

Effects of Short-Term Fasting in Male Sprague–Dawley Rats

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Fasting is a common procedure for animals in experiments. Although fasting may be necessary for scientific reasons, it should be minimized. In the current study, jugular-catheterized male Sprague–Dawley rats in metabolism cages were fasted for 0 to 24 h before measurement of various physiologic markers (serum chemistry, CBC analysis, serum corticosterone). When controlled for cohort, rats fasted for 6 and 16 h had significantly lower serum glucose than did nonfasted rats. Other values did not differ from controls. Only rats fasted for 24 h had elevated serum corticosterone levels. Therefore, fasting for as long as 16 h has fewer effects on rats than does fasting for 24 h. Fasting for 24 h or more therefore should receive appropriate consideration by both scientists and the IACUC in the experimental design and the animal-use protocol.

Abbreviation: CMS, chronic mild stress.

During experiments, laboratory animals may experience stress, defined as a nonspecific response of the body to external stimuli.^{1,31,35} Stress can become an experimental variable by changing blood parameters (stress leukogram, increased blood glucose), hormonal responses, wound healing rates, and other physiologic processes, including blood pressure and heart rate.^{26,30,31} These variables should be recognized and perhaps controlled to produce a sound experimental design. In addition, a more complete picture of the effects on the animal may benefit members of the IACUC when reviewing protocols or laboratory animal veterinarians when making recommendations.

One objective method to assess stress in rodents involves measurement of the hormone corticosterone.²⁶ Corticosterone, as one of the neuroendocrine mediators that result from activation of the hypothalamic–pituitary–adrenal axis, can be used to indicate stress.²² Handling of rodents can alter corticosterone release and may be considered stressful for rodents.²⁷ Other stimuli that have been used to induce stress include reversal of light:dark cycles, cold, restraint or immobilization, and forced running or swimming.^{9,15} Stress in these paradigms typically is assessed by measurement of corticosterone levels in serum^{2,13,20,37} or feces;^{24,26,30} organ weight analysis of spleen, thymus, or testes;¹² and various behavioral tests including self-stimulation, drug reward, open-field, and fine motor control.^{2,5,13,33} Of these, serum corticosterone concentration changes rapidly in response to stress and can easily be measured chronically.³⁰

Food manipulation can also create stress. Food manipulation often is performed prior to surgery, analysis of blood parameters, dosing of test compounds, behavioral testing, and other study-related procedures. Two main forms of food manipulation in-

clude restriction—reducing the amount of calories available to an animal—and deprivation—preventing access to food for a set period of time. Numerous studies in the literature evaluate chronic (typically greater than 24 h) caloric restriction^{5,7,12,13,33} and its effects on stress, behavior, and aging in rats and mice; however, studies on the stress effects of short-term food deprivation (less than 24 h) are less prevalent.

Chronic caloric restriction has been shown to affect various behavioral tests in animals. Activity in the open field increases,¹³ thresholds for rewarding stimulation are lowered,^{2,5} and fine motor function is decreased when animals are chronically food-restricted.³³ Food restriction (30% less than intake of ad libitum controls) has been associated with decreased liver and thymus weights and increased adrenal and testis weights.¹² Rats restricted to approximately 85% of the body weight of a free-feeding cohort had higher corticosterone but no increase in anxiety-associated behaviors, as measured by the elevated plus maze test and social interaction tests of anxiety.¹¹ In a study of B6C3F1 mice and Fischer 344 rats that were fed 60% of the ration of their ad libitum cohorts,⁷ the restricted rodents showed lower average body temperature, decreased frequency of trips to the food hopper, and a lower ratio of food:water consumption. These same restricted animals showed a greater range of body temperatures, greater average water consumption, and higher motor activity than did their ad libitum fed counterparts. Contradictory results have been reported in different strains of mice. In DBA/2J mice, animals with restricted access to food (2 h daily for 5 d) had increased wheel-running, whereas C57BL/6 mice reduced wheel-running.^{10,11}

Studies of food deprivation for 48 h or more have been performed. Young (5 mo) or old (20 mo) male Wistar rats deprived of food for 48 h did not have elevated serum corticosterone.¹⁸ Food deprivation for 2 d in rats led to no change in serum corticosterone concentrations; in contrast, deprivation for 10 d increased corticosterone in the serum, with subsequent refeeding resulting in a decline of corticosterone.³⁹ When rats were deprived of food for 5 d (60 h), corticosterone increased compared with basal levels.¹⁷

Received: 24 Jun 2010. Revision requested: 04 Aug 2010. Accepted: 29 Oct 2010.

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Measurements of corticosterone were not performed at less than 2 d of food deprivation in any of these studies, so no conclusions can be drawn on the effects of fasting on corticosterone levels earlier than 48 h.

Brief periods of food manipulation, in combination with multiple other stressors, are used in the chronic mild stress (CMS) model of depression.^{4,6,9,16,19,21,23} In this model, fasts of less than 24 h (with or without concurrent water deprivation) are used in series with light-cycle manipulation, forced running or swimming, and other low-level stressors. However, this chronic paradigm is very different from a single food deprivation of less than 24 h. The laboratory animal literature does not contain information about the stress induced by a single episode of acute food deprivation (defined for purposes of the current study as 24 h or less), regardless of the frequency with which this procedure is performed. Fasting of less than 24 h has been used as part of an unpredictable stress paradigm in a studies of memory, anhedonia, and depression;^{6,9,19,21,23,36} however, serum corticosterone changes as a result of this fasting have not been measured directly. Indeed, the stress induced by CMS is thought to be due to the unpredictability of the stressors, because all conditions are thought to be relatively mild stressors.⁴ Daily food deprivation with once-daily meal feeding is often used as a motivational tool in behavioral studies and has been for many years,^{2,11,25,28} but this paradigm typically is used chronically and more closely resembles food restriction rather than deprivation.

Because much of the literature describes fasts of 48 h or longer, the use of short-term fasting as a subset of a larger stress paradigm (such as in CMS paradigms), or the involvement of multiple short-term fasts as a motivational tool, the current study sought to describe the effects of a food fast of less than 24 h without specific concurrent stressors on commonly measured blood parameters in rats. Specifically, serum corticosterone and other blood analytes were measured during fasting periods from 2 to 24 h in male Sprague–Dawley rats. These time intervals for fasting were chosen to represent those commonly chosen for various experimental procedures that use fasting. Although past studies of food deprivation have shown no corticosterone increase after fasts of 48 h, we hypothesized that shorter durations of deprivation would increase serum corticosterone as compared with control animals.

Materials and Methods

Animals. Male Sprague–Dawley rats (213 to 278 g on arrival) surgically implanted with jugular catheters were obtained from Charles River Laboratories (Portage, MI). Catheters were made of polyurethane tubing (inner diameter, 0.025 mm; outer diameter, 0.040 mm) and exited from the animal between the scapulae dorsally, where they could be connected to a tether and swivel system, to minimize the stress of handling while obtaining blood samples during the study. Sentinel animals for these rats tested negative for Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, Kilham rat virus, Toolan H1 virus, rat parvovirus, rat minute virus, reovirus, *Mycoplasma pulmonis*, rat theilovirus, lymphocytic choriomeningitis virus, hantaviruses, mouse adenovirus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, *Bordetella bronchiseptica*, *Corynebacterium kitchneri*, *Salmonella* spp. *Streptobacillus moniliformis*, *Helicobacter* spp., *Klebsiella* spp. (*pneumoniae*, *oxytoca*), *Pasteurella* spp. (*multocida*, *pneumotropica*), *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneu-*



Figure 1. A rat in the jacket and tether system used in this project.

moniae, β *Streptococcus* group B, β *Streptococcus* group G, other β *Streptococcus* spp., Tyzzer disease virus, and endo- and ectoparasites. Male rats were used to avoid the cyclic hormonal changes in female rats that are associated with the estrus cycle and that could have confounded results.⁸

Housing and husbandry. On arrival, rats were transported to their housing location, weighed, and placed in jackets (Braintree Scientific, Braintree, MA) to protect the catheter for a 3-d period of acclimation before the study began (Figure 1). Rats were housed in metabolism cages for the duration of the study (Nalgene, Rochester, NY; Figure 2) so that their jugular catheters could be accessed without handling. Cages are designed for rats weighing 300 g or less and provide 50 in.² (320 cm²) of floor space. All rats were housed in the same room. As many as 8 cages were housed per rack, in rows of 4 cages. Municipal water, which was treated for human consumption with monochloramine (2.4 to 2.5 mg/L) and then passed through a 5.0- μ filter prior to distribution within the animal facility, was offered ad libitum. Powdered chow (Lab-Diet 5001, Purina Mills, St Louis, MO) was available ad libitum except as indicated by study procedures. Rats were acclimated to the powdered chow from the time of arrival. Room temperature was maintained between 20 and 23 °C. The health of the rats was checked daily by a member of the husbandry staff; there was no traffic into or out of the room except study personnel for sample collection. All animal procedures were performed under a protocol approved by the University Committee on the Care and Use of Animals in an AAALAC-accredited facility in accordance with the *Guide for the Care and Use of Laboratory Animals*.¹⁴

A 12:12-h reverse light cycle (lights on, 1900; lights off, 0700) was used to ensure fasting during the rats' nocturnal (active) period and to facilitate sample collection. For the time period prior to the study, catheter maintenance was performed every 2 to 3 d according to the supplier's instructions and locked with a heparinized glycerol solution (500 mg/mL heparin in a 1:1 mixture with glycerol). After 72 h of acclimation, a catheter extension with a 12-in. protective tether line (Braintree Scientific) was attached to the jugular catheter to allow blood sampling without handling the animal. The rats were allowed to acclimate for an additional 3 d after tether attachment.



Figure 2. The metabolic cage used to house the rats in this project. The water bottle (w), food hopper (h), and urine and feces cups (u and f) are visible in this photo.

Experimental procedures. The experiments were conducted in 3 cohorts to allow for manageable numbers of rats to be sampled and tested during each study segment. Rats were allocated into the experimental groups (6 rats per group) comprising controls (no fasting) and 2, 4, 6, 12, 16, and 24 h of fasting. Groups in each cohort were as follows: cohorts 1 and 2, control rats and those experiencing 4-, 6-, 12-, and 16-h fasts; cohort 3, control rats and those experiencing 2- and 24-h fasts. Blood samples in volumes of either 0.7 mL (for CBC, chemistry, and corticosterone) or 0.1 mL (for corticosterone only) were taken from the catheter without removing rats from their cage. CBC and chemistry analysis were only performed at 0, 6, 16, and 30 h to limit blood loss due to sample collection and avoid interference with corticosterone measurements. Blood samples for corticosterone measurement were taken at 0, 2, 4, 6, 12, 16, and 24 h. Because of catheter failure, not all blood samples could be obtained from all rats at some time points. In these cases, hematologic parameters were analyzed with fewer animals per group; the smallest number of animals analyzed in any group was 4. Because of irreparable catheter failure, 2 rats had to be euthanized before completion of the study.

Sample analysis. Hematology samples were run on a clinical hematology analyzer (Hemavet 1500FS, Drew Scientific, Waterbury, CT); serum chemistry samples were analyzed automatically (VetTest 8008, Idexx Laboratories, Westbrook, ME). Analytes

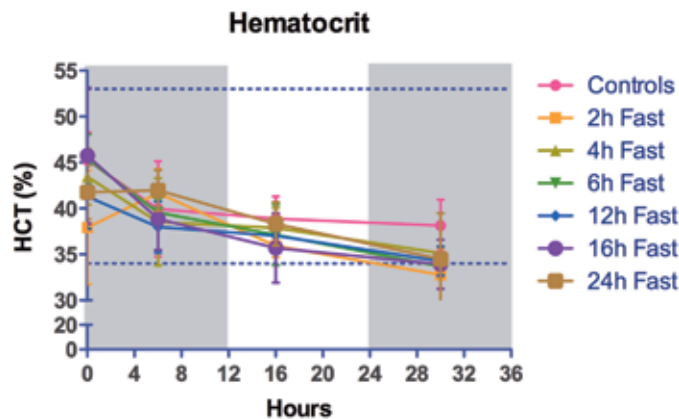


Figure 3. Hematocrit over the duration of the study. Dashed lines indicate normal range; shaded areas indicate the dark cycle.

measured in the serum chemistry panel were limited by the small sample size and included: alanine aminotransferase, alkaline phosphatase, blood urea nitrogen, creatinine, glucose, total protein, and creatine kinase. Serum corticosterone was analyzed by using a commercial colorimetric ELISA kit (Assay Designs, Ann Arbor, MI) in accordance with the manufacturer's instructions. Samples were run in duplicate and read at 405 nm. Results were extrapolated from standard curves.

Statistical analysis. Data were analyzed by using SAS software (version 9.1, SAS, Cary, NC) and GraphPad Prism 5 (GraphPad, San Diego, CA). Corticosterone, WBC count parameters (total cells, lymphocytes, and neutrophils), and glucose were assessed for normality by using the Univariate procedure. Variables that were not normally distributed were transformed to \log_{10} scale for subsequent statistical analysis with procedures that required the assumption of normality. Descriptive statistics (mean and 95% confidence interval or median and range) and plots were determined at the time points for which the outcome variables were measured.

Repeated-measures ANOVA was performed to assess whether the outcome measures were associated with fasting status over time, after controlling for intrasubject correlation. A class variable indicating the cohort to which the rat belonged was included in the analysis to control for any possible unmeasured confounding effects; the Mixed procedure was used. Fixed-effects independent variables used in the model initially included fasting status, time, cohort, and the fasting \times time interaction. Variables that were not moderately associated with the dependent (outcome) variable ($P > 0.200$ for F test based on Type III sums of squares) were dropped from the model, and a subsequent reduced model was refit. This process was repeated until all variables remaining in the model had a $P < 0.200$ (for F test based on Type III sums of squares). Fasting status was never removed from the model. The autoregressive (1) covariance matrix was used to model the covariance structure; this structure assumes measurement variability is constant over time and intrasubject correlations decrease as measurements are increasingly spaced over time.

For only those outcome measures that were significantly associated with fasting as determined by repeated-measures ANOVA, Student *t* test or 2-way ANOVA was used to determine the time points at which an outcome measure differed significantly between fasting and control groups. The test used was determined by the distribution of the outcome measure and whether cohort was associated with the outcome measure.

Table 1. Means and 95% confidence intervals for total WBC, lymphocyte, and neutrophil counts (in cells/ μ L)

		Control	Fasting
0 h	<i>n</i>	7	35
	Total WBC	10607.2 (8867.5, 12685.3)	10829.3 (9887.8, 11860.4)
	Lymphocyte	5793.0 (4762.1, 7046.9)	5925.2 (5236.0, 6591.7)
	Neutrophil	4370.0 (2783.0, 5957.0)	4471.1 (3847.5, 5094.8)
6 h	<i>n</i>	6	21
	Total WBC	10874.3 (8249.0, 14331.8)	12322.5 (11215.0, 13542.5)
	Lymphocyte	6714.3 (4619.6, 9761.1)	6241.6 (5589.8, 6969.5)
	Neutrophil	3670.0 (2515.1, 4824.9)	5749.0 (4705.2, 6792.9)
16 h	<i>n</i>	6	10
	Total WBC	15118.2 (13542.5, 16877.2)	15456.1 (13781.6, 17334.0)
	Lymphocyte	8525.1 (6996.8, 10384.8)	7890.4 (6406.2, 9720.8)
	Neutrophil	5838.3 (3413.5, 8263.2)	6893.0 (5074.2, 8711.8)

For all statistical tests, a *P* value of 0.05 or less was considered significant.

Results

Clinical chemistry values were generally within normal limits for all groups of rats throughout the study (alanine aminotransferase, 76.99 ± 13.51 U/L; alkaline phosphatase, 228.04 ± 64.24 U/L; blood urea nitrogen, 17.21 ± 3.88 mg/dL; creatinine, 0.32 ± 0.07 mg/dL; creatine kinase, 356.12 ± 151.39 U/L) and therefore were not analyzed further. Hematocrit decreased over time (Figure 3) during the study but remained within normal range and did not vary between treatment groups.

WBC parameters. Distributions of total WBC and lymphocyte counts were skewed high and did not pass tests for normality; therefore these parameters were transformed by using \log_{10} transformation for subsequent analyses. Neutrophil counts were normally distributed and remained untransformed for analyses.

For fasting and control rats, means and corresponding 95% 2-sided confidence intervals were computed for WBC parameters at each time point measured (Table 1). As expected at time 0, group means for the control and fasting groups for all parameters were almost identical. At subsequent time points, group means for total WBC and lymphocyte counts remained similar with more apparent variability between the groups than seen for neutrophil counts. For both groups, all parameters appeared to increase ($P < 0.0005$) from 0 to 16 h.

Repeated-measures ANOVA indicated no significant effect ($P = 0.2785$) of fasting on total WBC count in these rats. The time at which the sample was drawn had a significant ($P < 0.0005$) effect on total WBC count, which increased over time. Results of repeated-measures analysis for lymphocyte counts indicated no significant effect ($P = 0.4376$) of fasting in these rats. However, the time at which the sample was drawn had a significant ($P = 0.0012$) effect on lymphocyte count, which (as did total WBC count) increased over time. Repeated-measures analysis indicated that neutrophil counts increased significantly ($P < 0.0001$) over time and were significantly ($P = 0.0010$) associated with fasting status in these rats.

To determine at which time points neutrophil counts varied between groups, Student *t* tests were performed at each time

point. The mean differences (in cells/ μ L) between groups (control – fasted) and the respective *P* values for Student *t* tests at times 0, 6, and 16 h were -101.1 ($P = 0.8928$), -2079.0 ($P = 0.0433$), and -1054.7 ($P = 0.4207$), respectively, indicating an increase in neutrophils in the fasted rats at 6 hours.

Glucose. Glucose levels were normally distributed; means and standard errors for plasma glucose levels at each measured time point are presented in Table 2.

Repeated-measures analysis indicates a strongly significant ($P < 0.0001$) effect of fasting on plasma glucose levels, with fasting rats having lower glucose levels. In addition, time was significantly ($P = 0.0003$) associated with glucose levels, which were highest at 6 h for all rats.

To assess the relationship between glucose levels and fasting at each time point, a ANCOVA model was fit to the data at each time point, with glucose as the dependent variable and fasting status and cohort as independent covariables. Table 3 provides the mean difference in glucose levels between groups (control – fasted), a 95% 2-sided confidence interval for the mean difference, and the corresponding *P* value. At both 6 and 16 h of fasting, glucose levels are significantly ($P < 0.05$) higher for the control group relative to the fasting group.

Corticosterone. Rats typically have circadian variation of serum corticosterone,^{30,37} which peaks at or near the lights-off time and troughs at or near that for lights-on. This pattern is seen in comparing median lines (Figure 4), with the highest points near 0700 (time points 0 and 24 h [lights off]) and the lowest trough near 1900 (time point 12 h [lights on]).

The distribution of corticosterone was skewed high and did not pass tests for normality. A \log_{10} transformation of this variable was used to perform the repeated-measures analysis. Box and whisker plots of corticosterone levels at each time point for the fasting and control rats are presented in Figure 4. Repeated-measures analysis indicated that time of measurement was significantly ($P < 0.0001$) associated with corticosterone level, but fasting was not ($P = 0.0927$; Figure 4).

The 16-h time point was the only sample collected during the light (that is, rest) phase. As an exploratory analysis only, we repeated the repeated-measures analysis after excluding the 16-h time point, because we had not considered the possible differ-

Table 2. Plasma glucose levels

	Control			Fasting		
	<i>n</i>	mean	1 SD	<i>n</i>	mean	1 SD
0 h	7	129.7	4.83	34	127.1	3.68
6 h	6	154.8	4.58	20	122.0	2.17
16 h	6	135.0	7.77	9	104.2	3.34

Table 3. Mean difference (control – fasted) and 2-sided 95% confidence intervals for plasma glucose after controlling for cohort

	Mean difference	95% confidence interval	<i>P</i>
0 h	3.67	-8.38, 15.73	0.5406
6 h	33.26	23.17, 43.36	<0.0001
16 h	29.69	12.58, 46.80	0.0028

ential effects of sampling during the resting period in the study design. Results of this exploratory analysis indicated that fasting had a significant ($P = 0.0030$) effect on corticosterone levels measured during the active period; time continued to be significant ($P < 0.001$). These results indicate that fasting may affect corticosterone during an animal's active period, but that effect is absent in the resting period.

Discussion

To minimize negative experiences for research animals, we first must understand what constitutes a stressful experience. It previously had been assumed that although food restriction may be initially stressful, rats adapt with no adverse effects.³² This assumption, however, was made without any scientific data and could lead to false conclusions. The current study attempted to determine whether significant stress is induced by fasting of as long as 24 h, a common duration in experimental studies, to facilitate better-informed decisions regarding the animal welfare. As stated in *The Guide*, when restricting food "the least restriction that will achieve the scientific objective should be used."¹⁴ Therefore, investigators should be well-informed regarding the effects of fasting and the duration necessary to achieve the desired effects and should design experiments that take these factors into account.²⁹

Food deprivation is commonly used as a behavioral motivation tool^{25,28,38} and is also a component of the CMS model.^{16,19,21,23} In contrast to these paradigms, the current study investigated a single episode of fasting (unlike deprivation for behavioral motivation) without other concurrent stressors (unlike CMS).

WBC counts changed over the course of the experiment. Total WBC, lymphocyte, and neutrophil counts all increased over time regardless of group. Because rats were implanted with external catheters, an inflammatory response to this foreign body is the likely cause of the increased WBC numbers in all groups. In addition, fasted rats had higher neutrophil counts than did control rats, reaching significance ($P < 0.0010$) only at the 6-h time point. When the data were analyzed by repeated measures, fasted rats had significantly ($P < 0.0010$) higher neutrophil counts than did control rats. Taken together, these changes in WBC counts are suggestive of a stress response due to fasting (that is, a stress leukogram). The gradual decline in hematocrit among all groups most likely can be attributed to blood that was withdrawn during the

Corticosterone Level by Time
 Fasting vs Control

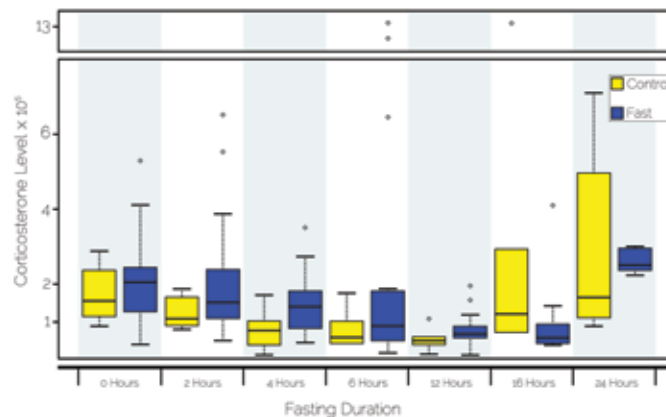


Figure 4. Box and whiskers plot of corticosterone, comparing fasted animals at each time point with control, nonfasted rats. *, $P = 0.0169$.

course of the study and was not significant between fasted and nonfasted control animals.

Serum glucose levels in the current study were significantly higher in nonfasted compared with fasted rats. Given that corticosterone was higher in fasted rats, a likely possibility for this finding is that higher serum glucose in nonfasted rats is related to food consumption. Lack of postprandial hyperglycemia may have masked this increase in fasted rats as compared with the nonfasted controls.

The circadian variation of corticosterone typical of rats was preserved in our experiment (Figure 4). Rats fasted for 24 h had significantly ($P = 0.0169$) higher serum corticosterone than did nonfasted controls, whereas a difference was not present at 16 h or earlier time points. Together these findings suggest that at some point between 16 and 24 h of food deprivation, rats generate a significant corticosterone response. Median corticosterone values for fasted animals were higher than those of controls at all time points except 16 h. The reversal of the pattern for the 16-h point is unexpected. The only recognizable difference between the 16-h sample and the other time points is photoperiod: the 16-h sample was the only one drawn during what would be the resting period for the rats (that is, lights on). Eliminating this time point results in a significant ($P = 0.003$) effect of fasting on corticosterone levels of fasted rats.

The animals in this study were singly housed in metabolism cages. No enrichment or bedding was provided. Although this design eliminated some possible confounding variables (such as any handling, competition over provided food, disruption of indwelling catheters by cagemates), translating these results to rats housed in solid-bottomed caging, with bedding or in groups should be done with caution. The current study, however, is a good starting point for considering the effect of food restriction during experimental design.

Variation was greater at the 24-h time point when compared with other time points (Figure 4). Reasons for this difference are unclear. However, without large samples sizes, 1 or 2 extreme values can increase variability dramatically. Perhaps 1 or 2 of the control rats experienced an unobserved stressful event, thereby resulting in increased variability at that time point.

Strains of mice vary in their responses to acute and chronic stress.³ Knowing this, further study of short-term fasting could be performed in other stocks and strains of rats to construct a more complete picture of the stress response to fasts of less than 24 h, which are often performed in laboratory settings. To simplify sample collection, urinary or fecal corticosterone could be examined as an indirect measure of serum corticosterone. Measuring urinary corticosterone as a ratio with urinary creatinine could be done to control for differing urine production rates over time between samples.³⁴ To determine the effect of sex, female rats would need to be ovariectomized or synchronously cycled to control for the changes in hormone concentrations that are associated with estrus and that can alter corticosterone levels or crossreact with corticosterone assays.⁸ Alternatively, assessing female rats at different points in their cycles could be performed, tracking the phase of estrus and examining differences between phases.

This study indicates that fasting for more than 16 h but less than 24 h induces elevated serum corticosterone and indications of a stress leukogram in rats. Other authors have indicated that feces or urine, which are collected noninvasively, may provide a better source for measurement of corticosterone metabolites than is serum corticosterone.³⁰ Subtle behavioral changes that are not detected easily may be indicators of stress (or distress) but were not measured in this study.²⁴ The current study does not differentiate between distress, eustress, or general arousal. To more fully examine the stress effect of fasting on rats, behavioral tests associated with stress or anxiety (such as an open-field test or elevated plus maze) could provide additional assessments.

Acknowledgments

We thank Patrick Kehres for his excellent care and handling of the animals during the study. We also thank Dr Jean Nemzek and Dr Omorodola Abatan for use of their laboratory and equipment and Dr Nancy Figler and Maya Hanna for assistance with manuscript preparation.

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