Comparative Medicine
Copyright 2019
by the American Association for Laboratory Animal Science

Vol 69, No 5
October 2019
Pages 337–349

Overview

Analytic and Interpretational Pitfalls to Measuring Fecal Corticosterone Metabolites in Laboratory Rats and Mice

Neil E Rowland[1] and Linda A Toth[2]

Minimization and alleviation of stress are generally viewed as desirable aspects of laboratory animal management and use. However, achieving that goal requires an unambiguous and valid measure of stress. Glucocorticoid concentrations are commonly used as a physiologic index of stress. Measurement of glucocorticoids in blood, serum or plasma clearly reflects many types of both acute and chronic stress. However, the rapid rise in concentrations of circulating glucocorticoids that occurs even with relatively simple manipulations such as handling has led to the increased use of fecal glucocorticoid metabolite (FCM) assays, which provide a temporally integrated measure that may allow a more accurate interpretation of chronic stressors. In this review, we consider 3 aspects of glucocorticoids as a measure of stress. First, we discuss the analytic and interpretational pitfalls of using FCM concentrations as an index of stress in mice and rats. Second, we consider evidence that some degree of stress may benefit animals by priming physiologic and behavioral adaptations that render the animals more resilient in the face of stress. Finally, we use 2 situations—social housing and food restriction—to illustrate the concept of hormesis—a biologic phenomenon in which a low dose or intensity of a challenge has a beneficial effect, whereas exposure to high doses or intensities is detrimental.

Abbreviations: CRH, corticotrophin-releasing hormone; EE, environmental enrichment; FCM, fecal corticosterone metabolites; GR, glucocorticoid receptors; MR, mineralocorticoid receptors; SAM axis, sympatho–adreno–medullary axis

DOI: 10.30802/AALAS-CM-18-000119

Interest in optimizing the humane care and use of laboratory rodents has led to an abundance of research intended to detect and quantify that vague condition known as stress. Readers are probably aware that not only the definition of stress but even the fundamental concept of what constitutes stress (as compared with beneficial homeostatic responses) vary substantially in the scientific literature. A large number of endogenous mediators are released in the body in response to many conditions that are viewed as stressful (Figure 1). These numerous mediators and their interactions lead to a wide variety of associated physiologic and behavioral outcomes that depend on the nature, magnitude and duration of the stressor (Figures 1 and 2) and that are evoked in complex temporal sequences after the onset of a stressor (Figure 3). This broad qualitative and temporal variation in stressors and the body's responses to different stressors make detection and quantification of stress a challenging undertaking. Comprehensive assessment of an individual's response to a particular perturbation, and by extrapolation, estimation of the severity of that perturbation, ideally requires serial measurement of multiple mediators or their downstream effects over a suitably long time period. However, rather than tackle this difficult undertaking, a common approach is to choose a single or a few mediators and time points. This selective approach can at best provide a limited assessment of an organism's response to a perturbation.

Animals have evolved a suite of behavioral and physiologic strategies that allow them to manage and survive the wide variety of challenges that they experience during life in natural settings.[77] The 2 major physiologic pathways that are invoked in response to psychologic or physiologic perturbations are the HPA system and the sympathetic–adreno–medullary (SAM) system. Serum or plasma concentrations of glucocorticoids and catecholamines, respectively, are correlated with and reflect activation of HPA and SAM systems.[56,75] Both of these systems directly or indirectly exert a wide variety of physiologic effects that develop and abate with different time courses.[38,56] Glucocorticoids are commonly measured in studies of both acute and chronic stress, and a decrease in glucocorticoid concentrations is often viewed as synonymous with the alleviation of stress. The pervasiveness of this perspective is reflected in the unfortunate tendency of many authors to state in their published work that they are measuring a 'stress hormone' or even 'stress' rather than precisely identifying the analyte as corticosterone or its metabolites.[35]

Several factors related to the SAM system and the nature of monoamines probably underlie the focus of stress-related research on the measurement of glucocorticoids. The predominant SAM mediators, norepinephrine and epinephrine, as well as brain monoamines, are released rapidly in response to the appropriate stimulus. With the exception of epinephrine,

Received: 31 Dec 2018. Revision requested: 12 Feb 2019. Accepted: 08 Apr 2019.
[1]Department of Psychology, University of Florida, Gainesville, Florida; and [2]Department of Pharmacology, School of Medicine, Southern Illinois University, Springfield, Illinois
**Corresponding author. Email: nrowland@ufl.edu*

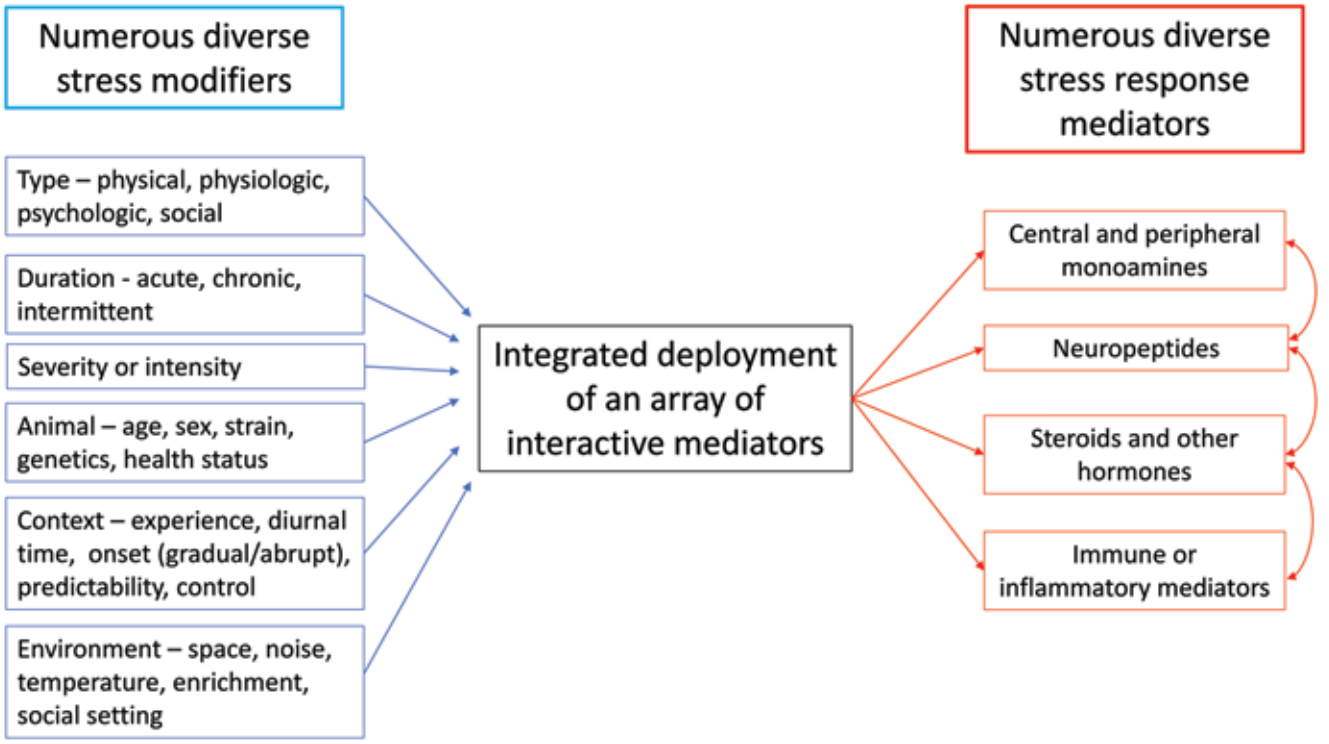

Figure 1. Integrated deployment of stress mediators.[38] The left side of the figure lists factors that can influence the specific response to stress; the right side lists several broad categories of stress response systems that are evoked and interact in a context- and time-dependent manner. Figure and legend are modified from reference 38.

Duration	General condition, model, or procedure	Representative examples
Short term (minutes to days)	Condition or study with acute or limited duration	Acute environmental perturbations (for example, cage change, occasional noise) Acute psychologic or physical stress (for example, acute limited pain, novel environment) Surgery or other acute intervention to create model or induce disease (for example, ovariectomy, tumor implantation) Drug administration (acute or limited regimen, including necessary associated restraint) Acute food or water deprivation (overnight or 24 h)
Long term (weeks to months)	Condition or study that is chronic, repeated, or long-term	Breeding colonies, natural aging, either group or individual housing Chronic environmental perturbations (for example, cold exposure, crowded living conditions, noise) Repeated or chronic food or water restriction Repeated or chronic drug administration Chronic disease condition (for example, cancer, diabetes, inflammatory disease, obesity, neurologic condition)

Figure 2. Common laboratory rodent experiences classified by duration of exposure.

they are released into synapses, where they are rapidly metabolized or cleared. Their synaptic sites of action make these compounds difficult to retrieve and quantify in a relevant time frame and at an accurate concentration at the effector site. These limitations can be accommodated to some extent by measuring secondary endpoints, such as sympathetic nerve activity or indices of peripheral effects such as changes in heart rate. However, in comparison with most monoamines, glucocorticoids have greater chemical and physiologic stability, have endocrine hormonal (as compared with neurotransmitter) properties, are easily collected from the circulation and other sites in the body, and are supported by commercially available immunoassays.

The primary glucocorticoid in rodents is corticosterone, which can be measured in blood, serum or plasma, urine, saliva and integumentary structures (for example, hair and feathers), and as metabolites in excreta (fecal corticosterone metabolites; FCM).[77] In plasma, only 3% to 5% of circulating glucocorticoids are unbound and therefore biologically active (capable of diffusing through cell membranes and binding to glucocorticoid and mineralocorticoid receptors).[53,60] Of the remainder, 80% to 90% is bound with high affinity, but low capacity to corticosteroid-binding globulin (CBG), and 10% to 15% is bound to albumin with high capacity but low affinity.[53,60] These binding proteins can, therefore, buffer the amount of free glucocorticoid during periods of high secretion and provide a reservoir of circulating

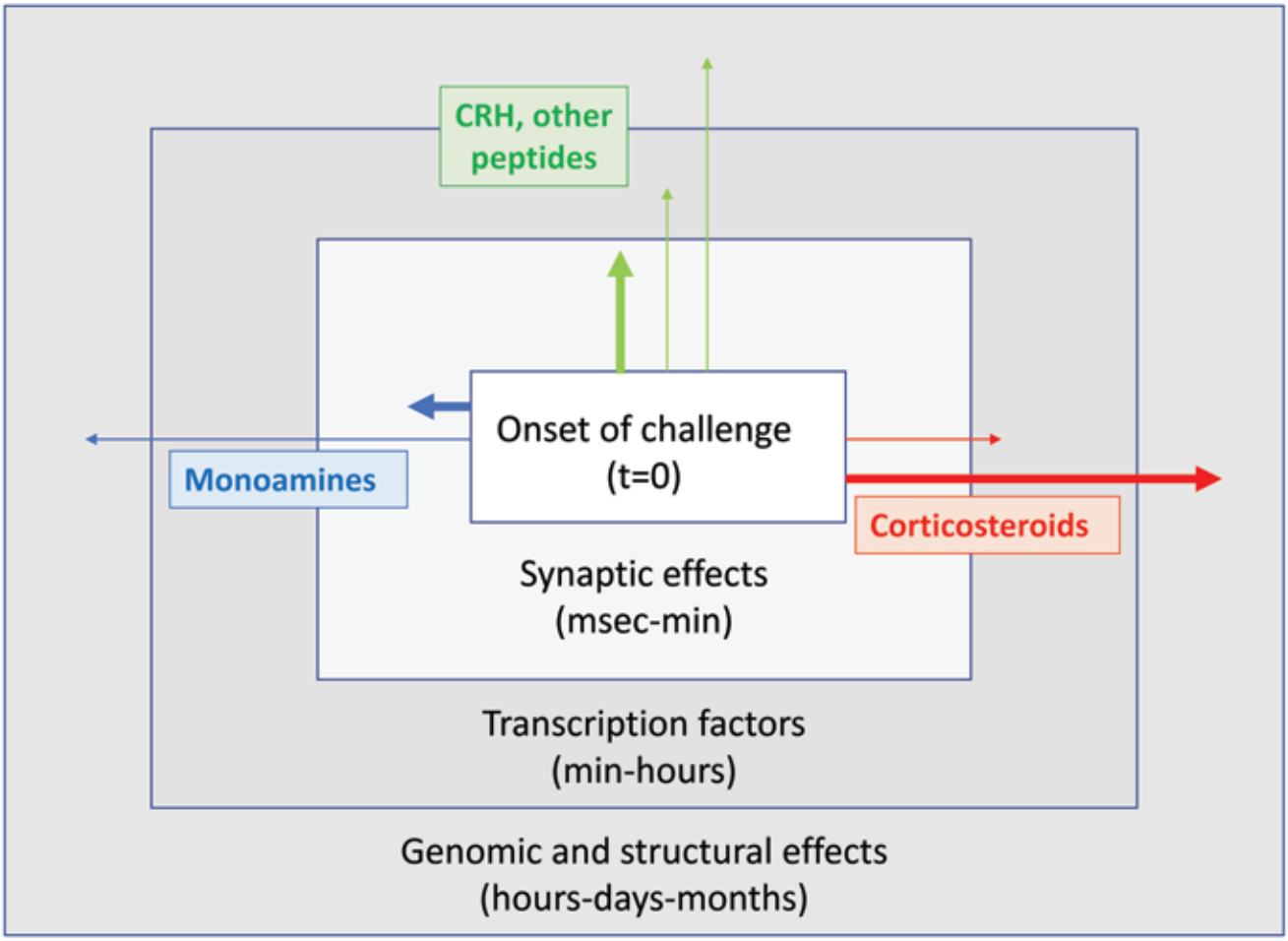

Figure 3. Functional and temporal domains of stress mediators.[38] The components of the stress response can be modeled as 3 temporal domains that represent distinct mechanisms. Monoamines and peptides have predominant rapid synaptic effects that trigger a rapid response to the situation. They also can affect transcription factors within seconds-to-minutes, leading to sustained genomic changes that prepare for long-term or recurrent stress. In contrast, corticosteroids can act in minutes through mineralocorticoid receptors responses to stress, yet their predominant effects are genomic and therefore relatively long-term and sustained. In the figure, the major temporal action and category of effect for each class of mediators is shown by the thick arrows, with thin arrows showing complementary or alternative molecular actions that occur in different temporal niches. Figure and legend are modified from reference 38.

protein-bound glucocorticoid that becomes active after dissociation of the bound complex.[53,60]

In rats and mice, corticosterone and its metabolites are excreted into the urine and via the bile into the feces, where they can be detected as FCM. In male rats, fecal excretion accounts for 75% to 90% of the corticosterone eliminated from the body, with the remainder excreted in urine.[5,47] After intraperitoneal injection of radioactive corticosterone in rats, peak radioactivity appears in urine after about 2 to 3 h and in feces after about 15 to 17 h, with detectable levels present in feces between 8 and 48 h.[5,47] Studies in male and female C57BL/6J mice report that males excrete significantly more FCM than do females, with higher proportions in the urine of females.[86] In both sexes, peak radioactivity was present in the first urinary samples collected after the injection (median, 2 h; range: 2 to 6 h) and subsequently decreased rapidly, whereas in feces peak radioactivity was detected about 10 h after the injection (median: 10 h, range: 8 to 12 h) and then fell rapidly.[86] In both rats and mice, the precise time courses of excretion were influenced by the time of day at which the radiolabeled corticosterone was administered.[5,86] Measurement of FCM has become a common approach to evaluating HPA activity because it is noninvasive and provides a relatively more stable and time-integrated picture of HPA activation than do circulating corticosterone levels.[77] The first part of this article reviews considerations relevant to the collection of samples and analysis of FCM in rats and mice.

An animal's physical environment in the research setting should be stable, physiologically and behaviorally supportive, and tailored to the species, thus allowing the animal to achieve and maintain physiologic homeostasis and general wellbeing with minimal effort or stress. Such living conditions promote not only healthy research subjects but also provide desirable uniformity among subjects, thereby increasing replicability across experiments or between different laboratories and supporting the use of lower numbers of animals by reducing interanimal variability in outcome measures. However, even though laboratory animal cage environments are often disparaged for insufficient space, absence of naturalistic features, social isolation, or lack of enrichment, social housing and enrichment can themselves create conditions that affect individual animals differently, thereby reducing uniformity among subjects. On the other hand, all of these situations likely create physiologic and psychologic challenges that may themselves have beneficial effects on overall animal wellbeing. The second part of this review discusses this conundrum with particular emphasis on the interpretation of glucocorticoid responses as a key factor in the animal's ability

to manage and tolerate social, environmental, and experimental perturbations. The remainder of this review provides examples in 2 areas: social housing and food availability.

As this introduction implies, the assessment or quantification of stress is fraught with complications. A major complication is that the precipitating stressful condition generally has complex interactive relationships with characteristics of the animal itself, with the many endogenous mediators whose release is altered by the condition, and with the physiologic and behavioral alterations that are invoked to allow the animal to cope with and/or alleviate the condition (Figures 1 and 3). Furthermore, the physiologic and behavioral responses to stress change over time in the context of acute stress, chronic stress, and acute stress embedded in a condition of chronic stress. This review does not delve deeply into these many complex relationships or into considerations of what does or does not constitute stress. For more information on these areas, the reader is referred to numerous excellent reviews on stress and its mediators and effects.[20,38,56,74,75] For purposes of the discussion here, a stressor will be considered as any perturbation (physical or psychologic, acute or chronic) that triggers a homeostatic response.[77] Our goal is to make readers aware of analytic pitfalls and alternative interpretations relevant to the use of FCM to identify and quantify a presumed state of stress in rodents. To that end, cited references have been selected to emphasize these issues. The following discussion is in no way intended to challenge or minimize the importance of studies aimed at optimizing the environments of research animals, enhancing animal wellbeing, or evaluating HPA activation as an index of physiologic state. Rather, such studies should not be undertaken lightly; they should be thoughtfully designed, appropriately analyzed, thoroughly reported, unbiased in their interpretation and conclusions, and undertaken with a firm understanding of both the benefits and the detriments of acute or chronic activation of HPA systems.

Measuring Fecal Corticosterone Metabolites

Many factors must be considered when establishing an assay for measurement of FCM. Several of these are summarized in Figure 4 and are discussed in more detail later.

Research animals are generally exposed to stress in 2 types of situations—intentional (experimental) and inadvertent (husbandry-related). In general, the housing environment of research animals should create stable conditions that are associated with regular diurnal rhythms of glucocorticoids and catecholamines. A stable and low baseline supports the sensitivity and reproducibility of studies that require measurement of glucocorticoid or catecholamine concentrations. However, catecholamines and glucocorticoids, like other serum analytes (for example, blood glucose, electrolytes), have physiologic normal ranges. Statistically significant decreases from a low baseline may be clinically and biologically insignificant and potentially hard to reproduce, particularly when the reduced level remains within the normal range.

Furthermore, levels below the physiologic normal range may be pathologic. As an extreme example of this, removal of the adrenal cortex or medulla results in extremely low levels of corticosterone and epinephrine, respectively, that are physiologically unhealthy and prevent the animal from coping appropriately with homeostatic challenges. In such a situation, one cannot assume that low levels mean the animal is experiencing a lower state of stress; on the contrary, the animal may be impaired in generating an appropriate ameliorative response to other challenges and may therefore experience more severe stress than would have occurred had these mediators been available. Meaningful floor and ceiling normal ranges must be established for any analytes before statistically significant changes can be considered biologically or clinically significant. Establishment of normal ranges appropriate for the subjects should also be an important aspect of FCM assessment.

The metabolism of glucocorticoids is complex. Steroids that are metabolized in the liver are excreted via the bile into the gut; however, enterohepatic recirculation may allow some degree of metabolite reabsorption and further hepatic degradation.[13,65,77,82] Intestinal microflora, environmental conditions, and male and female hormonal status can also alter the array of FCM.[22,26,77,82] The gallbladder, when present, acts both to concentrate bile and to serve as a bile reservoir. Some compounds may be present in the gallbladder at concentrations up to 10 times greater than those in hepatic bile.[29] Such high concentrations, coupled with biliary retention, may allow passive reabsorption of metabolites through the gallbladder mucosa.[29] Rats and mice obviously differ substantially in enterohepatic circulation and biliary excretion because mice have a gallbladder and rats do not.[29] In general, the biliary ducts are thought to assume the reservoir and concentrating functions of a gallbladder in species lacking that organ.[64,77] However, a classic publication[59] used quantification of bile pigment to compare the concentrating ability of the biliary system in rats and mice. The study found that in mice, bile collected from the gallbladder was more concentrated than that collected from the common duct of the same animal, and the bile collected from the ducts during stasis had a pigment concentration similar to that in the gallbladder.[59] In rats, however, bile in the ducts did not become more concentrated during stasis. Thus, the rat appeared to lack both the reservoir and the concentration functions of a gallbladder.[59] The publication further speculated that the small size of the ducts and the negligible tonus of the Sphincter of Oddi in rats appear to negate the function of the ducts as a reservoir.[59] To our knowledge, these factors have not been studied comparatively in rats and mice with regard to corticosterone metabolism and excretion but suggest that caution be used when comparing methodology or findings in different types of rodents or other species.

Establishment of normal ranges must consider the normal diurnal variation of corticosterone and FCM; this normal variation renders the collection period for fecal samples crucial to generating reliable and interpretable results.[13] For both chronic and acute studies, a 24-h collection period avoids the problems of circadian variation and the timing of metabolism and excretion relative to the time of the challenge. However, after defecation, factors such as temperature, humidity, and bacterial enzymes may influence the types and concentrations of immunoreactive FCMs in the sample.[84] Because of this situation, collecting and freezing samples at relatively short (for example, 3 h) intervals may be warranted. In addition, because concentrations of some substances may vary by as much as 40% between neighboring fecal pellets in rats,[66] all feces excreted during a designated test interval should be collected and processed to a homogeneous sample prior to extraction of metabolites for assay.[17] This precaution avoids errors due to sampling frequency or circadian shifts and can be repeated at appropriate intervals over an animal's experimental life.

Many studies identify a specific diurnal time period of fecal collection. However, the adequacy of this approach may be compromised if treatments generate diurnal phase shifts or changes in food intake or gut physiology. In a circadian study using C57BL/6J mice, maximal, and minimal concentrations of FCM respectively occurred during the first third of the dark phase and at the beginning of the light phase.[85] A comparison

1. Establish normal range (floor and ceiling)
2. Consider the influence of the circadian rhythm on normal ranges, defecation pattern, and food intake
3. Control for postdefecation metabolism of FCM
4. Obtain a sufficient and representative fecal sample
5. Consider the potential effects of species, strain, housing conditions, diet, gastrointestinal anatomy and physiology, and experimental use (for example, surgery that may alter defecation patterns, treatments that may change food intake between experimental groups)
6. Validate extraction method and assay for recovery, sensitivity, and consistency by using internal standards.
7. Include samples from biologic positive and negative control treatments.
8. Consider the optimal way to report FCM findings (concentration or absolute amount).
9. Use appropriate statistical analysis, including transformations as necessary.

Figure 4. Factors to consider in measurement of fecal corticosterone metabolites in laboratory rodents.

of 2 inbred mouse strains, C57BL/6 and BALB/c, found different strain-related diurnal patterns of defecation, with BALB/c mice having a more pronounced rhythm than did C57BL/6 mice.[40] This difference was accentuated by a high-fat diet, which displaced the rhythm in C57BL/6, but not BALB/c mice.[40] C57BL/6 mice on a high-fat diet also consumed more of their daily food intake during the light period, which could change the defecation pattern.[40] In rats, peak diurnal FCM values were detected 7 to 9 h after the peak plasma corticosterone concentration.[5,17,83] In a study of the impact of husbandry-related disturbances on FCM concentrations, higher levels were detected at the same clock time on the day after as compared with the day before the experimental perturbation, but because the perturbation included a 3-h delay in light onset, the possibility of a phase shift, as compared with a perturbation-induced increase, could not be discounted.[16] Therefore, what may appear to be an elevation in FCM excretion may, in fact, be a diurnal phase shift, a change in food intake or gut physiology, or another aspect of an experimental treatment.

The process of feces collection must be rigorously standardized to prevent the collection procedure itself from causing variation in the HPA response. Disturbing an animal for any reason (for example, weighing, handling during a cage change, an acute blood collection or administering an injection) could itself change levels of blood glucocorticoids within 2 to 3 min after the animal is first disturbed, with resultant changes in FCM.[85] In one study, handling mice for transfer to both clean and dirty cages significantly increased serum corticosterone at 15 min after transfer; however, at 60 min after transfer, concentrations were comparable to those of unmanipulated mice,[70] FCM were not measured in the cited study, but could potentially have been at least modestly elevated at some point after transfer. In another study, FCM concentrations in male C57BL/6 mice were significantly elevated at 24 h after handling and restraint to collect a baseline blood sample via puncture of the retro-orbital sinus.[28]

In some cases, rodents are housed in wire-bottom or metabolism cages to allow noninvasive collection of feces without disturbing the internal cage environment.[5,17,39,83] This advantage may be countered by the need for individual housing, a permeable cage floor, and perhaps a novel food formulation or differences in enrichment. BALB/c mice housed in metabolism cages for 21 d excreted approximately 10-fold higher amounts of FCM than did mice housed in standard caging.[39] In contrast, a study comparing rats housed for 3-d sequential periods in standard, metabolic, and again standard caging found that the rats produced significantly larger amounts of feces when housed in the metabolic caging, yet FCM excretion, expressed as nanograms per 24 h and per kg body weight, did not change.[21] Collection of feces from group-housed animals in solid-bottom cages by necessity entails some amount of animal disruption.

Nonetheless, this amount of disruption may be far less than that caused by blood sampling to measure plasma corticosterone. Measuring plasma corticosterone from group-housed rodents usually results in lower values in animals that were sampled first and higher values in the animals that were sampled subsequently,[87] indicating an on-going HPA response in animals that remained in the disrupted cage for longer periods after the initial disturbance. These findings led to the conclusion that sampling should be completed within 3 min after disturbance to measure accurate baseline concentrations.[87] Because the FCM level is a reflection of plasma levels during the hours prior to sampling, rapid confounding effects of disruption are avoided, even with group housing. Nonetheless, if group housing or housing on bedding is essential, the collection must be standardized. For example, animals could be relocated daily into clean cages so that all feces can be collected from the used cage, and all animals would experience a similar degree of disruption.

Several quality-control measures should be applied to assays for FCM. First, metabolite extraction from feces is necessary to prepare samples for use in an immunoassay. Because FCM is a mixture of different metabolites with a wide range of polarities, an appropriate extraction technique must be selected and validated.[13,24,84] An internal standard should be included in the extraction procedure for quantification of extraction proportions. Second, a given immunoassay should be validated with regard to at least 4 properties: 1) specificity (that is, the ability to detect compounds of interest in the sample matrix being analyzed); 2) parallelism of serial dilutions of sample with the standard curve; 3) accuracy in detecting exogenously added internal standards added to the sample prior to extraction and over a range of concentrations; and 4) limitations of the assay (for example, limits of detection, within- and between-assay variance).[77] The inclusion of a standard created from pooled samples that are frozen after extraction (ideally, one with high and one with low values found on the standard curve) can be used to test for assay reproducibility over time. With regard to measuring FCM, both radioimmunoassay and enzyme-linked assay kits designed to measure corticosterone in serum have been formulated and validated specifically for that compound in that matrix. However, such kits may not have been validated for measurement of FCM. Validation should determine that the antibody used has adequate crossreactivity for detection of FCM, as compared with high specificity for corticosterone itself.[1,17,77] Conversely, a given antibody detects different metabolites differentially, such that interanimal differences in metabolism and differences across experimental groups may increase both random and systematic variance, perhaps distorting the data and its interpretation.[24] Third, because species, strain, age, sex, and experimental treatment can all affect the types of FCM formed, both the extraction method and the assay system must be validated for measuring FCM under the conditions being tested.[84] In other words, animal characteristics or treatments may change the specific array of FCM being excreted, rather

than changing HPA activation and circulating corticosterone. Differences in the metabolite profile may influence extraction efficiency or antibody reactivity, thereby potentially influencing outcome measures disproportionately among experimental groups and potentially leading to misinterpretation.[17]

Fourth, both positive (ACTH) and negative (dexamethasone) control treatments should be tested to assure that the assay being used will detect expected outcomes.[77,84,85] For example, one study found that intravenous administration of low and high doses of ACTH to rats both resulted in a rapid and significant increase in circulating corticosterone in 3 min after injection, with levels significantly elevated for 90 min after the low dose and for 240 min after the high dose.[78] However, a significant increase in FCM was present for 8 h after the high dose but was not detected after the low dose.[78] The authors concluded that FCM could be used as a measure of exposure to a substantial stress (for example, surgery) but was not sensitive enough to reveal minor or short-duration stress.[78] An additional useful control is a biologically relevant test. in which the animal is exposed to a known stressor to document that the method will appropriately measure FCM in response to known in vivo stressors.[13,77] Fifth, FCM can be expressed in terms of concentration or an absolute amount.[48] These measures are correlated but do not necessarily respond comparably across animals and experimental situations. For example, in male rats, diurnal variation and the effects of ACTH and dexamethasone were reflected accurately by using either concentration or an absolute amount.[48] However, in female rats, dexamethasone-induced suppression of FCM was apparent using either measure, but ACTH-induced stimulation was detected only if the data were expressed in terms of concentration.[48] Therefore, care should be taken in determining how to best express data. Finally, FCM may show a log-normal distribution, such that data must be transformed for some types of statistical analysis.[40]

Concentrations of FCM vary not only with the amount of circulating corticosterone but also with diet, the metabolic capacity of the liver, gut contents, gut motility, and the amount of feces produced.[13,30,41] For example, feeding mice a high-fat diet lowered FCM excretion by about half as compared with mice on a standard diet and also reduced fecal mass to approximately one third.[40] Challenging mice with synthetic ACTH and dexamethasone produced the expected respective increase and decrease in FCM, but the magnitude of the drug effects was less than was that related to diet.[40] As another example, an animal that has been fasted and then subjected to surgery is likely to produce smaller amounts of feces in the early stages after operation, perhaps with associated higher concentrations of FCM.[30] Although a number of studies have suggested the use of FCM as a marker for pain,[2,36,90] other physiologic effects related to surgery (for example, postoperative ileus or reduced food intake) could contribute significantly to altered FCM concentrations. Therefore, caution is necessary when interpreting FCM data in association with changes in diet, food intake, or intestinal motility or absorption. If fecal output is reduced, then expressing FCM as output per mass of sample could result in potentially misleading conclusions.[40] In one study, mice underwent isoflurane anesthesia with or without vasectomy; behavior was evaluated for 8 h after surgery; and body weight, food and water intake, and FCM were measured for 3 d before and after the procedure.[36] Vasectomized mice showed postoperative behavioral changes and reduced food and water consumption and defecation, whereas anesthetized control mice—but not vasectomized animals—showed elevated FCM on the first day after anesthesia.[36] The authors concluded that food and water consumption and behavior were potentially useful markers of postoperative pain in vasectomized mice, but FCM concentrations were not.[36] Similarly, in mice subjected to arterial catheterization, plasma corticosterone levels were increased, and body weight fell after surgery, yet the total FCM excretion was significantly reduced during the 24 h after surgery, as was defecation.[80] Buprenorphine treatment significantly lowered the plasma corticosterone levels but had no effect on FCM excretion or body weight change.[80] The authors concluded that FCM excretion was not useful for assessment of postoperative stress in this model.[80]

To summarize, measurement of FCM in principle allows for a time-integrated measure of HPA activation after an acute stress or during prolonged stress but entails both procedural and interpretational pitfalls that must be recognized and managed to produce accurate and meaningful conclusions.

Interpreting Changes in Corticosterone and its Metabolites: Homeostasis, Allostasis, Hormesis, and Resilience

In their outstanding review, Sapolsky and colleagues present a compelling synthesis of 3 ideas: 1) Selye's classic view that stress-induced secretion of glucocorticoids enhances and mediates the physiologic and behavioral responses to stress, 2) Ingle's view that basal glucocorticoid levels are permissive of the stress response, and 3) abundant data on glucocorticoids as agents that limit and contribute to recovery from the stress response.[75] Sapolsky's review distinguishes between 2 classes of glucocorticoid actions: modulatory, which alter an organism's response to the stressor; and preparative, which alter the organism's response to a subsequent stressor or aid in adapting to a chronic stressor (Figure 5).[75] The modulatory actions of glucocorticoids are then subclassified as permissive, suppressive, and stimulating (Figure 5).[75] However, these actions and effects are complex; they occur in tandem as well as in series, and individual mediators interact with other mediators and their effects (Figures 1 and 3). Other considerations also complicate these effects and interactions. Dose–response relationships for glucocorticoids can be monotonic, with responses occurring in proportion to increasing glucocorticoid concentrations and receptor interactions, or, alternatively, may exhibit bell-shaped or biphasic relationships (Figure 6).[75] Furthermore, the timing and duration of glucocorticoid exposure, including circadian effects, also influences the effects of glucocorticoid exposure.[75] Sapolsky's review poses 4 questions that should be used to determine whether and how specific glucocorticoid actions contribute to a presumed stress response: 1) Does a particular glucocorticoid action enhance or reduce the effects of other stress-responsive hormones (conformity)? 2) When does the action appear relative to the onset of stress (time course)? 3) Is the physiologic stress response mitigated when the stress-induced rise in glucocorticoid activity is blocked, and, conversely, does replication of stress-induced secretion provoke the expected stress response (subtraction and replacement)? and 4) Does a particular glucocorticoid action permit, stimulate, or suppress the stress response, or prepare the organism for the next stressor (homeostasis)?[75]

Claude Bernard[9] was the first to propose that maintenance of relative constancy in the internal environment is essential for life. Walter Cannon subsequently introduced the term *homeostasis*, which can be defined as a state of constancy that is achieved through processes that resist change.[15] According to this view, deviations in external or internal environments are countered by opposing responses that restore the basal or initial conditions which, by default, are generally considered to be biologically

Category	Mediator	Timing	Effect	Systems affected
Permissive	GCs that are present before the onset of a stressor (basal levels); independent of a stress-induced increase in GC levels	Effects are expressed during the initial immediate response to stress	Prime defense mechanisms that allow an organism to respond to stress	Cardiovascular Immunologic Metabolic
Stimulatory	Stress-induced rise in GC concentrations	Effects appear minutes to hours more after the onset of the stressor	Enhance the effects of the first wave of hormonal responses to stress	Metabolic
Suppressive	Stress-induced rise in glucocorticoid concentrations	Effects appear minutes to hours after the onset of the stressor	Prevents stress-activated defense reactions from overshooting	Immune Metabolic Glucose transport and utilization in the brain Appetite
Preparative	Stress-induced rise in glucocorticoid concentrations	Prepare for response to a subsequent stressor	Modulate the response to a subsequent stressor	Glucose transport and utilization in the brain Appetite

Figure 5. Types of glucocorticoid actions that contribute to stress responses.Adapted from Figure 1 and Tables 1 and 2 in reference 75.

optimal. Going a step farther, Mrosovsky[62] explained examples of apparent lack of homeostasis by stating that "A change in the defended level of the internal environment is an elaboration, not a contradiction, of homeostasis" (p 13). This concept, which is now known as allostasis, refers to a situation in which the animal physiologically and/or behaviorally adapts to an environmental or physiologic challenge it experiences (for example, chronic cold, limited food availability).[68,79] These adaptive responses promote physiologic and behavioral coping and confer resilience in the face of such challenges. *Allostasis* and *homeostasis* (which functionally is a special case of allostasis) are both usually achieved at a cost in the form of energy or effort that is incurred to either make a change or to resist it. That cost has been termed 'allostatic load' and can be regarded as a stress to the organism. Laboratory environments typically provide few or deliberately avoid, situations that invoke resilience, with the potential exception of the low ambient temperature at which laboratory mice are often maintained.[18,27]

The term *hormesis* refers to a situation in which an organism's response to a low dose or intensity of a substance or stimulus is qualitatively different from the response to high-dose exposures.[52] As a biologic phenomenon, hormesis occurs when exposure to a low dose of an agent has a beneficial effect (for example, improved health, greater stress tolerance, extended longevity) whereas higher doses or exposures are deleterious, toxic or lethal. To a large extent, the effects of both glucocorticoids and stress, in general, are examples of hormesis, with both acting in a nonlinear, inverted U dose–response curve as a function of stress severity or glucocorticoid concentration (Figure 6).[46,74] For example, glucocorticoids may enhance target tissue sensitivity to a cytokine while simultaneously lowering the concentration of the cytokine.[46] An inverted-U effect of glucocorticoids can also result from a dose-dependent difference in the stimulation of mineralocorticoid receptors (MR) and glucocorticoid receptors (GR; Figure 6); for example, low corticosterone levels enhance T cell responses through MR but suppress them at high concentrations through GR.[46]

An important modulator of MR or GR action is the enzyme 11β-hydroxysteroid dehydrogenase, which exists in 2 isoforms and is often colocalized with these receptors. The type 2 enzyme transforms corticosterone into its inactive metabolite cortisone and thereby reduces the concentration of corticosterone at adjacent MRs.[91] In tissues that express the type 2 enzyme, such as kidney and salivary glands, this process prevents the activation of MRs (which otherwise have high affinity to glucocorticoids) by corticosterone and allows aldosterone to be the primary ligand. Although the type 2 enzyme is widely expressed in developing rodent brain, in adults it is limited to a few brainstem regions.[91] Therefore, in adult hippocampus, the MR that are expressed are normally fully occupied by even low or basal levels of glucocorticoids. In general, MR occupancy enhances synaptic plasticity, whereas GR occupancy impairs it, generating an inverted-U pattern from these opposing effects.[74]

Therefore, allostasis mediators like glucocorticoids can have beneficial adaptive effects in response to mild-to-moderate stressors that are relatively brief in duration, yet they can have maladaptive and damaging effects in 4 situations: 1) repeated challenges, particularly those that are unpredictable; 2) inability to adapt or habituate to repeated challenges; 3) failure to terminate the response when the challenge ends; and 4) failure to mount an adequate response to the challenge.[46] In general, acute stressors cause a rapid surge of neurotransmission, neuronal activation, and hormone release that is followed by rapid return to baseline levels; however, this transient response can trigger alterations in gene expression that may have longer-lasting effects) (Figure 3).[38] By contrast, chronic stress—sometimes defined as stress that lasts a week or more—can provoke sustained or progressive changes in the expression of particular genes, structural alterations in neurons, and neuronal firing patterns that may cause prolonged deviations from the normal network function.[38] The phenomenon of hormesis underscores the importance of establishing normal ranges for interpretation of the potential beneficial or harmful effects of glucocorticoids and, by extension, adaptive coping or maladaptive dysregulation.

The many types of stressors; the age, sex, strain, and genetic background of the animal; and contextual factors, such as the ambient temperature, the presence of conspecifics, or the point in the diurnal rhythm at which the stressful event takes place, all trigger the deployment of a wide variety of interactive signaling molecules that mediate the temporal and contextual response to the stress situation (Figures 1 and 3).[13,24,38,74] The deployment of these many modifiers is blurred in the measurement of FCM because rather than measuring the original active signaling molecule, this noninvasive approach instead measures unspecified metabolic end products of the hormone after it has been cleared

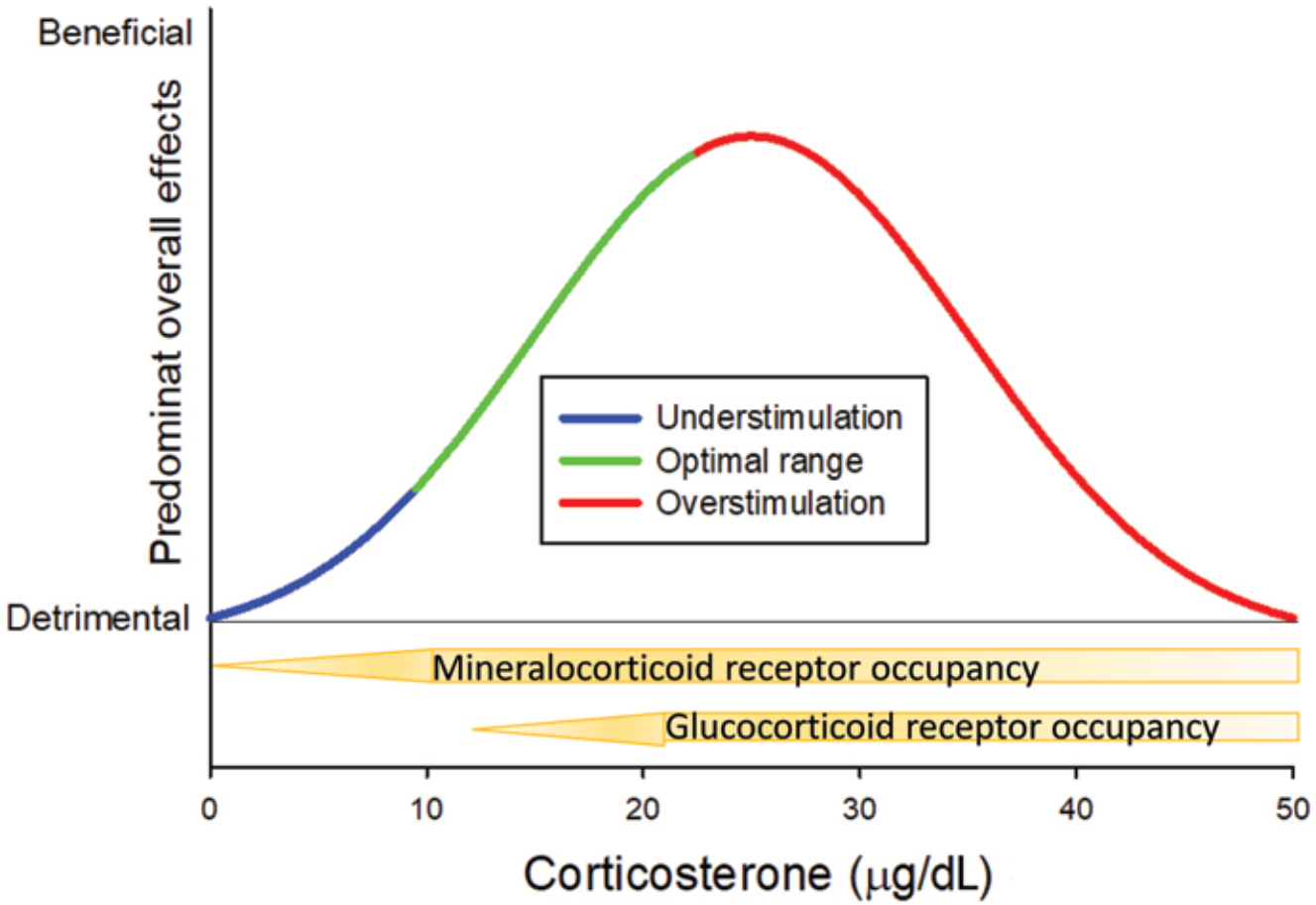

Figure 6. Conceptualization of inverted-U effects of corticosterone.[74] The mild-to-moderate range of circulating corticosterone concentrations is considered in this diagram to be approximately 10 to 20 μg/dL. Within this approximate range, corticosterone is viewed as being functionally beneficial, particularly if levels fluctuate in response to environmental conditions within this range. In contrast, excessively low or high levels, particularly if prolonged, have deleterious effects. This inverted-U pattern is related to the brain's dual receptor system for glucocorticoids; beneficial effects of corticosterone are heavily mediated by increasing occupancy of the high-affinity, low-capacity mineralocorticoid receptors, whereas deleterious effects are mediated by the low-affinity, high-capacity glucocorticoid receptors. Figure and legend are modified from reference 74.

from the circulation and extensively modified by bacteria in the gut.[24] Furthermore, individuals may differ with regard to the type and relative amounts of the hormone metabolites they form.[24] Because a given antibody detects different metabolites differentially, interanimal differences in metabolism, as well as differences across experimental groups, may increase both random and systematic variance, thereby perhaps distorting the data and its interpretation.[24]

In addition, even genetically and environmentally identical individuals of the same species can show highly divergent responses in the same situation, reacting, for example, as aggressive or submissive, bold or cautious, proactive or reactive, fight or flight or freeze.[46] Individual conspecifics may even differ with regard to whether a particular event or internal state is perceived as stressful.[74] Different individual responses reflect individual differences in the activation or suppression of the physiologic, neuroendocrine, and neural mechanisms that are invoked in the context of the ecologic situation.[46] Individual variability in perceived stress, resilience and vulnerability to stress-related disease is an important topic in today's world.[74] A critical question for both individuals and populations is the point along the axis of stress severity that is the tipping point of the inverted-U for any particular stressor, setting and context (Figure 5).[74]

To summarize, glucocorticoids have numerous functional effects that depend, in part, on whether HPA activation was reactive to or anticipatory of the stressor. In either situation, these effects are not inherently negative. Rather, they may benefit or facilitate adaption, coping, or resilience of an organism when faced with an environmental challenge. Therefore, the interpretation of the effects of elevated glucocorticoid concentrations on the organism is highly dependent on the environmental context in which the increase occurs.

Examples of Allostasis and Resilience

Social housing and environmental enrichment. Group housing of social animals is generally viewed as providing a positive contribution to welfare. However, the influence of social housing with regard to perceived stress is complex. In their natural situation, rodents often live in large underground burrow systems, with large and interconnected populations that are segregated into groups of either the same or mixed sex.[6,14] To study behavior in such environments, laboratory burrow systems have been developed in which a large enclosure is

subdivided into enclosed (simulating underground) chambers of varying sizes connected by tunnels and an uncovered (simulating surface) region.[4,11,58,67] Individual animals within the group can be studied using electronic surveillance. Despite supportive features of this environment (ample food and water, constant temperature, no predators), unrelated males fight and establish dominance hierarchies independent of the presence of females.[11] After initial fighting has waned, subordinate animals show persistent submissive behaviors, eat less and lose weight, and may die prematurely.[12] In one study, groups of 4 male and 2 female Long–Evans rats were placed together in the burrow system for 2 wk.[11] One dominant male emerged in each colony.[11] At the end of the 2-wk study, the dominant males had lost a small amount of body weight (approximately 5%) whereas the subordinate males had lost over 20%.[11] Furthermore, the basal plasma glucocorticoid levels of subordinates were 2- to 4-fold higher than those of dominants, and, regardless of dominance status, all males from the burrow system had glucocorticoid levels similar to or higher than those of singly housed male controls.[11] However, variation in weight and HPA status occurred most prominently in males when they were group-housed with females; this variation was generally minimal or absent in all-male colonies. Later studies included male–female pairs to allow for sexual interaction and to avoid social isolation.[58,81]

The variation in body weight and HPA status among mixed-sex group-housed rats in visible burrow systems shows that individual animals respond differently in a social situation depending on their social status and that the animals in the group can become to some extent physiologically distinct.[10,11,32] This basic conclusion has been corroborated in several studies of commonly-used laboratory strains of rats or mice in studies comparing individual and group housing: glucocorticoid levels are either the same or lower in singly housed animals,[7,33,35,42,45] despite some differences related to strains and housing density.[63,89] One study showed that after separation of group-housed female mice, the concentration of FCM decreased from day 1 to day 3 after separation and stabilized at these lower concentrations during days 4 to 7, suggesting an adaptation to individual housing that was associated with lower HPA reactivity.[44]

Some evidence suggests that group housing produces resilience to subsequent physical or psychologic stressors,[45,49] possibly by means of a direct priming effect of periodic mild stress that is present due to social interactions. For example, in one study, group-housed mice showed lower elevations in serum corticosterone in response to restraint than did singly housed mice.[49] Therefore, although group housing of social animals is commonly thought to reduce stress and thereby be beneficial, group housing may be beneficial precisely because it produces relatively low-level stress via short adversarial encounters that the animal can manage by either aggression, submission or escape. Such relatively mild stressful encounters may promote resilience.[57,72] The critical point here is that elevation of circulating corticosterone or FCM is likely stress-related, but the overall response is beneficial in the context of the animal's environment.

As with social housing, providing environmental enrichment (EE) is also commonly viewed as a positive strategy for enhancing rodent wellbeing. However, as with social housing, the effects of EE are also complex, and EE can be associated with apparent HPA and SAM activation in rats and mice.[8,45,50,61] Baseline plasma corticosterone levels were elevated in 2 rat stocks after 6 wk of EE.[45] In response to restraint stress, hormone levels in EE rats tended to peak earlier and approach or exceed baseline values more quickly than occurred in the comparable control groups.[45] In another study, EE rats had higher resting plasma corticosterone concentrations, larger adrenals, and a greater corticosterone release to buspirone challenge than did control rats.[61] Furthermore, EE rats also showed lower ACTH, corticosterone, and adrenaline responses in response to handling.[61] EE mice showed a shorter freezing time and no HPA reactivity in a stress paradigm, in contrast to mice housed under standard conditions.[8] In a study of male mice housed 4 per cage, EE was associated with increased aggressive behavior and significant elevations of plasma corticosterone concentrations and adrenal tyrosine hydroxylase activities, indicating activation of both HPA and SAM systems.[50] In these examples, HPA and SAM activation in EE animals does not necessarily reflect a negative condition but rather may prepare the animals to better adapt to new situations.[45,54,55,61]

Food deprivation and caloric restriction. The standard condition for management of research rodents (as compared with many other species of animals) is continuous free access to nutritionally balanced food, usually a grain-based, low-fat commercially available chow. However, in the long term, free access feeding of rodents is associated with excessive body fat gain, metabolic disease, and shortened life span.[51]

Chronic caloric restriction is well known to increase life and health span in rats, mice, and numerous other species[3,34,54,55] and provides a good example of hormesis in association with moderate HPA activation[52,73] as compared with activation induced in rodents by physical stress.[23,51,73,88] For example, restriction of Sprague–Dawley rats to about 45% of the food consumed by rats with free access to food resulted in daytime glucocorticoid levels that were less than 2-fold higher in restricted as compared with free-access rats.[71] Moderate early-life nutritional stress created in situations such as heavy competition for nursing or a low-protein maternal diet can produce a modest reduction in the growth of the pups but resistance to metabolic disease and increased life span in the adult animal, with glucocorticoid levels that were about 50% higher in the mature offspring of protein-restricted dams.[19,76] One report suggests that elevated corticosterone induced by long-term dietary restriction may be detrimental to learning but that this unfavorable effect is balanced by numerous protective mechanisms that are also induced by dietary restriction and that are reflected in improved long-term functional outcomes.[69]

Some studies suggest that HPA activation is stronger in imposed as compared with voluntary weight loss.[23,25] In one study, rats that were exposed to chronic variable stress for 2 wk voluntarily reduced their food intakes; these rats were compared with both control rats and weight-matched rats that received rationed food twice daily.[23] Exposure to 30 min of novel restraint elicited a significant increase in plasma corticosterone in all groups; however, basal levels were higher in the rationed group.[23] Food restriction reduced adrenal weight, whereas chronic variable stress induced adrenal hypertrophy.[23] A similar distinction between voluntary and imposed food restriction was observed when rats made obese by feeding high-fat diet were caused to lose comparable body weight either by either gastrectomy or by a restricted ration that was fed once daily for 30 d.[25] At the end of this time, both gastrectomy and rationed animals weighed about 15% less than obese rats with continued free access to the high-fat diet.[25] However, basal morning plasma glucocorticoid levels were 2.5-fold higher in the rationed animals than in free access and gastrectomy groups.[25] A parallel study found that hypothalamic corticotrophin-releasing hormone (CRH) mRNA was 3- to 4-fold higher in rationed rats as in free-access and gastrectomy groups, comparable in magnitude to their respective changes in basal glucocorticoid levels.[25] However, in both of

these studies,[23,25] the rationed group likely consumed their total ration at least 12 h before blood sampling, whereas groups with free access to food presumably had been eating through the night until shortly before the sampling time. Because the availability of the restricted food ration was not paired in time to the food consumption of the nonrestricted groups, the difference in HPA activity could reflect an interaction of acute (deprivation) and chronic (weight loss) causes.

Acute food deprivation is also associated with elevated circulating glucocorticoid levels, which in turn promote energy availability, including gluconeogenesis. Basal or nadir glucocorticoid levels generally have been reported to increase by up to approximately 5-fold after 1 to 2 d of total food deprivation. The importance of HPA activation in this outcome is supported by a study[37] of mice with a disrupted gene encoding CRH; this disruption rendered the mice unable to secrete corticosterone. When free access feeding was permitted, CRH-deficient mice had the same body weight and plasma glucose as CRH-replete mice. However, after 36 h of food deprivation, plasma glucose fell significantly more in CRH-deficient mice as compared with CRH-replete mice. Furthermore, rodents subjected to food deprivation show a marked reduction in hepatic metabolism of corticosterone.[31,43,89] In a study that used rats, food deprivation for 48 h increased plasma glucocorticoid by 34%; however, the clearance rate of a radioactive glucocorticoid bolus was concurrently reduced by approximately 30%, which was sufficient to explain the rise in level without change in secretion.[89] Another study in rats found approximately a 4-fold elevation in morning (nadir) glucocorticoid levels after 60 h food deprivation in rats; this increase was associated with a 27% decrease in CRH mRNA in the paraventricular nucleus of the hypothalamus, suggesting reduced HPA activation during food deprivation.[43] In mice, a 48-h period of food deprivation was associated with a 2-fold increase in plasma glucocorticoid and a 30% increase in hypothalamic CRH mRNA.[92] Increasing the duration of deprivation to 72 h did not further increase either dependent variable.[92]

To summarize, in interpreting food deprivation–related increases in circulating glucocorticoid levels, several factors should be considered: 1) the magnitude of these changes in glucocorticoids and related HPA variables after food deprivation is small in comparison to the effects of physical stressors; 2) the changes do not linearly reflect the period of deprivation; 3) the changes are not in any clear way related to physical or psychologic stress; 4) the increase is likely due in large part to reduced clearance, as compared with increased secretion; and 5) the changes likely allow the animal to maintain homeostasis during the period of deprivation by mediating compensatory and ameliorative physiologic adjustments.

Conclusions

Blood levels of corticosterone are highly variable. They naturally oscillate with circadian and ultradian rhythms and increase rapidly in response to a variety of stressors. The response to a stressor depends on many factors, including the time of diurnal phase of exposure to the stressor, the animal's prior experience with the stressor, its duration, and its perceived severity. These factors, together with the temporally changing nature of the physiologic response to stress, make single-time–point measures of corticosterone difficult to interpret as a diagnostic tool. The magnitude and duration of corticosterone secretion may be measured in terms of FCM, which may provide a somewhat time-averaged and therefore perhaps generally representative measure of the impact of a stressor on the HPA axis. However, as reviewed here, studies that use FCM measurements must be carefully designed, performed, analyzed, and interpreted. Furthermore, physiologic or behavioral adaptation may develop to allow adaptive coping to long-term perturbations.

Exposure to low or moderate intensity and brief or escapable stressors can promote physiologic and behavioral resilience, which is particularly relevant for animals in long-term studies. In such situations, the ideal housing may be a stable situation in which any stressors that the animals experience (for example, handling, administration of treatments, aggressive cage mates, low ambient temperature, limited food availability) are of a frequency, duration, and intensity that allow them to invoke adaptive physiologic or behavioral escape responses. Elevations in HPA activity and basal glucocorticoid levels may be crucial to such adaptation and are not necessarily indicative of excessive stress, but rather of managed stress.

As a side note, the literature on this topic is challenging to review due to the absence of key data from many studies (for example, the age, strain, or sex of the animals; features of the housing and environment; key analytic controls, and elements of statistical design and analysis). This lack of key information compromises the reader's ability to interpret the work and renders the studies difficult and perhaps impossible to reproduce, perhaps even by the same laboratory. Statistical power is rarely if ever discussed, nor are normal ranges for key analytes. A Cochrane-type review and/or a consensus statement by involved organizations would perhaps guide future research on these important topics.

Finally, this overview is in no way intended to challenge the importance of studies aimed at optimizing the environments and management of research animals, maximizing animal wellbeing, or evaluating HPA activation as an index of physiologic state. Rather, such studies should not be undertaken lightly; they should be thoughtfully designed; appropriately analyzed; thoroughly reported, unbiased in their interpretation and conclusions; and undertaken with a firm understanding of both the benefits and the detriments of acute or chronic activation of HPA systems. Recognizing that 1) we will likely never be able to completely prevent HPA activation in research animals; 2) accomplishing this might indeed be detrimental by limiting the animal's ability to manage stress; and 3) we can often recognize experimental design flaws in retrospect (including in our own work), we contend that the goal of research in this area should be to identify those features that make an environment supportive, predictable, and stable in the context of HPA and SAM activation. The normal biologic role of these systems is to mitigate or prevent adverse effects of transient and low-level perturbations of homeostasis, thereby promoting stable levels of animal resilience, health, and wellbeing. Finally, researchers who focus on assessing or mitigating stress in research animals should design the experiments and interpret the resultant data in light of current knowledge about the scientific underpinnings of homeostasis, allostasis, and hormesis, avoiding the simplistic view of glucocorticoids simply as simple markers of stress, and stress as an avoidable and fundamentally unhealthy component of life.

Acknowledgments

We thank Dr Eric Krause for his helpful comments during the preparation of this manuscript.

References

1. **Abelson KS, Kallikoski O, Teilmann AC, Hau J.** 2016. Applicability of commercially available ELISA kits for the quantification of faecal immunoreactive corticosterone metabolites in mice. In Vivo **30:**739–744. https://doi.org/10.21873/invivo.10989.

2. **Adamson TW, Kendall LV, Goss S, Grayson K, Touma C, Palme R, Chen JQ, Borowsky AD.** 2010. Assessment of carprofen and buprenorphine on recovery of mice after surgical removal of the mammary fat pad. J Am Assoc Lab Anim Sci **49:**610–616.
3. **Anson RM, Guo Z, de Cabo R, Iyun T, Rios M, Hagepanos A, Ingram DK, Lane MA, Mattson MP.** 2003. Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. Proc Natl Acad Sci USA **100:**6216–6220. https://doi.org/10.1073/pnas.1035720100.
4. **Arakawa H, Blanchard DC, Blanchard RJ.** 2007. Colony formation of C57BL/6J mice in visible burrow system: identification of eusocial behaviors in a background strain for genetic animal models of autism. Behav Brain Res **176:**27–39. https://doi.org/10.1016/j.bbr.2006.07.027.
5. **Bamberg E, Palme R, Meingassner JG.** 2001. Excretion of corticosteroid metabolites in urine and faeces of rats. Lab Anim **35:**307–314. https://doi.org/10.1258/0023677011911886.
6. **Barnett SA.** 1958. The rat: a study in behavior. Chicago (IL): University of Chicago Press.
7. **Bartolomucci A, Palanza P, Sacerdote P, Ceresini G, Chirieleison A, Panerai AE, Parmigiani S.** 2003. Individual housing induces altered immuno-endocrine responses to psychological stress in male mice. Psychoneuroendocrinology **28:**540–558. https://doi.org/10.1016/S0306-4530(02)00039-2.
8. **Benaroya-Milshtein N, Hollander N, Apter A, Kukulamsky T, Raz N, Wilf A, Yaniv I, Pick CG.** 2004. Environmental enrichment in mice decreases anxiety, attenuates stress responses, and enhances natural killer cell activity. Eur J Neurosci **20:**1341–1347. https://doi.org/10.1111/j.1460-9568.2004.03587.x.
9. **Bernard C.** 1878. [Leçons sur les phénomènes de la vie commons aux animaux et aux végetaux.] Paris: Ballière.[In French].
10. **Blanchard DC, Sakai RR, McEwen B, Weiss SM, Blanchard RJ.** 1993. Subordination stress: behavioral, brain, and neuroendocrine correlates. Behav Brain Res **58:**113–121. https://doi.org/10.1016/0166-4328(93)90096-9.
11. **Blanchard DC, Spencer RL, Weiss SM, Blanchard RJ, McEwen B, Sakai RR.** 1995. Visible burrow system as a model of chronic social stress: behavioral and neuroendocrine correlates. Psychoneuroendocrinology **20:**117–134. https://doi.org/10.1016/0306-4530(94)E0045-B.
12. **Blanchard RJ, Blanchard DC, Flannelly KJ.** 1985. Social stress, mortality and aggression in colonies and burrowing habitats. Behav Processes **11:**209–213. https://doi.org/10.1016/0376-6357(85)90062-2.
13. **Busso JM, Ruiz RD.** 2011.Excretion of steroid hormones in rodents: An overview on species differences for new biomedical animal research models. In: Diamanti-Kandarakis E, editor. Contemporary Aspects of Endocrinology. https:/doi.org/10.5772/17051 IntechOpen.
14. **Calhoun JB.** 1962. The ecology and sociology of the Norway rat. Bethesda (MD): US Dept Health Education and Welfare.
15. **Cannon WB.** 1929. Organization for physiological homeostasis. Physiol Rev **9:**399–431. https://doi.org/10.1152/physrev.1929.9.3.399.
16. **Cavigelli SA, Guhad FA, Ceballos RM, Whetzel CA, Nevalainen T, Lang CM, Klein LC.** 2006. Fecal corticoid metabolites in aged male and female rats after husbandry-related disturbances in the colony room. J Am Assoc Lab Anim Sci **45:**17–21.
17. **Cavigelli SA, Monfort SL, Whitney TK, Mechref YS, Novotny M, McClintock MK.** 2005. Frequent serial fecal corticoid measures from rats reflect circadian and ovarian corticosterone rhythms. J Endocrinol **184:**153–163. https://doi.org/10.1677/joe.1.05935.
18. **David JM, Chatziiannou AF, Taschereau R, Wang H, Stout DB.** 2013. The hidden cost of housing practices: using noninvasive imaging to quantify the metabolic demands of chronic cold stress of laboratory mice. Comp Med **63:**386–391.
19. **Davis K, Chamseddine D, Harper JM.** 2016. Nutritional limitation in early postnatal life and its effect on aging and longevity in rodents. Exp Gerontol **86:**84–89. https://doi.org/10.1016/j.exger.2016.05.001.
20. **de Kloet ER.** 2013. Functional profile of the binary brain corticosteroid receptor system: mediating, multitasking, coordinating, integrating. Eur J Pharmacol **719:**53–62. https://doi.org/10.1016/j.ejphar.2013.04.053.
21. **Eriksson E, Royo F, Lyberg K, Carlsson HE, Hau J.** 2004. Effect of metabolic cage housing on immunoglobulin A and corticosterone excretion in faeces and urine of young male rats. Exp Physiol **89:**427–433. https://doi.org/10.1113/expphysiol.2004.027656.
22. **Eriksson H, Gustafsson JA.** 1970. Steroids in germfree and conventional rats. Distribution and excretion of labelled pregnenolone and corticosterone in male and female rats. Eur J Biochem **15:**132–139. https://doi.org/10.1111/j.1432-1033.1970.tb00987.x.
23. **Flak JN, Jankord R, Solomon MB, Krause EG, Herman JP.** 2011. Opposing effects of chronic stress and weight restriction on cardiovascular, neuroendocrine and metabolic function. Physiol Behav **104:**228–234. https://doi.org/10.1016/j.physbeh.2011.03.002.
24. **Goymann W.** 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods Ecol Evol **3:**757–765. https://doi.org/10.1111/j.2041-210X.2012.00203.x.
25. **Grayson BE, Hakala-Finch AP, Kekulawala M, Laub H, Egan AE, Ressler IB, Woods SC, Herman JP, Seeley RJ, Benoit SC, Ulrich-Lai YM.** 2014. Weight loss by calorie restriction versus bariatric surgery differentially regulates the HPA axis in male rats. Stress **17:**484–493. https://doi.org/10.3109/10253890.2014.967677.
26. **Handa RJ, Weiser MJ.** 2014. Gonadal steroid hormones and the hypothalamo-pituitary-adrenal axis. Front Neuroendocrinol **35:**197–220. https://doi.org/10.1016/j.yfrne.2013.11.001.
27. **Hankenson FC, Marx JO, Gordon CJ, David JM.** 2018. Effects of rodent thermoregulation on animal models in the research environment. Comp Med **68:**425–438. https://doi.org/10.30802/AALAS-CM-18-000049.
28. **Harper JM, Austad SN.** 2000. Fecal glucocorticoids: a noninvasive method of measuring adrenal activity in wild and captive rodents. Physiol Biochem Zool **73:**12–22. https://doi.org/10.1086/316721.
29. **Haschek WM, Rousseaux CG, Wallig MA.** 2010. Gastrointestinal tract. Chapter 8. p 163–196. In: Haschek WM, Rousseaux CG, Wallig MA, editors. Fundamentals of toxicologic pathology. Burlington (MA): https://doi.org/10.1016/B978-0-12-370469-6.00008-8 Academic Press.
30. **Hau J, Kalliokoski O, Jacobsen K, Abelson K.** 2011. Interpretations of faecal concentrations of corticosteroid. Lab Anim **45:**129–130. https://doi.org/10.1258/la.2010.010125.
31. **Herbst AL, Yates FE, Glenister DW, Urquhart J.** 1960. Variations in hepatic inactivation of corticosterone with changes in food intake: an explanation of impaired corticosteroid metabolism following noxious stimuli. Endocrinology **67:**222–238. https://doi.org/10.1210/endo-67-2-222.
32. **Herman JP, Tamashiro KL.** 2017. The visible burrow system: a view from across the hall. Physiol Behav **178:**103–109. https://doi.org/10.1016/j.physbeh.2017.01.021.
33. **Horn MJ, Hudson SV, Bostrom LA, Cooper DM.** 2012. Effects of cage density, sanitation frequency, and bedding type on animal wellbeing and health and cage environment in mice and rats. J Am Assoc Lab Anim Sci **51:**781–788.
34. **Hubert MF, Laroque P, Gillet JP, Keenan KP.** 2000. The effect of diet, ad libitum feeding, and moderate and severe dietary restriction on body weight, survival, clinical pathology parameters, and cause of death in control Sprague–Dawley rats. Toxicol Sci **58:**195–207. https://doi.org/10.1093/toxsci/58.1.195.
35. **Hunt C, Hambly C.** 2006. Faecal corticosterone concentrations indicate that separately housed male mice are not more stressed than group housed males. Physiol Behav **87:**519–526. https://doi.org/10.1016/j.physbeh.2005.11.013.
36. **Jacobsen KR, Kalliokoski O, Teilmann AC, Hau J, Abelson KS.** 2012. Postsurgical food and water consumption, fecal corticosterone metabolites, and behavior assessment as noninvasive measures of pain in vasectomized BALB/c mice. J Am Assoc Lab Anim Sci **51:**69–75.
37. **Jeong KH, Sakihara S, Widmaier EP, Majzoub JA.** 2004. Impaired leptin expression and abnormal response to fasting in

corticotropin-releasing hormone-deficient mice. Endocrinology **145:**3174–3181. https://doi.org/10.1210/en.2003-1558.
38. **Joëls M, Baram TZ.** 2009. The neuro-symphony of stress. Nat Rev Neurosci **10:**459–466. https://doi.org/10.1038/nrn2632.
39. **Kalliokoski O, Jacobsen KR, Darusman HS, Henriksen T, Weimann A, Poulsen HE, Hau J, Abelson KS.** 2013. Mice do not habituate to metabolism cage housing—a 3 week study of male BALB/c mice. PLoS One **8:**1–11. https://doi.org/10.1371/journal.pone.0058460.
40. **Kalliokoski O, Jacobsen KR, Teilmann AC, Hau J, Abelson KS.** 2012. Quantitative effects of diet on fecal corticosterone metabolites in 2 strains of laboratory mice. In Vivo **26:**213–221.
41. **Kalliokoski O, Teilmann AC, Abelson KS, Hau J.** 2015. The distorting effect of varying diets on fecal glucocorticoid measurements as indicators of stress: a cautionary demonstration using laboratory mice. Gen Comp Endocrinol **211:**147–153. https://doi.org/10.1016/j.ygcen.2014.12.008.
42. **Kamakura R, Kovalainen M, Leppäluoto J, Herzig KH, Mäkelä KA.** 2016. The effects of group and single housing and automated animal monitoring on urinary corticosterone levels in male C57BL/6 mice. Physiol Rep **4:**1–9. https://doi.org/10.14814/phy2.12703.
43. **Kiss A, Jezova D, Aguilera G.** 1994. Activity of the hypothalamic pituitary adrenal axis and sympathoadrenal system during food and water deprivation in the rat. Brain Res **663:**84–92. https://doi.org/10.1016/0006-8993(94)90465-0.
44. **Kolbe T, Palme R, Tichy A, Rülicke T.** 2015. Lifetime dependent variation of stress hormone metabolites in feces of 2 laboratory mouse strains. PLoS One **10:**1–11. https://doi.org/10.1371/journal.pone.0136112.
45. **Konkle AT, Kentner AC, Baker SL, Stewart A, Bielajew C.** 2010. Environmental-enrichment-related variations in behavioral, biochemical, and physiologic responses of Sprague–Dawley and Long Evans rats. J Am Assoc Lab Anim Sci **49:**427–436.
46. **Korte SM, Koolhaas JM, Wingfield JC, McEwen BS.** 2005. The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neurosci Biobehav Rev **29:**3–38. https://doi.org/10.1016/j.neubiorev.2004.08.009.
47. **Lepschy M, Touma C, Hruby R, Palme R.** 2007. Non-invasive measurement of adrenocortical activity in male and female rats. Lab Anim **41:**372–387. https://doi.org/10.1258/002367707781282730.
48. **Lepschy M, Touma C, Palme R.** 2010. Faecal glucocorticoid metabolites: how to express yourself - comparison of absolute amounts versus concentrations in samples from a study in laboratory rats. Lab Anim **44:**192–198. https://doi.org/10.1258/la.2009.009082.
49. **Liu X, Wu R, Tai F, Ma L, Wei B, Yang X, Zhang X, Jia R.** 2013. Effects of group housing on stress induced emotional and neuroendocrine alterations. Brain Res **1502:**71–80. https://doi.org/10.1016/j.brainres.2013.01.044.
50. **Marashi V, Barnekow A, Ossendorf E, Sachser N.** 2003. Effects of different forms of environmental enrichment on behavioral, endocrinological, and immunological parameters in male mice. Horm Behav **43:**281–292. https://doi.org/10.1016/S0018-506X(03)00002-3.
51. **Martin B, Ji S, Maudsley S, Mattson MP.** 2010. "Control" laboratory rodents are metabolically morbid: why it matters. Proc Natl Acad Sci USA **107:**6127–6133. https://doi.org/10.1073/pnas.0912955107.
52. **Masoro EJ.** 2007. The role of hormesis in life extension by dietary restriction. Interdiscip Top Gerontol **35:**1–17.
53. **Mattos GE, Heinzmann JM, Norkowski S, Helbling JC, Minni AM, Moisan MP, Touma C.** 2013. Corticosteroid-binding globulin contributes to the neuroendocrine phenotype of mice selected for extremes in stress reactivity. J Endocrinol **219:**217–229. https://doi.org/10.1530/JOE-13-0255.
54. **Mattson MP, Duan W, Lee J, Guo Z.** 2001. Suppression of brain aging and neurodegenerative disorders by dietary restriction and environmental enrichment: molecular mechanisms. Mech Ageing Dev **122:**757–778. https://doi.org/10.1016/S0047-6374(01)00226-3.
55. **Mattson MP, Duan W, Wan R, Guo Z.** 2004. Prophylactic activation of neuroprotective stress response pathways by dietary and behavioral manipulations. NeuroRx **1:**111–116. https://doi.org/10.1602/neurorx.1.1.111.
56. **McCarty R.** 2016. Learning about stress: neural, endocrine and behavioral adaptations. Stress **19:**449–475. https://doi.org/10.1080/10253890.2016.1192120.
57. **McEwen BS, Gray J, Nasca C.** 2015. Recognizing resilience: learning from the effects of stress on the brain. Neurobiol Stress **1:**1–11. https://doi.org/10.1016/j.ynstr.2014.09.001.
58. **McEwen BS, McKittrick CR, Tamashiro KL, Sakai RR.** 2015. The brain on stress: Insight from studies using the Visible Burrow System. Physiol Behav **146:**47–56. https://doi.org/10.1016/j.physbeh.2015.04.015.
59. **McMaster PD.** 1922. Do species lacking a gall bladder possess its functional equivalent? J Exp Med **35:**127–140. https://doi.org/10.1084/jem.35.2.127.
60. **Moisan MP, Castanon N.** 2016. Emerging role of corticosteroid-binding globulin in glucocorticoid-driven metabolic disorders. Front Endocrinol (Lausanne) **7:**1–5. https://doi.org/10.3389/fendo.2016.00160.
61. **Moncek F, Duncko B, Johansson BB, Jezova D.** 2004. Effect of environmental enrichment on stress related systems in rats. J Neuroendocrinol **16:**423–431. https://doi.org/10.1111/j.1365-2826.2004.01173.x.
62. **Mrosovsky N.** 1990. Rheostasis: the physiology of change. Oxford(United Kingdom): Oxford University Press.
63. **Nicholson A, Malcolm RD, Russ PL, Cough K, Touma C, Palme R, Wiles MV.** 2009. The response of C57BL/6J and BALB/cJ mice to increased housing density. J Am Assoc Lab Anim Sci **48:**740–753.
64. **Oldham-Ott CK, Gilloteaux J.** 1997. Comparative morphology of the gallbladder and biliary tract in vertebrates: Variation in structure, homology in function and gallstones. Microsc Res Tech **38:**571–597. https://doi.org/10.1002/(SICI)1097-0029(19970915)38:6<571::AID-JEMT3>3.0.CO;2-I.
65. **Palme R.** 2005. Measuring fecal steroids: guidelines for practical application. Ann N Y Acad Sci **1046:**75–80. https://doi.org/10.1196/annals.1343.007.
66. **Pihl L, Hau J.** 2003. Faecal corticosterone and immunoglobulin A in young adult rats. Lab Anim **37:**166–171. https://doi.org/10.1258/00236770360563822.
67. **Pobbe RL, Pearson BL, Defensor EB, Bolivar VJ, Blanchard DC, Blanchard RJ.** 2010. Expression of social behaviors of C57BL/6J versus BTBR inbred mouse strains in the visible burrow system. Behav Brain Res **214:**443–449. https://doi.org/10.1016/j.bbr.2010.06.025.
68. **Power ML.** 2004. Viability as opposed to stability: an evolutionary perspective on physiological regulation. p 343–364. In: Schulkin J, editor. Allostasis, homeostasis, and the costs of physiological adaption. Cambridge (United Kingdom): Cambridge University Press. https://doi.org/10.1017/CBO9781316257081.012
69. **Qiu G, Spangler FL, Wan R, Miller M, Mattson MP, So KF, de Cabo R, Zou S, Ingram DK.** 2012. Neuroprotection provided by dietary restriction in rats is further enhanced by reducing glucocortocoids. Neurobiol Aging **33:**2398–2410. https://doi.org/10.1016/j.neurobiolaging.2011.11.025.
70. **Rasmussen S, Miller MM, Filipski SB, Tolwani RJ.** 2011. Cage change influences serum corticosterone and anxiety-like behaviors in the mouse. J Am Assoc Lab Anim Sci **50:**479–483.
71. **Robertson KL, Rowland NE, Krigbaum J.** 2014. Effects of caloric restriction on nitrogen and carbon stable isotope ratios in adult rat bone. Rapid Commun Mass Spectrom **28:**2065–2074. https://doi.org/10.1002/rcm.6994.
72. **Russo SJ, Murrough JW, Han MH, Charney DS, Nestler EJ.** 2012. Neurobiology of resilience. Nat Neurosci **15:**1475–1484. https://doi.org/10.1038/nn.3234.
73. **Sabatino F, Masoro EJ, McMahan CA, Kuhn RW.** 1991. Assessment of the role of the glucocorticoid system in aging processes and in the action of food restriction. J Gerontol **46:**B171–B179. https://doi.org/10.1093/geronj/46.5.B171.
74. **Sapolsky RM.** 2015. Stress and the brain: individual variability and the inverted-U. Nat Neurosci **18:**1344–1346. https://doi.org/10.1038/nn.4109.
75. **Sapolsky RM, Romero LM, Munck AU.** 2000. How do glucocorticoids influence stress responses? Integrating permissive,

suppressive, stimulatory, and preparative actions. Endocr Rev **21:**55–89.

76. **Sebaai N, Lesage J, Breton C, Vieau D, Deloof S.** 2004. Perinatal food deprivation induces marked alterations of the hypothalamo-pituitary-adrenal axis in 8-month-old male rats both under basal conditions and after a dehydration period. Neuroendocrinology **79:**163–173. https://doi.org/10.1159/000078098.
77. **Sheriff MJ, Dantzer B, Delehanty B, Palme R, Boonstra R.** 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia **166:**869–887. https://doi.org/10.1007/s00442-011-1943-y.
78. **Siswanto H, Hau J, Carlsson HE, Goldkuhl R, Abelson KS.** 2008. Corticosterone concentrations in blood and excretion in faeces after ACTH administration in male Sprague–Dawley rats. In Vivo **22:**435–440.
79. **Sterling P.** 2011. Allostasis: a model of predictive regulation. Physiol Behav **106:**5–15. https://doi.org/10.1016/j.physbeh.2011.06.004.
80. **Sundbom R, Jacobsen KR, Kalliokoski O, Hau J, Abelson KS.** 2011. Post-operative corticosterone levels in plasma and feces of mice subjected to permanent catheterization and automated blood sampling. In Vivo **25:**335–342.
81. **Tamashiro KL, Nguyen MM, Fujikawa T, Xu T, Yun Ma L, Woods SC, Sakai RR.** 2004. Metabolic and endocrine consequences of social stress in a visible burrow system. Physiol Behav **80:**683–693. https://doi.org/10.1016/j.physbeh.2003.12.002.
82. **Taylor W.** 1971. The excretion of steroid hormone metabolites in bile and feces. Vitam Horm **29:**201–285. https://doi.org/10.1016/S0083-6729(08)60050-3.
83. **Thanos PK, Cavigelli SA, Michaelides M, Olvet DM, Patel U, Diep MN, Volkow ND.** 2009. A non-invasive method for detecting the metabolic stress response in rodents: characterization and disruption of the circadian corticosterone rhythm. Physiol Res **58:**219–228.
84. **Touma C, Palme R.** 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann N Y Acad Sci **1046:**54–74. https://doi.org/10.1196/annals.1343.006.
85. **Touma C, Palme R, Sachser N.** 2004. Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. Horm Behav **45:**10–22. https://doi.org/10.1016/j.yhbeh.2003.07.002.
86. **Touma C, Sachser N, Möstl E, Palme R.** 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocrinol **130:**267–278. https://doi.org/10.1016/S0016-6480(02)00620-2.
87. **Vahl TP, Ulrich-Lai YM, Ostrander MM, Dolgas CM, Elfers EE, Seeley RJ, D'Alessio DA, Herman JP.** 2005. Comparative analysis of ACTH and corticosterone sampling methods in rats. Am J Physiol Endocrinol Metab **289:**E823–E828. https://doi.org/10.1152/ajpendo.00122.2005.
88. **Wan R, Camandola S, Mattson MP.** 2003. Intermittent food deprivation improves cardiovascular and neuroendocrine responses to stress in rats. J Nutr **133:**1921–1929. https://doi.org/10.1093/jn/133.6.1921.
89. **Woodward CJH, Hervey GR, Oakey RE, Whitaker EM.** 1991. The effects of fasting on plasma corticosterone kinetics in rats. Br J Nutr **66:**117–127. https://doi.org/10.1079/BJN19910015.
90. **Wright-Williams SL, Courade JP, Richardson CA, Roughan JV, Flecknell PA.** 2007. Effects of vasectomy surgery and meloxicam treatment on faecal corticosterone levels and behaviour in 2 strains of laboratory mouse. Pain **130:**108–118. https://doi.org/10.1016/j.pain.2006.11.003.
91. **Wyrwoll CS, Holmes MC, Seckl JR.** 2011. 11β-hydroxysteroid dehydrogenases and the brain: from zero to hero, a decade of progress. Front Neuroendocrinol **32:**265–286. https://doi.org/10.1016/j.yfrne.2010.12.001.
92. **Yadawa AK, Chaturvedi CM.** 2016. Expression of stress hormones AVP and CRH in the hypothalamus of *Mus musculus* following water and food deprivation. Gen Comp Endocrinol **239:**13–20. https://doi.org/10.1016/j.ygcen.2016.03.005.