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Circadian pattern of total and free corticosterone concentrations, corticosteroid-binding globulin, and physical activity in mice selectively bred for high voluntary wheel-running behavior

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Abstract

In vertebrates, baseline glucocorticoid concentrations vary predictably on a diel basis, usually peaking shortly before the onset of activity. Presumably, circadian patterns in glucocorticoid secretion have evolved to match predictable rises in energetic need. In mice from lines selectively bred for high voluntary wheel-running, previous studies have reported that baseline plasma corticosterone concentrations at two different times during the photophase are elevated twofold above those of non-selected control lines. Here, we tested the hypothesis that the elevated daytime corticosterone levels could be explained by a shift in the circadian pattern of corticosterone levels. We measured baseline total plasma corticosterone levels, corticosteroid-binding globulin (CBG) capacity, and calculated free corticosterone levels (corticosterone not bound to corticosteroid-binding globulin and potentially biologically active) at six points during the 24-hour cycle in males on a 12:12 photoperiod. We also examined the daily pattern of both wheel-running and home-cage activity. Based on combined analysis of all six points, the circadian pattern of total corticosterone, corticosteroid-binding globulin, and free corticosterone levels did not significantly differ between high-runner and control mice (linetype $*$ time interaction $P = 0.56$, 0.45, and 0.55, respectively); however, all varied with time (all $P < 0.0001$) and mice from the selected lines had significantly elevated total ($P = 0.0125$) and free ($P = 0.0140$) corticosterone, with no difference in CBG binding capacity ($P = 0.77$). All mice were active primarily during the dark phase, and the factorial increase in activity of selected relative to controls lines was 2.33 for total daily wheel revolutions and 2.76 for total daily home-cage activity. The onset of the active period for both measures of locomotor activity coincided with peak total and free corticosterone levels in both selected and control lines. These findings lend support to our hypothesis that elevated circulating corticosterone levels have evolved as an adaptation to support increased locomotor activity in the selected lines.

Keywords: Artificial selection; Circadian rhythm; Corticosterone; Corticosteroid-binding globulin; Experimental evolution; Locomotor activity

1. Introduction

Adrenal glucocorticoid hormones have highly integrated effects on both energy balance (Dallman et al., 1993, 2007; Pecoraro et al., 2005, 2006) and behavior (Breuner and Wingfield, 2000; Pecoraro et al., 2005, 2006; Dallman et al., 2007). Under baseline conditions, plasma glucocorticoid (GC) levels vary predictably across a 24 h period (circadian variation) and, in some species, across the year in a seasonal pattern (for a review see Romero, 2002). Both circadian and seasonal patterns in GC secretion may have evolved to meet predictable rises in energy requirements (Romero, 2002; Pecoraro et al., 2006). For example, GC concentrations are highest around the time of arousal on a daily basis (morning for diurnal species and evening for

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nocturnal species), whereas seasonal peaks in baseline GC levels occur around the time of reproduction in several species of vertebrates, when energetic needs are often highest (Romero, 2002).

Acute elevations of circulating corticosterone (CORT) levels are superimposed on daily and seasonal fluctuations. In mammals (Lin et al., 1988, 1989; Coleman et al., 1998; Girard and Garland, 2002) and birds (Breuner et al., 1998; Lynn et al., 2003), CORT increases acutely in association with increases in locomotor activity. For example, in laboratory mice housed with access to wheels, plasma CORT concentration is significantly correlated with the number of wheel revolutions in the 20 min prior to blood sampling (Girard and Garland, 2002). Sparrows fed mealworms enriched with CORT display increased perch-hopping behavior (Breuner and Wingfield, 2000). Furthermore, ablation and replacement studies in rats have shown that CORT is necessary for rats to display schedule-induced wheel-running (Lin et al., 1988, 1989).

In a recent study, we reported that baseline plasma CORT levels of mice from lines that had been selectively bred for high levels of voluntary wheel-running are elevated twofold above those of their non-selected control lines (Malisch et al., 2007). Because of the known physiological effects of CORT that may support aerobically sustained exercise, such as increased lipolysis, proteolysis, and gluconeogenesis, with a simultaneous glycogen-sparing effect (Tharp, 1975; Coderre et al., 1992), we hypothesized that the increase in baseline CORT is an evolved (i.e., cross-generational) adaptation to support the high levels of wheel-running (nearly threefold higher than control mice). In addition, increased circulating CORT may promote wheel-running by increasing motivation to run. Running is a rewarding behavior (Belke and Garland, 2007; Brené et al., 2007) and elevation in plasma CORT increases the reward value of some behaviors. For example, increases in plasma CORT have been associated with increased self-administration of drugs, increased ingestions of saccharine, sucrose, and fats, and even increased self-administration of glucocorticoids (Piazza et al., 1993; Piazza and Le Moal, 1996, 1997, 1998; Bhatnagar et al., 2000; la Fleur et al., 2004; Pecoraro et al., 2004, 2005).

Increased circulating CORT levels as a correlated response to selective breeding for high locomotor activity (Girard and Garland, 2002; Malisch et al., 2007) are an important finding from the perspective of evolutionary endocrinology (e.g., see Garland and Carter, 1994; Finch and Rose, 1995; Goymann et al., 2004; Ketterson et al., 2005; John-Alder and Cox, 2007; Zera et al., 2007), but downstream modulators could negate or amplify any effects of CORT on target tissues. For example, corticosteroid-binding globulin (CBG) circulates in the plasma and binds CORT with high affinity (Hammond, 1995). Although the exact function of CBG is unknown, one hypothesis is that CORT bound to CBG is biologically inactive (Mendel, 1989; Breuner and Orchinik, 2002). Like CORT, CBG levels are not static, and in mammals they can vary seasonally (Tinnikov, 1999), daily (Friaria et al., 1988; Hsu and Kuhn, 1988), and in response to stress (Tinnikov and Oskina, 1994; Fleshner et al., 1995; Spencer et al., 1996; Deak et al., 1999).

Here, we examine baseline total CORT, CBG levels, and calculated free CORT (the putatively biologically active fraction) at multiple points across the daily cycle. A finding that CBG is increased in HR mice would suggest that it might be buffering elevated CORT, and hence that elevated CORT may be a maladaptive byproduct of the selection regimen. In contrast, a decrease or no change in CBG levels would be consistent with the hypothesis that elevated CORT in HR mice may be an adaptation to promote wheel-running. We also compared the circadian pattern of total CORT, free CORT, and bound CORT with the circadian pattern of two measures of activity, home-cage and wheel-running.

2. Materials and methods

2.1. Study animals

Adult (8- to 10-week-old) male *Mus domesticus* were obtained from an ongoing experiment in which four replicate lines of house mice are bred for high levels of voluntary running on days 5 + 6 of a 6-day exposure to wheels attached to standard housing cages (Swallow et al., 1998). Four replicate non-selected lines are maintained as controls (Swallow et al., 1998; Garland, 2003). Progenitors of these mice were from the outbred Hsd:ICR strain.

At weaning (21 days old), mice were toe-clipped for identification and housed randomly in same-sex groups of four. One week prior to any experimental procedure, mice were housed individually in an adjacent room to minimize disturbance. At all times, mice were on a 12:12 h light:dark cycle with lights on at 07:00 h Pacific Standard Time, and had ad lib food and water. Because mild novelty, including cage cleaning, can increase CORT levels and possibly alter activity patterns (Hennessy et al., 1977; Hennessy and Foy, 1987; Hennessy, 1991), a new cage and fresh bedding were provided 7 days prior to blood sampling and collection of behavioral data. Blood sampling may be stressful and may affect behavior, and physically demanding behaviors, such as wheel-running, may affect hormone levels; therefore, different individuals were used for behavioral studies than those used for hormone and binding globulin studies. To achieve adequate sample sizes, mice were obtained from multiple generations of the selection experiment. All animals used in these experiments were housed and maintained in accordance with NIH animal care guidelines, and all procedures were approved by the IACUC of the University of California, Riverside (protocol #0212042), an AAALAC-accredited institution.

2.2. Blood sampling for CORT and CBG

Males [$N = 192$, 24 from each of the four replicate HR lines and four replicate control lines] from generation 39 were used. Two mice were chosen at random from each of 12 different families per line. Animals were sampled in random order at one of six times: 02:00, 06:00, 10:00, 14:00, 18:00, and 22:00 h. To keep samples within ±30 min of the nominal time, mice were sampled in one of two separate batches. Mice were anesthetized with methoxyflurane, and blood samples (150 μl) were obtained by retro-orbital sinus puncture with heparinized microhematocrit tubes (Hoff, 2000). Data in Coleman et al. (1998; see their Fig. 5) for Hsd:ICR mice indicate no significant difference between CORT samples taken within 60 s after mice were removed from a home-cage versus after 2 min of treadmill running (two-way ANOVA on $\log_{10}$-transformed values, with time and sex as factors, P for time = 0.164: $N = 3$ females at time zero,

$\log_{10}$ mean ± SD = 1.16 ± 0.11 ng/ml; $N = 4$ males at time zero, 1.27 ± 0.21; $N = 4$ females after two min on treadmill, 1.37 + 0.35; $N = 4$ males after 2 min, 1.36 + 0.31). Therefore, in the present study, all blood samples were collected within 2 min of the initial disturbance to the animal (initial movement of cage). Samples were centrifuged (11,700 RPM) at room temperature for 5 min, hematocrit (HCT) was determined, and the plasma was stored at −80 °C.

2.3. Wheel-running activity

Wheel-running was recorded as part of the routine selective breeding protocol (Swallow et al., 1998) during the 43rd generation of selection. In brief 7- to 9-week-old mice (79 C line males and 112 HR line males) were allowed access to Wahman-type activity wheels (1.12 m circumference; Lafayette Instruments, Lafayette, IN, USA) for 6 days. Activity wheels were attached to standard housing cages, and running was measured as the total number of revolutions in 1-min blocks for 23 h (13:00–12:00 h) by an automated system (San Diego Instruments, San Diego, CA, USA). Here, we analyze running from the sixth day of wheel access to allow direct comparisons with home-cage activity (see next section).

2.4. Home-cage activity

Home-cage activity was measured in 10- to 12-week-old males from generation 43 (siblings to the wheel-running mice). Each individual ($N = 48$, six from each of the four HR and four C lines) was obtained from a different family. Home-cage activity was measured using four motion and activity detector (MAD-1) units from Sable Systems© (Las Vegas, Nevada, USA) interfaced to a Macintosh computer equipped with an A–D converter and LabHelper software (Warthog Systems, Riverside, CA, USA; http://www.warthog.ucr.edu). MAD-1 units are designed to measure activity of small animals in standard housing cages; activity is measured as changes in voltage output from isometric force transducers. Five days prior to measurement, mice were housed individually to mimic conditions during the wheel-testing selection protocol. Cages with food and water bottles were weighed, and cage mass was adjusted with bedding to 390 ± 0.1 g. Mice were minimally disturbed for five days. On day six, each cage was weighed and placed on the center of a MAD-1. Average activity in each half second interval was recorded 11:00–10:00 h the following morning for a total of 23 h (165,600 data points per mouse). LabAnalyst software (Warthog Systems) was used to convert results to normalized activity units (NAU) to ensure comparability between animals, MAD-1 units, and days. To compute NAU, we first eliminated drift using baseline correction, then output voltages (in volts), including both positive and negative values, were transformed to absolute values, normalized by dividing by the average of the lowest consecutive 10 min (1200 data points) of activity (presumed to be sleep), and then adjusted by subtracting 1.0 from the result (so that sleep periods had an average activity value of zero). For simplicity in figures, the summed 20-min activity bins were divided by 100.

2.5. Corticosterone assay

We determined total plasma levels of CORT in triplicate aliquots using an enzyme immunoassay kit from Assay Designs© (Ann Arbor, Michigan, USA). The CORT assay was optimized for use with our plasma samples following the methods of Wada et al. (2007), except that we used a 1:60 plasma dilution and 2% concentration of steroid displacement buffer (alterations based on results of the optimization assay). Average assay sensitivity was 1.7 ng/ml, and intra- and inter-assay coefficients of variation of a plasma pool assayed in triplicate in each assay were 9.45% and 16.65%, respectively.

2.6. Corticosterone-binding globulin assay and determination of free CORT

CBG affinity and capacity were determined following the methods of Breuner et al. (2003) but optimized for mice. In brief, plasma was incubated with dextran-coated charcoal for 20 min to remove endogenous steroids; supernatant was then removed and assayed at a final dilution of 1:1089. All assays were incubated for 2 h at 4 °C in 50 nM Tris (pH 7.4). For saturation analysis, pooled plasma was incubated with 0.15–9 nM ^{3}H-CORT in the presence or absence of unlabelled CORT to determine non-specific binding. A single binding site was found for mouse CBG ($K_d = 1.343$ nM; see Fig. 2.1 in Malisch, 2007). Based on the results of saturation analysis, point samples for CBG were incubated with 15 nM [^{3}H]CORT. Following incubation, assays were terminated and CBG bound to [^{3}H]CORT was separated from unbound by rapid vacuum filtration (Brandel Harvester, Gaithersburg, MD, USA) over glass fiber paper (GF/B, Brandel) that had been soaked for 1 h in 25 mM Tris plus 0.3% polyethylenimine. Filters were rinsed with 9 ml ice-cold 25 mM Tris. Following filtration, radioactivity of filters was determined with standard liquid scintillation spectrophotometry. All samples were run in triplicate and intra- and inter-assay variation were 15.62% and 21.97%, respectively. Individual CBG capacity estimates obtained reflected 92% occupancy of total binding sites; therefore all CBG values are corrected to 100% for statistical analysis. Free CORT concentrations were calculated using the equation of Barsano and Baumann (1989) as described by Deviche et al. (2001). This equation is commonly used to estimate free hormone levels for several binding globulin systems (e.g., growth hormone, glucocorticoids, androgens, thyroid hormones) and has been shown to correlate highly with direct measures of free CORT in mammals (Taymans et al., 1997; Adcock et al., 2006).

2.7. Statistical analyses

CBG affinity and capacity were determined by fitting untransformed data to appropriate equations using iterative, least-squares, curve-fitting techniques in GraphPad Prism (San Diego, CA, USA). The circadian patterns of total CORT, CBG concentrations, and free CORT were analyzed with a two-way, mixed-model analysis of covariance (ANCOVA) using SAS PROC MIXED (SAS Institute, Cary, NC, USA). The primary grouping factors were linetype and time, both fixed effects. Replicate lines were a random effect nested within linetype, and in addition family was nested within line. Degrees of freedom for testing the linetype effect were 1 and 6. The time effect and the time × linetype interaction were tested over the time × line-within-linetype interaction, again with 1 and 6 df. The animal's age and hematocrit were used as covariates in the model. For analyses of CORT and free CORT, latency from initial disturbance of animal to termination of the blood sampling procedure was also included as a covariate. The date on which animals were sampled (bleed batch) and assay batch were included as random cofactors in the model. All P values presented are 2-tailed and statistical significance was judged at $P < 0.05$ unless otherwise mentioned. (Analyses of individual timepoints are presented in Malisch (2007).)

Graphs of wheel-running activity and of home-cage activity were constructed using simple means ± SE of summed activity for 20-min time blocks. Statistical differences in total daily activity level (samples summed across 22 h; 13:00–11:00 h, divided by 158400) as well as average activity for nine 2-hour time blocks (average wheel revolutions/min for wheel-running and average normalized activity per half second for home-cage activity) were analyzed using a one-way mixed-model ANCOVA. As above, replicate lines were a random effect nested within linetype, and family was a random effect nested within line. Age was included as a covariate, and models were run both with and without body mass as an additional covariate. Mass was never a significant predictor of activity, and P values for the effect of linetype were similar; therefore, results are only shown for analyses without body mass as a covariate. For wheel analysis, family and measurement batch were additional random effects, and wheel-freeness (an inverse measure of wheel resistance) was used as an additional covariate in the model. For analysis of activity in 2-hour time blocks, statistical significance was judged at $P < 0.0056$ (i.e., 0.05/9) to correct for multiple comparisons.

3. Results

3.1. Diel pattern of total CORT, CBG binding, and free CORT

For both high-runner and control lines, total CORT levels followed the expected diel pattern, with highest levels at 19:00 h, just prior to lights out (Fig. 1). Thus, time of day was a highly significant predictor of total CORT ($P_{time} < 0.0001$). Linetype was also a significant predictor of total CORT ($P_{linetype} = 0.0125$), but the time * linetype interaction was not significant ($P = 0.560$), indicating that the daily pattern of CORT secretion does not statistically differ between HR and C males. In this analysis, mouse age, bleed delay time, and HCT were not significant (all $P > 0.29$).

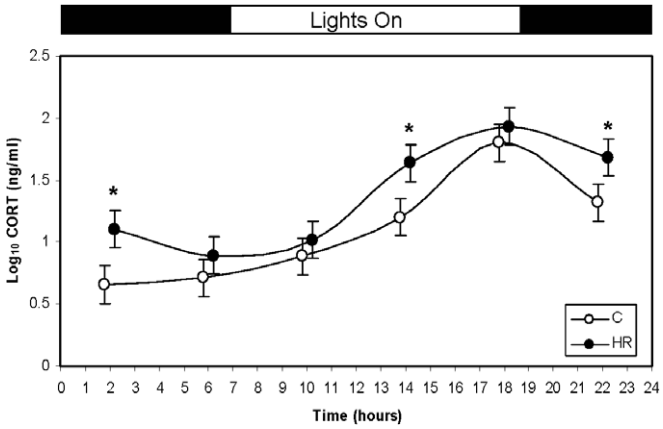

Fig. 1. Least square means ± SE of $\log_{10}$-transformed total plasma CORT concentrations (ng/ml) by time of day in hours since midnight for male mice from high-runner (HR) lines (open circles) and Control (non-selected) lines (closed circles). These values are from a two-way (linetype, time, linetype × time) ANCOVA with covariates of bleed delay time (seconds from initial disturbance until the end of blood sampling), age, and hematocrit. Plate (equivalent to assay batch), bleed batch, and family were also included in the model as random effects. For this model, both time ($P < 0.0001$) and linetype ($P = 0.0125$) were significant, but the time * linetype interaction was not ($P = 0.5595$). $N = 193$. Asterisks designate significant differences from one-way ANOVA models from analyses of individual timepoints (see Malisch, 2007).

CBG specific binding also followed a daily pattern ($P_{time} < 0.0001$), with a pronounced dip at 14:00 h (Fig. 2.3 in Malisch, 2007). Linetype ($P_{linetype} = 0.7735$) was not a significant predictor of CBG nor was there a significant time * linetype interaction ($P_{time * linetype} = 0.4509$). In this analysis, mouse age was not significant ($P = 0.54$) but HCT was almost a significant positive predictor of CBG binding capacity (2-tailed $P = 0.0510$). However, as each sampling time involved a separate assay (inter-assay variation = 21.97%), the overall circadian pattern should be interpreted with caution.

Free CORT levels followed a similar pattern to that of total CORT, with highest levels at 19:00 h (Fig. 2.4 in Malisch, 2007). Both time of day ($P_{time} < 0.0001$) and linetype were significant predictors of free CORT ($P_{linetype} = 0.0140$), but the lack of a time * linetype interaction ($P_{time * linetype} = 0.5474$) indicates that the daily pattern did not differ statistically between HR and C males. In this analysis, mouse age, bleed delay time, and HCT were not significant (all $P > 0.38$).

3.2. Diel pattern of locomotor activity

When examined in 2-hour time blocks, neither wheel-running activity nor home-cage activity differed statistically between HR and C males during the lights-on phase (Tables 1 and 2). As shown in Figs. 2 and 3, respectively, both HR and C mice showed a distinct onset of wheel-running and home-cage activity at lights off (19:00 h). During the active phase, HR males were significantly more active than C animals for the 8 h following lights off (Figs. 2 and 3; Tables 1 and 2). Neither body mass nor age consistently predicted either type of activity. Wheel-freeness never had a significant effect in analyses of wheel-running activity.

Total activity (summed across 22 h, 13:00–11:00 h) also differed significantly between HR and C males. HR were more active than C in both wheel-running ($P_{linetype} = 0.0006$) and home-cage activity ($P_{linetype} = 0.0006$). The HR/C difference in least squares means for total wheel revolutions was 9186.2/3939.9 = 2.33. For total home-cage activity, we computed the total normalized values (for

Table 1
Least square means and standard errors of average wheel revolutions/min for nine 2-hour bins

Time	N	Transform	Control	High runner	Linetype	Age	Freeness
1300–1500	191	Rank	105.230 ± 6.076	89.243 ± 5.627	0.102	0.730−	0.782−
1500–1700	191	Rank	101.350 ± 14.090	91.351 ± 13.892	0.609	0.403−	0.351−
1700–1900	191	Rank	101.560 ± 12.025	89.643 ± 11.724	0.505	0.636	0.437−
1920–2120	191	None	7.081 ± 0.988	17.120 ± 0.943	0.003	0.313−	0.730
2120–2320	191	None	5.749 ± 1.381	16.411 ± 1.344	0.002	0.621−	0.497
2320–0120	191	None	5.994 ± 1.462	14.399 ± 1.423	0.005	0.498−	0.539−
0120–0320	191	None	3.971 ± 1.194	11.813 ± 1.125	0.003	0.171−	0.700−
0700–0900	191	Rank	115.840 ± 9.596	80.638 ± 9.205	0.025	0.622	0.417−
0900–1100	191	Rank	107.120 ± 10.697	85.687 ± 10.331	0.200	0.935	0.463

Significance levels (*P* values) are shown for HR vs. control lines (linetype) and for the covariates age and wheel freeness. Family and measurement batch were also included as random factors (results not shown). A minus sign following a *P* value indicates a negative effect. For time periods that were not rank transformed, the least square mean values reported here can be made comparable to the simple means shown in Fig. 2 by multiplying the former by 20.

Table 2
Least square means and standard errors of normalized activity units (average score per sample) for home-cage activity for nine 2-hour bins

Time	N	Transform	Control	High runner	Linetype	Age
1300–1500	48	Log_{10}	0.210 ± 0.154	0.373 ± 0.157	0.487	0.126–
1500–1700	48	Log_{10}	0.240 ± 0.110	0.252 ± 0.111	0.942	0.005–
1700-1900	48	Log_{10}	0.551 ± 0.067	0.424 ± 0.068	0.231	0.628
1920–2120	48	Log_{10}	0.931 ± 0.057	1.456 ± 0.060	0.001	0.028
2120–2320	48	Log_{10}	0.726 ± 0.070	1.390 ± 0.073	0.001	0.128
2320–0120	48	Log_{10}	0.797 ± 0.060	1.334 ± 0.063	0.001	0.472
0120–0320	48	Log_{10}	0.669 ± 0.075	1.170 ± 0.077	0.004	0.219
0700–0900	48	Log_{10}	0.563 ± 0.106	0.418 ± 0.109	0.378	0.745
0900–1100	48	Log_{10}	0.251 ± 0.092	0.203 ± 0.094	0.730	0.840

Significance levels (P values) are shown for HR vs. control lines (linetype) and for the covariate age. A minus sign following a P value indicates a negative effect. These least square mean values can be made comparable to the simple means shown in Fig. 3 by antilogging the former and then multiplying by 24.

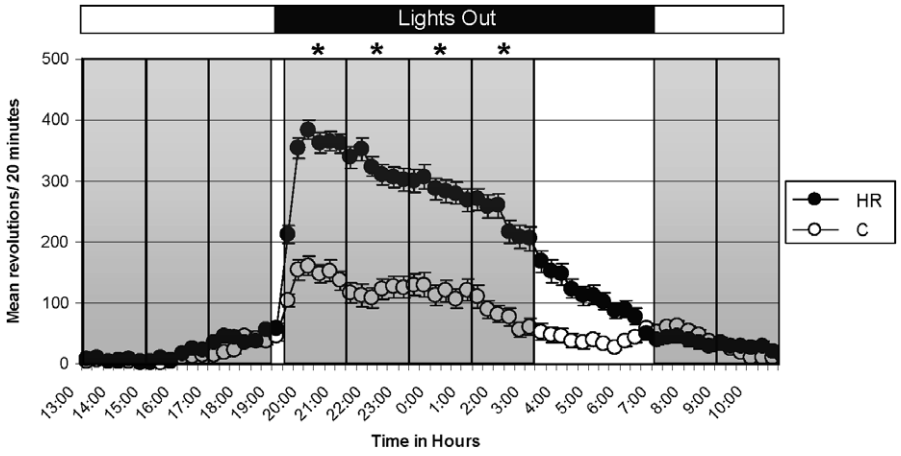

Fig. 2. Simple means ± SE of wheel-running activity for 20-min bins as a function of time in hours since midnight for male mice. Open circles represent HR lines and closed circles represent control lines. Grey columns represent 2-hour time blocks that were analyzed for statistical differences in activity level. Asterisks denote significant differences from time-block analyses (see Table 1). $N = 191$.

158,400 data points; 2 samples per second for 22 h) for each mouse. The factorial difference in least squares means for home-cage activity (backtransformed from log_{10}) was $18{,}676/6768 = 2.76$.

4. Discussion

Values measured for circulating CORT levels (see also Malisch, 2007) are similar to those from previous studies (Coleman et al., 1998; Malisch et al., 2007). Although the study of Malisch et al. (2007) on both sexes and that of Girard and Garland (2002) on females both indicated that HR mice have elevated baseline CORT relative to C mice, they examined only a few times. In addition, a recent study of HR males (at 10 and 18 months of age) indicated plasma CORT levels during the middle of the light phase that was not statistically higher than those from C (Vaanholt et al., 2007). Therefore, the possibility that elevated CORT in HR mice was attributable to a shifted daily pattern could not be dismissed, because a circadian pattern of CORT secretion has been well established in vertebrates including house mice (Ottenweller et al., 1979; Nichols and Chevins, 1981; Montano et al., 1991; Droste et al., 2003). In the present study, both HR and C males exhibited the expected pattern of total CORT concentrations (Fig. 1), with the highest levels just prior to lights out and lowest near lights on. Two-way ANOVA indicated a significant effect of both time and linetype, but no time * linetype interaction ($P_{\text{time * linetype}} = 0.560$). Therefore, the null hypothesis of no difference in daily pattern of total circulating CORT concentration cannot be rejected.

Daily fluctuations in CBG binding capacity have been reported for a number of vertebrate taxa, including humans (Angeli et al., 1978; Friaria et al., 1988), rats (Ottenweller et al., 1979; D'Agostino et al., 1982; Calvano and Reynolds, 1984; Hsu and Kuhn, 1988; Meaney et al., 1992), mice (Ottenweller et al., 1979), guinea pigs (Fujieda et al., 1982), and birds (Meier et al., 1978; Lynn et al., 2003). However, CBG does not seem to follow as consistent a daily pattern across taxa as compared with total CORT. Because CBG levels did not differ statistically between HR and C males, the circadian pattern of free CORT resembles that of total CORT (Fig. 1), with peak levels occurring at lights out (onset of the active period) and a nadir near the time of lights on.

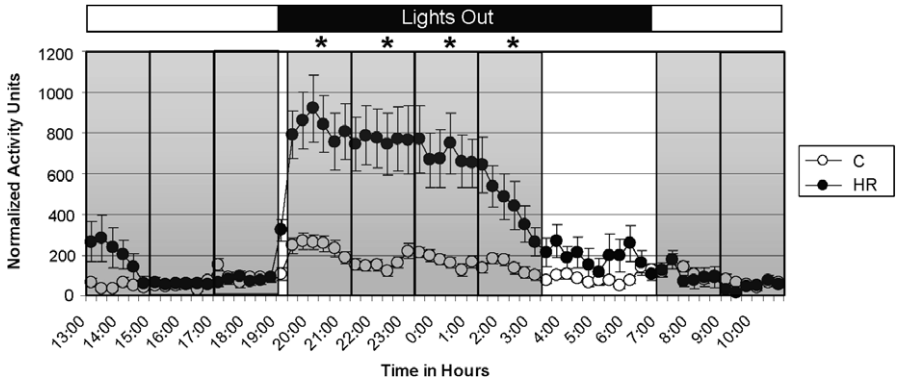

Fig. 3. Simple means ± SE of total (summed) normalized home-cage activity units for 20-min bins as a function of time in hours since midnight for male mice (all values have been divided by 100 for graphical convenience). Open circles represent HR lines and closed circles represent control lines. Grey columns represent 2-hour time blocks that were analyzed for statistical differences in activity level. Asterisks indicate significant differences from time-block analyses (see Table 2) $N = 48$.

An evolutionary increase in total and/or free CORT could promote wheel-running by increasing available energy to facilitate the behavior, increasing motivation to perform the behavior, or a combination of the two (Dallman et al., 1993, 2007; Pecoraro et al., 2006). Therefore, results of the present study are consistent with the hypothesis (Malisch et al., 2007) that elevated CORT levels are an adaptation to support high activity levels. On the other hand, the only published information on plasma glucose levels in these lines of mice indicated no difference between HR and C males from generation 17 measured at rest during the day (Dumke et al., 2001).

Of course, the elevated CORT levels of HR mice might simply be a consequence of increased activity levels, because locomotor activity increases CORT secretion in mammals and birds (Lin et al., 1988, 1989; Breuner et al., 1998; Coleman et al., 1998; Girard and Garland, 2002; Lynn et al., 2003). However, acute differences in locomotor activity cannot explain the significant difference in CORT level seen at 14:00 h (see Fig. 1 and Malisch, 2007), when mice are typically inactive. Although Fig. 3 suggests a difference in home-cage activity at this time, it is not statistically significant (Table 2); furthermore, when activity state (active or asleep) was included in the analysis for the 14:00 timepoint the linetype difference remained statistically significant and indeed the P value for linetype was reduced from 0.030 to 0.003 (Malisch, 2007). This increase in activity in the HR lines between 13:00 and 15:00 h is not seen in the graph of wheel-running (Fig. 2), and we suspect the increase in home-cage activity might reflect an increase in sensitivity to disturbance in the HR lines. Other factors that may interact with circulating CORT levels are body fat and rate of food consumption, but these were not recorded. Previous studies have reported that HR males have less body fat as compared with C (Dumke et al., 2001; Swallow et al., 2001; Vaanholt et al., 2007) and that they eat more on a body mass-adjusted basis, even when housed without access to wheels (Swallow et al., 2001; Vaanholt et al., 2007).

Although elevated CORT levels may be adaptive in the context of selective breeding for high locomotor activity, they may also have negative effects (see Sapolsky et al., 2000; Sapolsky, 2002 for general reviews). Indeed, our previous studies suggest that elevated CORT suppresses growth (Swallow et al., 1999; Girard and Garland, 2002; Malisch et al., 2007) and possibly immune function (Bunkers et al., 2004; Malisch, 2007) in HR mice.

An important issue left unresolved from our previous research was whether HR lines exhibit elevated locomotor activity when housed in standard cages without running wheels, and after they have been habituated (reviewed in Rhodes et al., 2005). If not, then it could be argued that the elevated wheel-running is a very specific behavior that may not parallel innate variation in voluntary activity levels. Here, we show for the first time that male HR mice exhibit elevated activity in standard housing cages, in the absence of running wheels, even when they have been habituated to this environment for several days. This is crucial for establishing the validity of the HR lines as a putative general model for variation in activity levels (e.g., see Mill et al., 2002).

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