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The distorting effect of varying diets on fecal glucocorticoid measurements as indicators of stress: A cautionary demonstration using laboratory mice

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ABSTRACT

The physiological stress response is frequently gauged in animals, non-invasively, through measuring glucocorticoids in excreta. A concern with this method is, however, the unknown effect of variations in diets on the measurements. With an energy dense diet, leading to reduced defecation, will low concentrations of glucocorticoids be artificially inflated? Can this effect be overcome by measuring the total output of glucocorticoids in excreta? In a controlled laboratory setting we explored the effect in mice. When standard mouse chow – high in dietary fiber – was replaced with a 17% more energy-dense diet, fecal mass was significantly reduced. As circulating levels of corticosterone and the total output of corticosterone metabolites over time remained unaffected, the result was an overestimation – more than a doubling – of the corticosterone metabolite excretion if expressed as concentrations. Similar results were obtained for testosterone metabolites. Although measuring the total output is not feasible in, for example, wildlife studies, the present findings highlight the perilousness of relying on concentrations of hormones in excreta with no associated information of the dietary intake as even moderate changes can exert a great influence.

1. Introduction

As a non-invasive method for assessing stress in wild (e.g., Sheriff et al., 2011), captive (e.g., Keay et al., 2006), production/livestock (e.g., Palme et al., 1999), and laboratory animals (e.g., Touma et al., 2004), the utilization of quantitative measures of fecal glucocorticoids as a proxy of hypothalamic–pituitary–adrenal (HPA) axis activity has been proven effective. In the prototypical stress response, the primary effector glucocorticoids (cortisol or corticosterone, depending on vertebrate species (Sheriff et al., 2011)) are released by the adrenals en masse in response to a stressful event (for reviews, refer to e.g., Axelrod and Reisine, 1984; Sapolsky et al., 2000) (and in other physiologically taxing situations, e.g., sexual behavior (Koolhaas et al., 2011; Madliger and Love, 2014)). With a fairly short half-life (measured in minutes rather than hours), these steroids are converted to form inactive/less bioactive metabolites both in peripheral tissues and centrally in the liver (Iyer et al., 1990; Michael et al., 2003; Nixon et al., 2012; Palme et al., 2005; Tsilchorozidou et al., 2003). The metabolites are subsequently cleared from circulation (Marandici and Monder, 1985), some being passed through the bile into the digestive tract to, ultimately, be excreted in feces (Mostl and Palme, 2002). It is this subset of metabolites that has been proven useful to quantify for a non-invasive, short-term cumulative record of preceding levels of stress (Goymann, 2005). A point of contention, however, is whether the excreted metabolites should be related to the elapsed time or the mass of matter passed through the gastro-intestinal tract (Hau et al., 2011; Hayssen et al., 2002; Lepschy et al., 2010). What is a useful unit of measure?

It has been hypothesized that hepatic blood flow is the limiting factor for glucocorticoid clearance: as more substance is passed through the gastro-intestinal (GI) tract, hepatic blood flow increases, putatively proportionally to the loading of the GI tract, and so, it is speculated, does the glucocorticoid clearance (Lepschy et al., 2010; Rabiee et al., 2001). With glucocorticoid metabolites purportedly being cleared through the intestinal route proportionally to the amount of matter passing through the GI tract, it follows that the excreted metabolite levels should be expressed as concentrations per fecal mass (dry/wet weight) to be reflective of preceding HPA axis activity (Mostl and Palme, 2002; Palme et al., 2013). This hypothesis – although rarely more than alluded to – has the largest following; a majority of published papers utilizing fecal glucocorticoids as a measure of stress express the excreted metabolite levels as concentrations per fecal mass.

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Although expressing glucocorticoid metabolite excretions as concentrations makes for a convenient measure we have, by contrast, argued that the evidence for the glucocorticoid metabolite clearance from circulation being proportional to fecal production is lacking (Dijkstra et al., 1991; Esteller, 2008; Hau et al., 2011). Instead, the residence times of the glucocorticoids in circulation appear independent of systemic concentrations (Kalliokoski et al., 2010; Windle et al., 1998). If no accumulation occurs except within the GI tract – and we know of no evidence to this effect – cortisol/corticosterone metabolites must be excreted in feces with a rate that is proportional to the studied time window (Hau et al., 2011; Kalliokoski et al., 2012). Expressing metabolite levels as concentrations would thereby only make sense when defecation occurs at a constant rate. It also follows that a change in diet intake has the potential to critically skew results if expressed as concentrations (Goymann, 2012; Goymann et al., 2006; Kalliokoski et al., 2012). We have (Hau et al., 2011; Kalliokoski et al., 2012; Pihl and Hau, 2003) (among others (Cavigelli et al., 2005; Chelini et al., 2006; Cook, 2012; Goymann, 2012; Goymann and Trappschuh, 2011; Goymann et al., 2006; Hayssen et al., 2002; Paramastri et al., 2007; Turner et al., 2014)) therefore advocated measuring the total amount excreted in a given time frame.

Using the common house mouse (*Mus musculus*) as a model species we aimed in the present study to demonstrate the perilousness of relying on concentrations of fecal glucocorticoids in the face of changing diets. We hypothesize that the change from a standard rodent diet, very high in dietary fiber, to a more energy-dense sucrose-enriched diet, will artificially inflate the measured concentrations of fecal corticosterone metabolites (FCM_{conc})[1] but not the total fecal corticosterone metabolite output (FCM_{abs}) over time (i.e., the excretion rate). We further posit that this artificial elevation will be absent in serum corticosterone (CORT), as there is no actual increase in HPA axis functioning, and that there will be a similar inflation of other hormones/hormone metabolites measured in feces – in the present study illustrated with testosterone.

2. Material and methods

2.1. Ethics statement

The study was approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture and Fisheries (license number 2012-15-2934-00186). All of the procedures were performed in accordance with the EU directive 2010/63/EU in a fully AAALAC accredited facility and under the supervision of a local animal welfare committee. Care was taken to never inflict undue pain or suffering.

2.2. Animals and housing conditions

Group housed, 50–70 days old, male BALB/cAnNCrl mice (littermates; $n = 44$) were obtained from Charles River Laboratories Inc. (Sulzfeld, Germany) and pair-housed in Eurostandard type III cages in individually ventilated cage systems (Tecniplast, Buguggiate, Italy) on arrival. The mice were acclimatized for 3 weeks prior to the actual experiments. The first week the animals were subjected to standard housing conditions: cages were lined with aspen chips (Tapvei Oy., Kortteinen, Finland), with plastic houses (JAKO shelter; Molytex, Glostrup, Denmark) and cardboard tubes (Lillico, Horley, UK) for shelter; nest-building material (Lillico) and aspen bite bricks (Tapvei Oy.) were provided as enrichment; cage temperatures were kept at 22 °C (range: 20–24 °C), air humidity at approximately 55%, with the air changes set to 63 h^{-1}; lights were kept on a 12/12 h light/dark cycle with lights on at 6:00 am and with a 30 min period of reduced lighting at transitions. Pelleted diet (Altromin 1314F; Brogaarden, Gentofte, Denmark) and acidified tap water (pH adjusted to 3 with citric acid) were provided *ad libitum*. The second week of acclimatization the bedding of the cages was changed to larger aspen chips ("Anibedding" Rodent Chips, 9–16 mm shaving size; AsBe-wood GmbH, Buxtehude, Germany) to better facilitate later collection of fecal matter – all other parameters were kept unaltered. On the final week of acclimatization half of the animals ($n = 22$) were randomly allotted (using a random number generator) to receive a sucrose-rich diet (D12450J; Research Diets Inc., New Brunswick, NJ, USA). For a comparison of the metabolizable energy composition of the two diets, refer to Table 1. At no point during the study did we observe aggression between any of the pair-housed mice.

2.3. Experimental procedures

On the first day of the experiment, 2 h before entering the dark period, all animals were transferred onto fresh large chip bedding and their feed was weighed. All animals retained their shelters and enrichment in order to maintain olfactory cues in the cages. Five pairs ($n = 10$) of animals on a sucrose-rich diet and five pairs ($n = 10$) of animals on a regular diet were injected subcutaneously with 50 μg synthetic adrenocorticotropic hormone ($ACTH_{1-24}$; Polypeptide Laboratories, Strasbourg, France) delivered in 100 μl sterile isotonic saline solution. These animals were subsequently boosted with the same dose of ACTH 16, 24 and 40 h later. All of the animals were euthanized 48 h after initiating the experiment and serum was collected: The cages were taken, one at a time, to an adjacent room; working quickly, the animals were concussed and decapitated, trunk blood being collected in the process. The remaining feed was weighed, the blood was centrifuged and the serum was stored at −20 °C until further analysis. Bedding samples, collecting 48 h of fecal matter, from the cages were similarly stored until further processing.

2.4. Hormone analyses

Hormone metabolites were extracted according to a protocol modified from what has previously been described (Sundbom et al., 2011). Briefly, feces were separated from the bedding by coarse sorting on an automated sieve-shaker (Retsch AS 400 control; Retsch GmbH, Haan, Germany) and further manually sorted. All of the sorted fecal matter, constituting a 48 h sample, was weighed and submerged in 96% (v/v) ethanol (7.5 ml/g solid matter). The samples were extracted overnight on a rocking table, centrifuged to pellet solids (at 3000g), and the supernatants were collected. 5 ml of the ethanol extracts were transferred to borosilicate vials and evaporated to dryness under vacuum at room temperature (approximately 90 min; Genevac EZ-2 personal [centrifugal] evaporator; Genevac Ltd., Stone Ridge, NY, USA). The

Table 1
Energy composition of the two diets used in the study. Values have been obtained from the suppliers.

	Regular diet	Sucrose-rich diet
Metabolizable energy breakdown	Altromin 1314F	Research diets D12450J
Calories from proteins (%)	27	20
Calories from fat (%)	13	10
Calories from carbohydrates (%)	60	70
Total energy (MJ/kg diet)	12.5	16.1

[1] We have chosen to adopt the terminology of Lepschy et al. (2010) for concentrations and absolute amounts [excreted in a given time frame] of fecal corticosterone metabolites: FCM_{conc} and FCM_{abs}, respectively.