TECHNICAL COMMENT

Methodological Considerations for Using Fecal Glucocorticoid Metabolite Concentrations as an Indicator of Physiological Stress in the Brown Bear (*Ursus arctos*)

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ABSTRACT

Reliable methods to measure stress-related glucocorticoid responses in free-ranging animals are important for wildlife management and conservation. Such methods are also paramount for our ability to improve our knowledge of the ecological consequences of physiological processes. The brown bear (*Ursus arctos*) is a large carnivore of ecological and cultural importance and is important for management. Here, we provide a physiological validation for an enzyme immunoassay (EIA) to quantify glucocorticoid metabolites in brown bear feces. We also provide an evaluation of the effects of sample exposure to ambient temperature on measured fecal glucocorticoid metabolite (fGCM) concentrations. We evaluated three EIA systems: a cortisol assay, an 11-oxoetiocholanolone assay, and an 11β-hydroxyetiocholanolone assay. Of these, the cortisol assay provided the best discrimination between peak fGCM concentrations detected 1–4 d after injections of synthetic adrenocorticotrophic hormone and preinjection baseline concentrations in four individual brown bears. The time of exposure to ambient temperature had substantial but variable effects on measured fGCM concentrations, including variation both between samples from the same individual and among samples from different bears. We propose that the validated EIA system for measuring fGCM concentrations in the brown bear could be a useful noninvasive method to monitor stress in this species. However, we highlight that this method requires that fecal samples be frozen immediately after defecation, which could be a limitation in many field situations.

Keywords: stress, ACTH challenge, noninvasive hormone monitoring, steroid stability, bear, *Ursus*, carnivore.

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Introduction

Physiological stress in animals is a response to physical or physiological (e.g., injury or disease; Ganswindt et al. 2010) or external (e.g., predation risk; Clinchy et al. 2013) stimuli. Stress is a complex neurological and physiological process, but a distinct component of a physiological stress response is the secretion of glucocorticoids by the hypothalamic-pituitary-adrenal axis (Sapolsky 1992). Acute secretion of glucocorticoids has no deleterious effects (Joseph and Linley 2007; but see Clinchy et al. 2013 for an alternative view). However, chronically elevated glucocorticoid concentrations can be pathological and have been associated with severe conditions, such as suppressed reproductive function, ulcers, muscle atrophy, and immune suppression (Munck 1984). Chronically elevated glucocorticoid responses to stressors can therefore have ecological consequences (Romero 2004) and subsequently be important for wildlife conservation and management (Wingfield et al. 1997).

Physiological stress responses in animals can be evaluated either invasively by measuring circulating levels of glucocorticoids in blood or noninvasively by measuring metabolites in urine, feces, or saliva (Sheriff et al. 2011). Apart from the logistical difficulties in collecting blood from free-ranging animals, measurements of circulating glucocorticoid levels are further complicated by the typically pulsatile release of hormones into the bloodstream (Axelrod and Reisine 1984). Hence, an individual sample may not reflect long-term endocrine status (von Holst 1998). An additional benefit of noninvasive techniques is that animals are usually not disturbed during sample collection, so that potential endocrine effects caused by the sampling itself are avoided (Sheriff et al. 2011). Because of these advantages, there has been a rapid increase in the use of noninvasive methods for wildlife applications (Palme 2019).

In addition to standard biochemical validation for immunoassays (Cekan 1975), noninvasive measurements must be physiologically validated to ensure that results are biologically interpretable (Touma and Palme 2005). This validation is necessary to confirm that the antibody of the assay at hand is recognizing metabolites that accurately reflect the circulating concentrations of the parent hormone (Palme 2019). Furthermore, bacteria and their enzymes can alter steroid metabolites in feces, so that detectable metabolite concentrations may shift with time following excretion (Möstl and Palme 2002; Lexen et al. 2008). Therefore, it is preferable to freeze samples as soon after deposition as possible. Since reluctance to disturb study animals or logistical constraints may make this difficult (Dloniak et al. 2004), the potential effects of exposure time on fecal glucocorticoid metabolite (fGCM) concentrations may be an important factor to take into account when planning the collection of samples in the field (e.g., Hulsman et al. 2011; Palme et al. 2013; Ganswindt et al. 2014; Webber et al. 2018).

The brown bear (*Ursus arctos*) is a large carnivore with a circumpolar distribution in the Northern Hemisphere (Pasitschniak-Arts 1993). The species is globally listed as of least concern, with several populations increasing in recent years, although some small and fragmented populations in the southern part of its range are still threatened by extinction (McLellan et al. 2017). The species is of ecological (Hilderbrand et al. 1999) and cultural (Kruuk 2002) relevance and is important for management (Clark and Rutherford 2014; Penteriani et al. 2018). Previous studies have focused on stress in bears in relation to foraging habits (Bryan et al. 2013), anthropogenic disturbance (Bourbonnais et al. 2013), and relocation to captive environments (Narayan et al. 2018). However, methods to examine stress by analyzing related glucocorticoid patterns are currently restricted to studies using unvalidated assays for the measurement of cortisol concentrations in hair (Koren et al. 2019), studies using microarrays for the measurement of stress-related proteins in skin (Carlson et al. 2016), and studies using radioimmunoassays (RIAs) for the measurement of fGCM concentrations (Hunt and Wasser 2003; White et al. 2015).

The aim of this study was to provide a physiological validation for an enzyme immunoassay (EIA) for measuring glucocorticoid metabolites in fecal samples of brown bears. To evaluate the utility of using fGCM monitoring as a noninvasive tool to examine physiological stress in brown bears, we also quantify the effects of exposure time on measured concentrations of fGCM.

Methods

Study Animals

We conducted the study on bears in a facility in Proaza, Asturias, Spain, managed by Fundación Oso de Asturias, as well as on bears in Wilhelma Zoo, Stuttgart, Germany. In Spain, we collected samples from two females and one male. The two females were sisters originating from the Cantabrian Mountain population in Spain. The male was born in captivity but with a central European origin. The females were kept in an outdoor enclosure (1,224 m^2) that was separated from the male outdoor enclosure (902 m^2). In Germany, we similarly conducted an adrenocorticotrophic hormone (ACTH) challenge on one female and one male. These two bears were of the Syrian subspecies (*Ursus arctos syriacus*) and were housed in outdoor enclosures in the bear and mountain animal complex according to institutional regulations. All experiments were carried out with permissions from Consejería de Medio Ambiente, Ordenación del Territorio e Infraestructuras, Gobierno del Principado de Asturias (permit 3849; April 15, 2011) in Spain, the regional government in Stuttgart, Germany (reference no. 35-9185.81/0361), and the Leibniz Institute for Zoo and Wildlife Research Agent of Animal Welfare (permit date, May 25, 2012) in Germany.

ACTH Challenges

We injected two bears in Asturias, one female and the male, and each of the bears housed in Germany with undiluted synthetic ACTH (Nuvachten Depot, Vademecum, Madrid, Spain; Synacthen Depot, Novartis, Wehr, Germany). Intramuscular injections were administered with a remote injection gun (Dan-Inject, Kolding, Denmark) in Spain and with a blowpipe in

Germany. Dosages were 0.5 mg for the female in Spain and the two bears in Germany and 1.5 mg for the male in Spain. These doses were determined according to respective estimated body weights. The injections were administered on April 16, 2011, in Spain and on October 1, 2012, in Germany. Before and after the ACTH challenge experiment, the animals stayed in separated enclosures.

We collected two fecal samples daily from each bear in Spain from 3 d before to 5 d after the ACTH injection, with the following exceptions: from injection to 48 h after injection, we collected all located samples, and we located only one sample for the male 2 and 4 d after injection. Samples were collected in the morning (0700–1000 hours) and in the afternoon (1500–1800 hours). We collected samples twice daily from the bears in Germany from 7 d before to 7 d after the injection. Samples in both locations were located opportunistically. To avoid contamination from urine or other potentially interfering exogenous agents and to account for sequential secretion of metabolites, we collected only a thoroughly homogenized aliquot of the interior of each feces.

Evaluation of Effects of Exposure Time on fGCM Concentrations

We collected five fecal samples from each of the three bears in Spain in July and August 2016. These samples were collected immediately (<5 min) after defecation whenever we observed an individual defecate. We homogenized each sample thoroughly before collection and immediately froze a subsample of ~10 mL at −20°C. The remainder of the homogenized sample was placed outdoors in the shade at ambient temperature for 14 d (temperature range: 10°–25°C). From each outdoor sample, we collected and froze (−20°C) a subsample of ~10 mL according to the following temporal sequence expressed as time after defecation: 0.5, 1, 2, 4, 8, 16, 32 h, 3, 7, and 14 d.

fGCM Extraction and Analysis

We extracted fGCMs directly from wet feces by defrosting the samples at room temperature for 90 min, after which an aliquot of 0.50–0.60 g was vortexed for 2 min in 5 mL of 80% methanol. The samples were centrifuged for 15 min at 2,500 *g*, and the supernatant was stored at −20°C until analysis. Extraction of fGCM from wet feces provides results comparable to those provided from extraction from dried or lyophilized feces and avoids additional laboratory work associated with drying or lyophilizing (Palme et al. 2013; Palme 2019).

Immunoreactive fGCM concentrations of samples from the ACTH challenges were determined using three different EIAs: a cortisol assay (Palme and Möstl 1997), an 11-oxoaetiocholanolone assay (detecting fGCMs with a 5β,3α-ol-11-one structure; Möstl et al. 2002), and an 11β-hydroxyaetiocholanolone assay (detecting fGCMs with a 5β,3α,11β-diol structure; Frigerio et al. 2004). Each assay was subject to standard validation criteria and evaluated for parallelism and accuracy (Cekan 1975). Full descriptions of assay components and cross-reactivities are provided by respective references listed above. Sensitivities of the EIAs were 2 ng/g feces for the cortisol assay, 6.6 ng/g for the 11-oxoaetiocholanolone assay, and 4.4 ng/g for the 11β-hydroxyaetiocholanolone assay. According to the samples from the ACTH challenges, intra- and interassay coefficients of variation (CV) of quality controls were <10% and <15%, respectively, for all three assays. We analyzed the samples for the evaluation of the effects of exposure time only with the cortisol assay, as this was regarded as the most appropriate method (see "Results"). All laboratory analyses were conducted at the Unit of Physiology, Pathophysiology, and Experimental Endocrinology, Department of Biomedical Sciences, University of Veterinary Medicine in Vienna, Austria.

Data Analyses

We calculated the median fGCM concentrations for all samples of each individual before the ACTH injection as the preinjection baseline. We expressed the samples from the ACTH injections as proportional deviations from the individual baselines for each EIA separately. Suitable EIAs were identified according to the increase in fGCM concentration by comparing the response from the highest fGCM peak after the ACTH injection with the median baseline fGCM concentration.

We used a mixed effects linear model to evaluate potential alterations in fGCM concentrations after defecation. In the model, we used the absolute values of the proportional deviation in fGCM concentration of each sample compared with that of the immediately frozen subsample as a response variable. We added time until freezing as a factorial predictor, raw concentration of the initial sample as a continuous covariate, their two-way interaction as fixed effects, and individual sample nested within bear identification as a random effects structure. We log transformed the response variable to achieve homogenized variances. We used package nlme (Pinheiro et al. 2019) for the statistical environment R for statistical analyses (ver. 3.5.3 for Linux; http://www.r-project.org).

Results

Physiological Validation

The cortisol assay detected peak fGCM concentrations that were 12.9 (Spanish female), 3.3 (Spanish male), 3.9 (German female), and 9.6 (German male) times higher than respective individual preinjection baseline concentrations (fig. 1*A*–1*D*). These concentrations occurred in the fourth (Spanish female; fig. 1*A*), third (Spanish male; fig. 1*B*), second (German female; fig. 1*C*), and fourth (German male; fig. 1*D*) samples after injection, which were collected 1 d after injection for the Spanish bears and the German female and 4 d after injection for the German male.

The 11-oxoaetiocholanolone assay detected peak fGCM concentrations that were 1.6 (Spanish female; fig. 1*E*), 2.1 (Spanish male; fig. 1*F*), 3.2 (German female; fig. 1*G*), and 8.0 (German male; fig. 1*H*) times higher than preinjection baselines, whereas the 11β-hydroxyaetiocholanolone assay detected peak fGCM

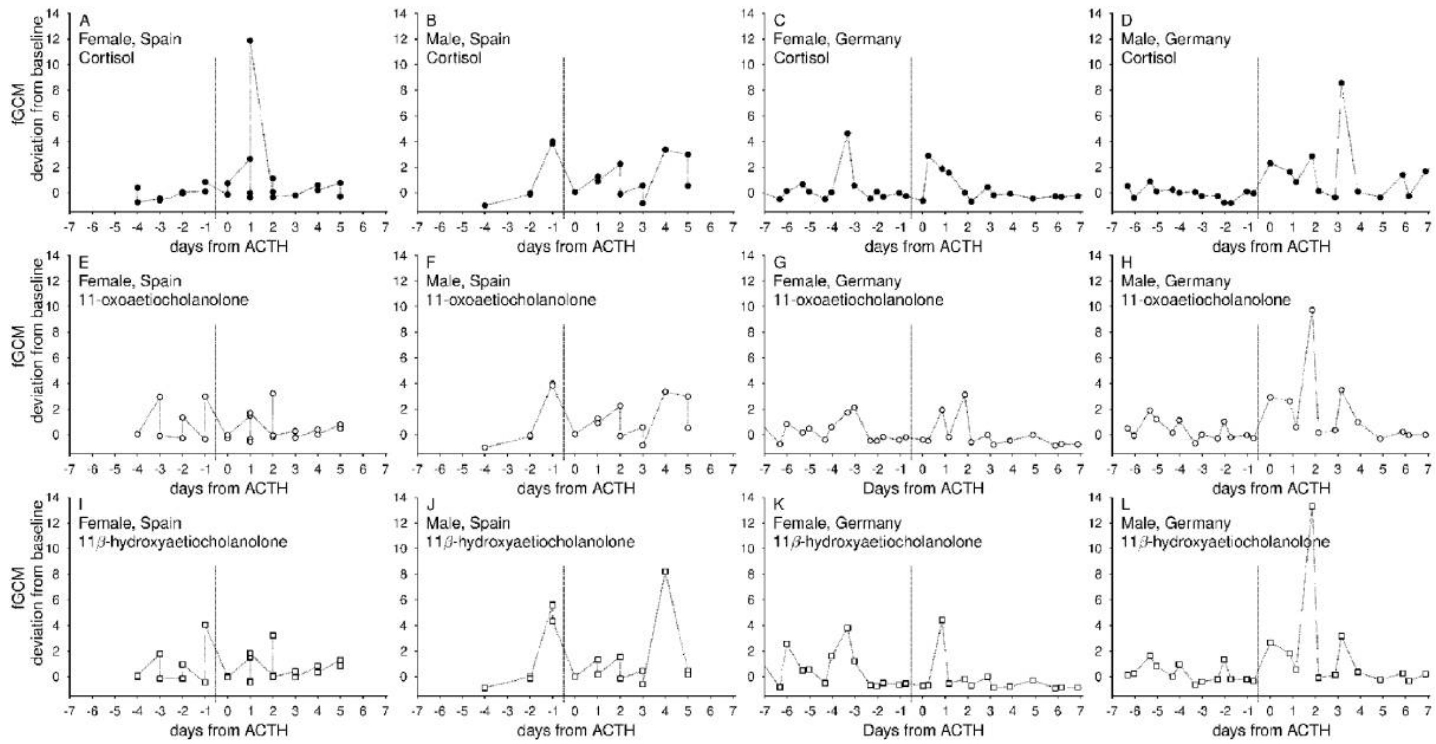

Figure 1. Profiles of fecal glucocorticoid metabolites (fGCMs) of four captive brown bears housed in Asturias, Spain, and in Wilhelma Zoo, Stuttgart, Germany. Measurements were taken from 1 wk before to up to 1 wk after injection of synthetic adrenocorticotrophic hormone (ACTH; doses were 0.5 mg for the Spanish female and the German bears and 1.5 mg for the Spanish male) using a cortisol enzyme immunoassay (EIA; *A–D*), an 11-oxoetiocholanolone EIA (*E–H*), and an 11ß-hydroxyaetiocholanolone EIA (*I–L*). Concentrations are expressed as proportional deviations from the preinjection baseline, which was calculated as the median of all samples collected before the injections.

concentrations that were 2.6 (Spanish female; fig. 1*I*), 3.7 (Spanish male; fig. 1*J*), 3.7 (German female; fig. 1*K*), and 11.5 (German male; fig. 1*L*) times higher than preinjection baselines. The samples with peak concentrations did not coincide with the samples containing peak concentrations using the cortisol assay (fig. 1).

Effects of Exposure Time on fGCM Concentrations

There was a significant effect of exposure time on the proportional deviation in fGCM concentrations compared with the initially frozen subsample ($F_{9,108} = 2.40$, $P = 0.02$) but no

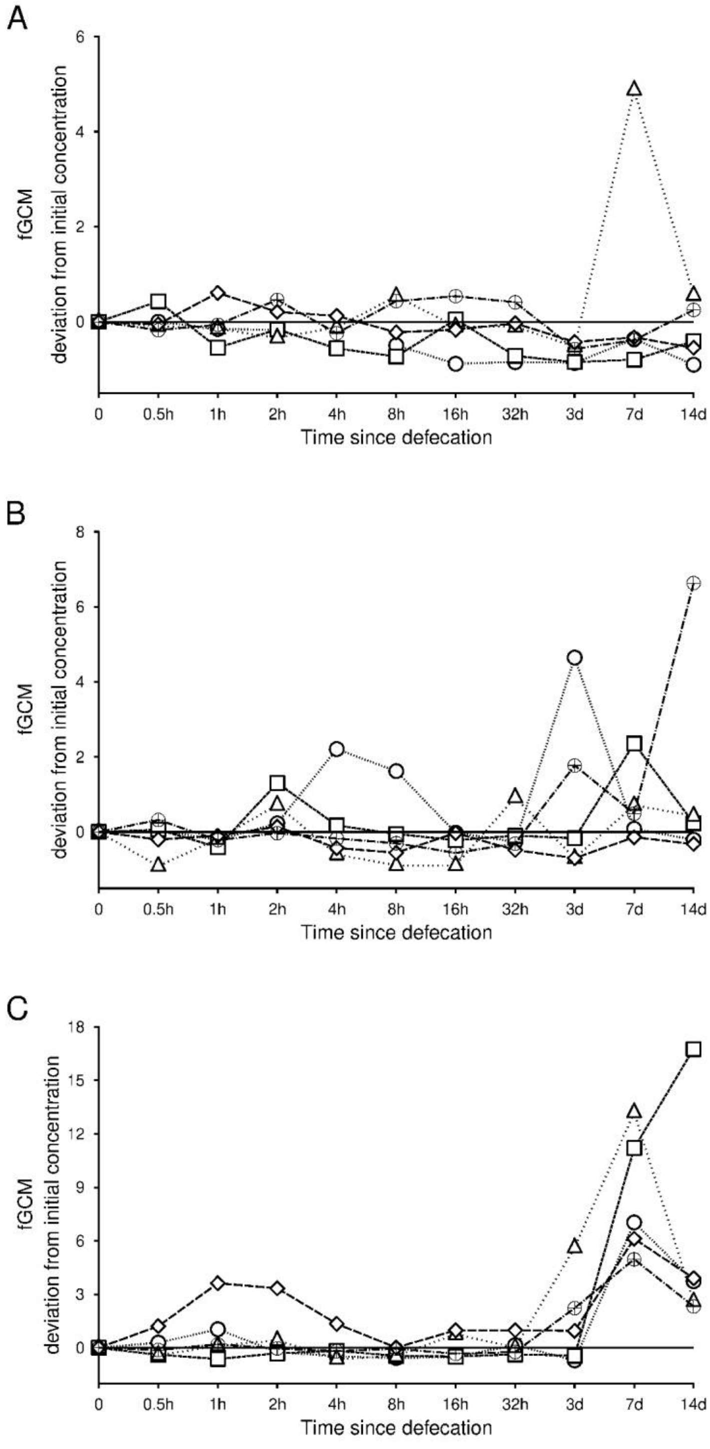

Figure 2. Proportional deviations from initial concentration of fecal glucocorticoid metabolites (fGCMs) in subsamples of five different feces each from two female (*A*, *B*) and one male (*C*) brown bear. Each subsample was frozen at varying times after defecation, ranging from 30 min to 2 wk. Note that the time scale on the *X*-axis is not linear.

significant interaction effect of exposure time and the initial fGCM concentration ($F_{9,108} = 1.30$, $P = 0.24$). Controlling for exposure time, the proportional deviation in fGCM concentrations compared with the initially frozen subsample varied considerably, both between samples from the same individual (SD = 0.60) and among samples from different bears (SD = 0.28; fig. 2). Although we noted an initial decline in steroid concentrations with increasing exposure time, this decline was not uniform over time among samples from the same bear or among samples from different bears. Fecal samples from all three bears declined as well as increased in their fGCM concentrations up until 4 h after defecation. If frozen more than 3 d after defecation, one sample from one female (fig. 2A), three samples from the other female (fig. 2B), and all samples from the male (fig. 2C) had higher fGCM compared with the initially frozen subsamples.

Discussion

We interpret our results as a successful physiological validation for an EIA measuring adrenocortical activity in the brown bear, although we acknowledge that we did not provide any temporal control samples for our ACTH challenges. From all assays evaluated, we regard the cortisol assay to be the most appropriate for this species because it consistently detected more distinct increases in fGCM concentrations after injection than the other two tested assays. It also showed the lowest tendency to detect unusually high fGCM concentrations among the baseline samples. While we are aware of multiple validations for RIA systems for measuring fGCM concentrations in brown bears (Hunt and Wasser 2003; Stetz et al. 2013; White et al. 2015), we are not aware of any published validations for EIA systems. EIAs may be beneficial in comparison with RIAs because they do not require the handling of radioactive markers (Sheriff et al. 2011). Therefore, the assay presented here provides an efficient and modern alternative to measure endocrine stress responses in the brown bear. Interestingly, the cortisol that the EIA identified as the most suitable for brown bears in our results has also been shown as the most suitable for the closely related polar bear (*Ursus maritimus*; Hein et al. 2020).

We observed a large variation in the effects of exposure time on fGCM levels. This variation included a larger variation within samples from the same bear than between samples from different bears, as well as inconsistent but strong effects of exposure time on measured fGCM concentrations. Although we did observe initial declines in some but not all subsamples during the first 24 h after defecation, we also noted marked and consistent increases at exposure times of 3 d to 2 wk. Using an RIA assay, Stetz et al. (2013) similarly found consistent increases in fGCM concentrations with increasing exposure times for brown bears. In contrast, studies on brown hyaenas (*Hyaena brunnea*; Hulsman et al. 2011), sheep (*Ovis aries*; Lexen et al. 2008), and African elephants (*Loxodonta africana*; Webber et al. 2018) have all indicated consistent declines in measured fGCM concentrations with time after defecation. These latter studies used an EIA that detected metabolites with a 5β-3α-ol-11-one structure. With different assays, fGCM concentrations have been shown to be relatively stable up to 30 d for baboons (*Papio ursinus*; Beehner et al. 2004), up to 6 d for leopards (*Pathera pardus*; Webster et al. 2018), up to 72 h for mountain hares (*Lepus timidus*; Rehnus et al. 2009), and up to 24 h for African wild dogs (*Lycaon pictus*; Crossey et al. 2018). We interpret these inconsistencies as strong support for both assay- and species-dependent effects on metabolite stability (Palme 2019), as well as possibly for additional external factors such as gut and environmental bacteria. We therefore reiterate previous recommendations for freezing samples immediately after defecation (Möstl and Palme 2002; Hulsman et al. 2011). If this is not possible, evaluation of metabolite stability may be necessary for any given species (Palme et al. 2013; Palme 2019).

Our results highlight that this method to monitor stress hormones in brown bears using feces requires samples to be frozen immediately after defecation. In cases where this is not feasible—for instance, when bears cannot be directly observed or to avoid disturbance—measurements in inert matrices, such as hair, may be more appropriate. However, no physiological validations have yet been carried out for these matrices in brown bears (reviewed in Koren et al. 2019). We therefore recommend further studies providing physiological validations for the measurement of glucocorticoids or their metabolites in inert matrices as a complement to existing noninvasive stress-monitoring tools in this species.

In conclusion, we have provided a physiological validation for an EIA system to measure fGCM concentrations in the brown bear. However, although we regard the validated EIA system to be an efficient and modern method to measure endocrine stress responses in this species, the method requires samples to be frozen immediately after defecation. Because this may be a limitation in many situations, we recommend further evaluation of metabolite stability under varying environmental conditions as well as using assays detecting different metabolites.

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