recovery levels for virtually all species tested so far.[15,16,26]

Usually, the quantification of fecal steroid metabolites is then performed by using an aliquot of the extract in a radioimmunoassay (RIA) or an enzyme immunoassay (EIA).[15,17,25,26,29] Often, commercially available cortisol or corticosterone kits are applied (for examples, see Refs. 32 and 43). However, the antibodies used in these assays might have some shortcomings, because they are produced primarily to measure the respective unmetabolized steroid in the plasma. As alternatives to these assay kits, different EIAs have been developed that are especially designed for measuring groups of steroid metabolites usually present in the feces (e.g., 11,17-dioxoandrostanes[15,26,75]). These EIAs use so-called group-specific antibodies, which have several advantages for the analysis of fecal GCMs.[15,16,26]

However, there are considerable species- and sex-specific differences in the types of GCMs formed, resulting in a characteristic pattern of GCMs present in the feces of a given species.[15,16,30] Accordingly, it is important to select an appropriate assay system that includes an antibody capable of detecting most, or at least a considerable proportion, of the respective GCMs present in the feces of the species investigated.[15–17,25,26,29,30]

In addition, after defecation, several factors, such as temperature, humidity, and other environmental conditions, may influence concentrations of immunoreactive GCMs in the sample.[15,17,29,54,76–78] Moreover, bacterial enzymes are reported to increase or decrease levels of immunoreactive fecal GCMs if samples are not frozen shortly after voidance.[15,29,54,59,76,77,79] Heat, alcohol, or other preservatives, such as acids, are therefore frequently used, especially in the field, where direct freezing of the samples to avoid further metabolism of the steroids is difficult.[25,29,42,76–81] However, because adding alcohol already starts the extraction, and because fecal GCM concentrations of samples preserved in this manner were also reported to change over time,[29,42,76,81] a careful evaluation of each sampling and storage protocol is necessary and strongly recommended.[17]

Taken together, a careful validation of all protocols, including sample storage, extraction procedures, and the immunoassays used to analyze fecal steroid metabolites, is crucial. In particular, the assay systems (including the respective antibody) should be characterized in analytical terms regarding sensitivity, accuracy, precision, and cross-reactivity with the reduced steroid metabolites present in the feces.

It is also necessary to use the correct nomenclature. In the literature, the term "fecal cortisol/corticosterone" or "fecal glucocorticoid" is often used for the substances measured by immunoassays. However, this implies that the measured substances are biologically active, which is neither known nor proved. Hence, the term is mislead-

ing and should be avoided, because the native, unmetabolized GC (cortisol or corticosterone) circulating in the blood is not present in the feces, but rather their 5α/5β reduced metabolites.[15,16] Instead, these metabolites should be referred to as "fecal glucocorticoid metabolites," or they should be labeled according to the group of metabolites detected by the respective antibody used in the assay.[15,17,26]

IMPORTANCE OF VALIDATION

For a reliable monitoring of adrenocortical activity in mammals and birds using fecal GCM analyses, it is of crucial importance to carefully validate the techniques used.

As pointed out previously, technical and analytical issues, such as sample preservation and stability, extraction procedures, and antibodies used in the assays (RIA or EIA), must be considered for each species (including both sexes). Furthermore, the importance of proving the biological relevance of the technique—that is, if the assay system can detect biologically meaningful alterations in the endocrine status of the animals—cannot be overestimated. As is pointed out in the following paragraphs, experiments dealing with the physiological and biological validity of fecal GCM analyses are essential and must be performed before applying the technique in a given species.

Physiological and Biological Validation

Physiological validation of the technique means to pharmacologically induce physiological changes in circulating GC levels and to evaluate whether these changes are reflected in measured concentrations of fecal GCMs afterward. In this respect, the most widely used experiment to stimulate adrenocortical activity (i.e., increase circulating GC levels) is the so-called ACTH challenge test (see TABLE 1). Ideally, fecal samples are collected frequently a certain time before and after the injection of ACTH, which should result in a significant increase of plasma GC concentrations. This pattern of sharply increasing (and decreasing again later) GC levels should be clearly reflected in the concentration of fecal GCMs after a certain lag time. Examples of studies describing ACTH challenge experiments involving various species of mammals and birds are compiled in TABLE 1. Although there are more than 140 articles published in peer-reviewed journals dealing with fecal GCMs in more than 70 species of mammals and birds, by the time of the writing of this review, convincing validation experiments have been performed on only a few species (see TABLE 1). This situation is especially dramatic in primates, for which only a single article describes ACTH challenge tests in long-tailed macaques and yellow baboons.[32] On the other hand, many such studies have been described in carnivores (including some of the earliest[82]) and ruminants (mainly domestic livestock). In birds, ACTH challenge tests have been performed very recently on only a few species (see TABLE 1).

Similar to findings in the plasma, authors who investigated a substantial number of animals reported considerable individual variation, both in basal and ACTH-induced levels of fecal GCMs (e.g., long-tailed macaque,[32] laboratory mouse,[35] do-

mestic dog,[44] cattle and domestic sheep,[57] domestic goose,[62] domestic chicken[65] see also TABLE 1). In fact, there are a few examples in the literature stating that in some species, certain individuals showed the expected pattern of fecal GCM concentrations after stimulation, whereas inconsistent results were obtained in others (e.g., long-tailed macaque,[32] black-footed ferret,[50] domestic pig[54]). Thus, for a proper physiological validation of the technique, it is strongly recommended to use enough individuals (of both sexes) and not to rely upon results obtained from only one or two individuals of a given species. Furthermore, each animal can be used as its own control, thereby minimizing the problems of individual differences in basal and peak levels of fecal GCMs (i.e., absolute differences or percent increases can be calculated; see also recommendations in Ref. 8).

A second experiment to physiologically validate the measurement of fecal GCMs is to perform the so-called dexamethasone (Dex) suppression test (see examples compiled in TABLE 1). Dex is an artificial steroid that mimics endogenous GCs and reduces circulating corticosteroid levels via the negative-feedback mechanism of the HPA axis.[1,4,5] Therefore, after injection of Dex, a suppression of adrenocortical activity (i.e., decreased concentrations of circulating GCs) is expected and should be reflected in reduced fecal GCM concentrations for a certain period (largely depending on the dosage of Dex).

The Dex suppression test is also very important to analytically discriminate between true GCM measurements and blank values. Because synthetic GCs and their fecal metabolites usually do not cross-react with the antibodies used in the respective immunoassays, the concentrations of naturally occurring GCMs in the feces should be very low after Dex treatment. Therefore, measured concentrations in those samples can be referred to as blank values, or they can reflect contributions of other cross-reacting steroid metabolites, probably of gonadal origin. Although only successfully included in a small fraction of studies in mammals (laboratory mouse,[35] domestic dog,[44] domestic cat,[44] domestic horse,[54] cattle,[57] domestic sheep,[57] roe deer[61]), a physiological validation of the technique using the Dex suppression test is strongly recommended. In birds, a Dex suppression test has been performed in the domestic chicken only. However, for elusive reasons, the expected effects could be detected neither in plasma nor in feces.[65,66] Whether this was caused by a different biological activity of Dex in birds or whether the injected dose was too low still needs to be investigated.

Besides these two pharmacological treatments, it is often argued that a strong positive correlation between concentrations of plasma GCs and GCMs measured in the feces indicates that the applied assay system is valid. However, although this may be true for the relatively slowly changing plasma levels of progesterone, the situation is quite different for GCs and androgens. Diurnal rhythms, as well as episodic fluctuations, result in considerable changes of circulating GC concentrations, even within short periods.[4,5,8] In addition, as will be discussed below, a potentially varying time delay of fecal excretion renders it difficult to select pairs of samples for a meaningful correlation.

Another aspect that can be covered by physiological validation experiments is to investigate the biological sensitivity of an assay used for fecal GCM measurements in a given species; that is, to evaluate which (small) alterations in adrenocortical activity, and thus plasma GC levels, can be reliably detected in the feces. This can be achieved by administration of different dosages of ACTH or Dex, respectively (dose-

response effects). However, up to now, such experiments have been described only for laboratory mice (low and high doses of ACTH and Dex, respectively[35]), cattle (a range of different ACTH doses, 0.06–3 mg[57,83]), and mourning doves (two different doses of ACTH[73]). In cows, Palme *et al.*[57,83] also reported that the percent increase of fecal GCMs above basal levels (but not the absolute values of fecal GCM concentrations, and neither absolute nor increase of plasma cortisol) was correlated with the administered dose of ACTH. This finding suggests that fecal GCM concentrations reflected the amount of secreted GCs better than plasma levels. This is especially important because very high plasma GC levels (induced by extremely high dosages of ACTH, as have been used in several studies in the literature) may very well be reflected in the concentration of fecal GCMs, but this might not be the case for smaller or moderate stimulations ("iceberg effect").

In addition to a careful physiological validation, experiments proving the biological validity of the technique are also important. That is, serial samples before and after a known stressful event like capture, immobilization, or transportation can be used to evaluate the biological relevance of an established technique. Such experiments have been described for a number of species from various taxa (see TABLE 1). Besides the previously mentioned procedures, others, such as anesthesia,[33,43] confinement/restraint,[39,61,72] disturbances caused by the presence of humans,[40,60,84] novelty,[38] agonistic encounters/social challenges,[41,48,62,63,85] different housing conditions,[53,83,86] and translocation[41,48,59,87] were reported to influence fecal GCM levels (see also TABLE 1). As an experiment to investigate the biological validity of the technique, it can also be useful to assess effects of injection procedures (e.g., injecting saline solution) or blood sampling.[35,60] Furthermore, measuring whether the naturally occurring diurnal variation of GCs is also reflected in the feces of a given species can indicate biological relevance.[35,37]

In endangered or intractable species, however, a rigorous physiological validation might not be possible. Nevertheless, even under these constraints, at least experiments to biologically validate the assay technique must be performed to produce reliable results (see also recommendations in Refs. 17 and 30).

Taken together, immunoassays for the assessment of fecal GCMs must be extensively validated. Besides analytical issues, such as demonstrating that the antibodies used cross-react to a considerable extent with GCMs present in the feces, experiments scrutinizing the physiological and biological validity of the technique must be performed. For a physiological validation, it is recommended to use pharmacological stimulation and suppression of adrenocortical activity (by injecting different doses of ACTH and Dex, respectively), inducing specific changes in circulating GC concentrations that should be reflected in fecal GCM concentrations afterward. Furthermore, a biological validation should be performed using different stressors relevant to the animal (e.g., restraint, injections, blood sampling, transportation, agonistic interactions), demonstrating that the technique can detect biologically meaningful changes in circulating GC levels.

As described in the Introduction, various factors can affect the levels of GCs in plasma and concentrations of GCMs in feces of animals. Besides differences between species, effects of age, social status, or early life experiences (prenatal or postnatal), gender differences as well as diurnal and seasonal variations (including life history stages) on GC levels are of special importance for the noninvasive monitoring, and are therefore addressed further in the following sections.

Gender Differences

Males and females differ with respect to various physiological and behavioral aspects. Several studies have shown pronounced gender differences regarding baseline levels of GCs as well as the reactivity of the HPA axis to stressors (e.g., laboratory mouse,[88] laboratory rat,[89] rabbit,[4] arctic ground squirrel,[90] guinea pig,[91] domestic sheep,[92] European starling,[93] Inca dove[19]).

Consequently, these differences in plasma concentrations also affect concentrations of GCMs assessed in fecal samples. Similar to findings in the plasma, several studies investigating fecal GCM levels in both sexes report higher concentrations in females (common marmoset,[94] northern muriqui,[95] laboratory mouse,[35] European hare,[40] domestic dog,[44] African wild dog,[47] domestic cat,[44] cheetah[86]), males (laboratory rat,[37] Steller sea lion,[96] domestic chicken[65]), or no difference between the sexes (wolf,[45] black rhinoceros,[56] white rhinoceros,[56] elk/red deer,[97] mourning dove[73]).

Different factors may be responsible for these gender-specific differences. First, higher plasma values (observed mostly in females) should also result in higher fecal GCM concentrations. This effect, with females having higher plasma GC levels than males, is thought to be brought about by a higher capacity of steroid-binding globulins expressing certain affinities to GCs;[98] that is, in females, circulating GCs are also bound to a considerable extent to gonadal steroid binding globulins, and therefore the total GC concentration can be higher. This might also be why GC concentrations vary significantly across the female's estrous cycle, as changing concentrations of estrogens and progesterone are known to influence the expression and the occupancy of plasma steroid-binding globulins.[3,98] In laboratory rats, for example, significant alterations of plasma GCs as well as fecal GCMs were reported across the estrous cycle, with highest levels occurring on the day of proestrus.[37]

Second, the level of metabolites excreted via the urine or via the feces might differ significantly between males and females. Touma *et al.,*[99] for example, showed in laboratory mice that males excreted about 73% of radioactive corticosterone via the feces, whereas females excreted only about 53% via the feces. Hence, females eliminated a larger fraction via the urine. Similar findings were reported for horses and cats.[23,44]

Third, the GCMs formed might differ significantly between males and females. High-performance liquid chromatography immunograms performed with fecal samples from different species revealed considerable gender-specific differences regarding the structure as well as the quantity of fecal GCMs (laboratory mouse,[99] laboratory rat,[37] domestic chicken,[65] European stonechat[71]). Because the cross-reactivity of the antibodies used in a given assay strongly depends on the biochemical structure of the steroid, these differences are likely to bring about different concentrations of immunoreactive GCMs in males and females (see also Ref. 16).

Moreover, especially when males with high plasma levels of androgens are investigated (e.g., dogs[44] or elephants in musth[52,100]), it should be carefully monitored that the antibody used in the respective assay does not cross-react with androgen metabolites present in the feces. In such cases it might be advisable to use different assays for males and females (e.g., domestic cat,[43] African elephant[52,53]).

Taken together, possible gender-specific effects should be carefully evaluated and gender differences should be seriously considered when measuring fecal GCMs in

males and females of a given species. This is especially important in field studies, when samples from unknown individuals are collected, and therefore fecal GCM concentrations might be biased by the gender of the animals.

Diurnal Variations

Well-defined circadian rhythms of plasma GCs (with peak levels 5–10 times higher than trough levels) have been described in most vertebrate species (e.g., laboratory mouse,[101] laboratory rat,[102] tree shrew,[4] squirrel monkeys,[103] domestic chicken,[104] white-crowned sparrow,[105] house sparrow,[106] European starling[93]). Usually, the peak of hormone secretion occurs toward the end of the dark period in primates and other diurnal animals, whereas in primarily nocturnal animals like most rodents and cats, there is a peak toward the end of the light period. Therefore, it is obviously important to sample GCs at the same time of day if repeated measurements are to be made on different days or if comparing different groups/populations of animals.

This diurnal variation of GCs should also be taken into account for the monitoring of hormone metabolites from fecal samples. So far, only a few studies have addressed this point, but diurnal variations of GC metabolites have been observed in fecal samples of some mammalian and bird species (common marmoset,[94] long-tailed macaque,[108] laboratory mouse,[35] laboratory rat,[36,37,107] domestic goose,[62] great tit[70]). Particularly in small animals, which usually defecate more frequently (i.e., providing a higher temporal resolution in the feces), a distinct circadian rhythm of fecal GCM excretion is expected and has been documented in detail for mice and rats.[35,37] However, in species with a relatively long gut passage time (e.g., hind-gut fermenters) or animals that defecate rather infrequently (e.g., most carnivores and reptiles), it might be impossible to detect diurnal changes of circulating GC levels in the feces.

Thus, information on the animals' activity rhythm, gut passage time, and defecation rate should be considered when planning the fecal sampling regimen for a given species. Complex interactions between these parameters may also exist. For example, in mice, the amount of feces produced varied during the course of the day (in accordance with the animals' activity pattern) and thereby influenced the lag time of fecal GCM excretion.[99] Similar effects are likely to exist in other species as well.

To consider diurnal changes and to avoid possible effects of the time of sampling, an option is to collect and combine all samples defecated by an individual over 24 h. These pooled samples are likely to represent the hormonal status of an animal more accurately, because individual differences or shifts in activity patterns and/or excretion profiles are compensated for (cf. Ref. 109).

In other cases, however, the diurnal variation of GCs might be an important parameter to monitor. In humans, for example, several pathologic states have been associated with alterations in the circadian rhythm of different endocrine parameters including GCs (e.g., depression, anxiety disorders, Parkinson's disease, or Alzheimer's disease[3,110,111]). Perturbations of the GC rhythm have also been reported well before other symptoms of the disease appeared.[112] Thus, the noninvasive technique to assess similar changes by means of fecal hormone metabolite analyses in, for example, laboratory mice and rats, which are the most commonly used animal models

for human diseases, can be a unique opportunity and might open new perspectives in biomedical research (cf. Ref. 109).

Taken together, circadian rhythms of GC secretion should be seriously taken into account. Fecal samples should be collected at the same time each day, or all samples voided over a 24-h period should be pooled to avoid fluctuations caused by diurnal variations in GCM concentrations.

Seasonal Variations and Life History Stages

Under natural conditions, GC concentrations vary significantly in most vertebrate species studied so far.[20] The basal activity as well as the reactivity of the HPA axis to stressors is modulated; that is, baseline GC concentrations and the magnitudes of stress responses might vary depending upon the time of year (e.g., squirrel monkey,[103] rabbit,[4] yellow-pine chipmunk,[113] arctic ground squirrel,[90] mountain chickadee,[114] snow bunting,[115] house sparrow[106]). Although the underlying mechanisms and the functional significance of the annual GC rhythm are still poorly understood, in reptiles, amphibians, birds, and at least some mammals, the annual cycle of GCs tends to peak during the breeding season, indicating biological relevance and effects of life history stages.[20,116]

Besides effects of variations in the level of plasma corticosteroid binding globulin (CBG) and a changing sensitivity of target tissues to GCs, three potential explanations for the seasonal modulation of GCs are discussed in detail by Romero (the energy mobilization hypothesis, the behavior hypothesis, and the preparative hypothesis[20]).

Therefore, alterations of the general activity and reactivity of the HPA axis in response to seasonal changes or different life history stages should be carefully taken into account when assessing GC concentrations in plasma as well as in fecal samples of wild animals.

Significant effects of season or weather conditions such as temperature, humidity, and availability of food and water on fecal GCM concentrations have been shown for several species of mammals and birds, with most studies reporting higher levels during harsher conditions in winter or during the dry season (chacma baboon,[117] northern muriqui,[118] ring-tailed lemur,[34] red-backed vole,[39] deer mouse,[39] grizzly bear,[119] African elephant,[120] elk/red deer,[84,97,121] greylag goose,[122] mourning dove[73]).

Similarly, times with higher intraspecific competition for food or mating partners, associated with higher levels of aggression, also correlated with elevated fecal GCM concentrations (northern muriqui,[95] capuchin monkey,[123] ring-tailed lemur,[34] wolf,[45] greylag goose[63]).

The reproductive status of a female is another seasonal/life history event that significantly influences the concentration of fecal GCMs. Similar to findings in plasma samples, the phase of the estrous cycle or the stage during pregnancy was found to be associated with alterations in fecal GCM levels (chacma baboon,[117] ring-tailed lemur,[34] laboratory rat,[37] spotted hyena[85]). Increased metabolic demands—for example, during late pregnancy and lactation in mammals or during egg production, laying, and incubation periods in birds—are likely to influence GCM concentrations as well (cf. Refs. 30 and 116). GCs are also known to rise significantly near term in most mammalian species because they actually trigger the cascade resulting in par-

turition.[26,87] Furthermore, the placenta can produce large amounts of androgens or their derivatives, which can influence the levels of immunoreactive metabolites in the feces (due to their cross-reactivities with the antibody used in the assay[87]).

Taken together, fecal GCM concentrations in mammals and birds often vary seasonally and can be largely influenced by life history stages. Therefore, knowledge about these seasonal variations should be carefully incorporated in the study design and considered for the interpretation of the results.

CONCLUSION

The monitoring of adrenocortical activity by means of fecal GCM analysis offers several advantages and has been successfully applied to various species of mammals and birds. Because the sampling is completely noninvasive, the animal's behavior and endocrine state as well as physiological functions, like the circadian GC rhythm, are not affected by stress responses associated with capture, restraint, or blood sampling. Therefore, frequent sampling of the same individual is possible (even over extended periods), allowing the monitoring of short-term as well as long-term endocrine changes. In addition, due to pooling effects in the gut, concentrations of fecal GCMs represent a more integrated measure of adrenocortical activity, dampening episodic fluctuations, or pulsatile secretion patterns of GCs. Because only the unbound fraction of circulating GCs is readily metabolized by the liver and excreted via the bile into the gut, levels of GCMs measured in feces might also reflect the biologically active fraction more accurately. Thus, this noninvasive technique has tremendous potential for diverse investigations in laboratory, companion, farm, zoo, and wild animals.

However, because clear differences regarding the metabolism and excretion of GCMs exist, the technique needs to be extensively validated for each species and gender investigated. In analytical terms, the protocols of sample storage, extraction procedure, and immunoassay performance should be carefully evaluated (including sensitivity, accuracy, and precision of the assay used, as well as the cross-reactivity of the respective antibody with fecal steroid metabolites).

Furthermore, it is crucial to scrutinize the physiological as well as the biological validity of fecal GCM measurements in a given species, that is, the relevance of the technique to detect biologically meaningful alterations in adrenocortical activity. Because significant effects of the animals' gender, the time of day, season, and different life history stages (involving various behavioral and physiological alterations) have been shown in plasma and feces, possible differences between the sexes, diurnal, and seasonal variations should be seriously considered when measuring fecal GCMs.

Thus, besides analytical and technical issues, comprehensive information on the animals' biology and stress physiology should be carefully taken into account and thoroughly included in the study design. Together with an extensive physiological and biological validation of the technique, this will ensure that the measurement of fecal GCMs can be used as a powerful tool to assess adrenocortical activity in mammals and birds.

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