Baseline and Stress-Induced Plasma Corticosterone Concentrations of Mice Selectively Bred for High Voluntary Wheel Running

Jessica L. Malisch[1]
Wendy Saltzman[1]
Fernando R. Gomes[1,*]
Enrico L. Rezende[1,†]
Daniel R. Jeske[2]
Theodore Garland Jr.[1,‡]
[1]Department of Biology, University of California, Riverside, California 92521; [2]Department of Statistics and Statistical Consulting Collaboratory, University of California, Riverside, California 92521

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ABSTRACT

The hypothalamic-pituitary-adrenal (HPA) axis is important in regulating energy metabolism and in mediating responses to stressors, including increasing energy availability during physical exercise. In addition, glucocorticoids act directly on the central nervous system and influence behavior, including locomotor activity. To explore potential changes in the HPA axis as animals evolve higher voluntary activity levels, we characterized plasma corticosterone (CORT) concentrations and adrenal mass in four replicate lines of house mice that had been selectively bred for high voluntary wheel running (HR lines) for 34 generations and in four nonselected control (C) lines. We determined CORT concentrations under baseline conditions and immediately after exposure to a novel stressor (40 min of physical restraint) in mice that were housed without access to wheels. Resting daytime CORT concentrations were approximately twice as high in HR as in C mice for both sexes. Physical restraint increased CORT to similar concentrations in HR and C mice; consequently, the proportional response to restraint was smaller in HR than in C animals. Adrenal mass did not significantly differ between HR and C mice. Females had significantly higher baseline and postrestraint CORT concentrations and significantly larger adrenal glands than males in both HR and C lines. Replicate lines showed significant variation in body mass, length, baseline CORT concentrations, and postrestraint CORT concentrations in one or both sexes. Among lines, both body mass and length were significantly negatively correlated with baseline CORT concentrations, suggesting that CORT suppresses growth. Our results suggest that selection for increased locomotor activity has caused correlated changes in the HPA axis, resulting in higher baseline CORT concentrations and, possibly, reduced stress responsiveness and a lower growth rate.

Introduction

Glucocorticoid hormones are end products of the hypothalamic-pituitary-adrenal (HPA) axis, a highly conserved vertebrate endocrine system that is integral to energy balance during predictable circadian changes in activity as well as during periods of stress (for a review, see Jacobson 2005). Glucocorticoid release from the adrenal cortex is modulated in part by the hypothalamus and the anterior pituitary, which secrete stimulating hormones (e.g., corticotrophin-releasing hormone and adrenocorticotropic hormone, respectively) when an energetic need is perceived and decrease production of stimulating hormones when energetic needs are perceived as stable (Sapolsky 2002). Under baseline (i.e., resting and nonstressed) conditions, plasma glucocorticoid levels vary predictably across a 24-h period (circadian variation) and, in some species, across the year in a seasonal pattern (circannual variation; for a review, see Romero 2002). Presumably, circadian and circannual patterns in glucocorticoid secretion have evolved to match predictable rises in energetic need.

Circulating glucocorticoid concentrations rise dramatically above baseline levels in response to psychological and physical stressors. The definition of biological "stress" and "stressors" is complicated and somewhat controversial, but for purposes of this study, psychological stressors can be characterized as stimuli that evoke such emotional responses as fear or anxiety. A physical stressor, on the other hand, triggers preprogrammed responses automatically, such as when a fall in blood glucose triggers an increase in the release of adrenocorticotropic hormone and corticosterone (CORT). Physical stressors include internal or external noxious stimuli, such as extreme temper-

* Present address: Departamento de Fisiologia, Instituto de Biociencias, Universidade Estadual Paulista, 18618-000 Botucatu, Brazil.
† Present address: Integrative Ecology Group, Estacion Biologica Doñana, Consejo Superior de Investigaciones Científicas, Apartado 1056, E-40180 Sevilla, Spain.
‡ Corresponding author; e-mail: tgarland@ucr.edu.

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atures, increased physical activity, and injury (Reeder and Kramer 2005).

Typically, both psychological and physical stressors are associated with increased energetic needs, and it is generally presumed that the stress response is adaptive and has evolved to help meet these needs. However, long-term exposure to stressors and elevated circulating CORT concentrations may be associated with multiple deleterious effects, including growth suppression, muscle wastage, immune suppression, and neuronal death (Sapolsky 2002). Therefore, evolved differences in baseline glucocorticoid concentrations and/or the magnitude of their elevation in response to a stressor must reflect a balance between their beneficial and deleterious effects. Various investigators have documented differences in baseline and/or stress-induced glucocorticoid levels among species or populations of vertebrates and have developed hypotheses to explain these differences (e.g., Coe et al. 1992; Dunlap and Wingfield 1995; Martin et al. 2005). However, as detailed elsewhere (e.g., Garland and Adolph 1994; Leroi et al. 1994), comparative studies are inherently correlational in nature and can be viewed as nondefinitive with respect to the actual adaptive significance of interspecific variation in such traits as glucocorticoid levels. Therefore, we have employed the alternative, an experimental evolution approach, and we provide the first example of an apparently adaptive change in baseline glucocorticoid concentrations in response to a well-defined selective regime favoring increased levels of locomotor activity in mice (see also Girard and Garland 2002).

Physical exercise requires increased energy and elicits acute increases in circulating glucocorticoid concentrations in mammals (Tharp 1975; Kraemer et al. 1993; Viru et al. 1994; Coleman et al. 1998). We have been investigating the correlated physiological, morphological, and behavioral responses to selection for high voluntary activity levels in house mice (Swallow et al. 1998). After 16 generations, mice from four replicate lines selectively bred for high voluntary wheel-running behavior (HR mice) ran 2.7 times more than those from four randomly bred control lines (C mice), and this difference has been maintained or slightly increased through 28 subsequent generations (T. Garland, unpublished data). HR mice show numerous differences from C mice, including smaller body size and reduced body fat, altered hindlimb bone dimensions, higher maximal oxygen consumption during forced treadmill exercise, higher predatory aggression, and altered sensitivity to drugs that target dopamine function (see, e.g., Rhodes et al. 2001, 2003, 2005; Garland et al. 2002; Gammie et al. 2003; Garland 2003; Rhodes and Garland 2003; Kelly et al. 2006; Rezende et al. 2006).

The only previous study of CORT concentrations in the HR and C lines involved only females and found that for mice housed without wheels, plasma CORT was significantly higher in the HR lines in one of three daytime samples (Girard and Garland 2002). The purpose of the present study was to examine baseline plasma CORT concentrations in both males and females from a later generation, when any difference might be larger, and to determine whether the CORT response to a novel stressor differed in HR and C lines. We investigated both sexes because females of both HR and C lines run more and faster than males (e.g., Swallow et al. 1998, 1999, 2001, 2005; Garland 2003). We hypothesized that both sexes of HR mice (1) would have elevated baseline CORT concentrations, as compared with C mice, and (2) would exhibit an exaggerated CORT response to a commonly employed nonexercise stressor, physical restraint. Because chronic CORT elevation might be associated with hypertrophy of the adrenal cortex, we also examined adrenal mass as a gross index of HPA activity.

Material and Methods

Study Animals

Adult (7–9-wk-old) male and female house mice (*Mus domesticus*) derived originally from the Hsd:ICR strain were used. Mice were obtained from the thirty-fourth generation of an artificial selection experiment in which house mice are bred for high levels of voluntary wheel running (Swallow et al. 1998; Garland 2003; Rhodes et al. 2005). Individuals ($N = 112$, seven of each sex from each of four replicate HR lines and four C lines) were chosen at weaning (21 d of age), with one female and one male chosen at random from each of seven different families per line. At weaning, mice were toe-clipped for identification and housed randomly in same-sex groups of four individuals for 2 wk. Mice were housed individually and moved to an adjacent room 2 wk before any experimental procedure in order to minimize unnecessary disturbance. Mice were maintained on a 12L : 12D cycle with lights turned on at 0700 hours and were provided with food and water ad lib. Because mild novelty, including cage cleaning, can increase CORT concentrations in laboratory mice (Hennessy and Levine 1977; Hennessy et al. 1977; Hennessy and Foy 1987; Hennessy 1991), mice were provided with a new cage and fresh bedding 10 d before blood sampling. Thereafter, no further cage cleaning was performed until after blood sampling. All animals used in these experiments were housed and maintained in accordance with National Institutes of Health animal care guidelines, and all procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Riverside (IACUC 0212042), an institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Experimental Design

One baseline and one postrestraint blood sample were obtained from each mouse. For baseline sample collection, animals were sampled in random order between 1600 and 1800 hours (i.e., 1–3 h before lights were turned off), when mice are typically inactive and CORT concentrations are near the circadian peak (Montano et al. 1991). Mice were anesthetized by methoxy-

flurane, and blood samples (150 μL) were obtained by retro-orbital sinus puncture using heparinized microhematocrit tubes (Hoff 2000). Increases in circulating CORT concentrations can be detected 2 min after disturbance in rats (Davidson et al. 1968). Therefore, baseline blood samples were collected within 2 min of the initial disturbance to the animal (initial movement of cage). Samples were centrifuged at room temperature for 5 min, hematocrit (Hct; percentage of plasma that comprises red blood cells [RBC]; % RBC) was determined, and the plasma was stored at −80°C until analysis.

Because CORT levels can fluctuate predictably across the estrous cycle in mice (Nichols and Chevins 1981), we determined the stage of estrus by vaginal lavage within 2 h after blood sampling. We scored lavage samples according to the five stages outlined by Rugh (1968; see also Girard and Garland 2002). After blood sampling, mice were weighed, placed in a new cage with fresh bedding, provided with fresh water, and returned to the housing room.

Ten days after baseline blood sampling, mice were subjected to a 40-min restraint stressor (see below), and a blood sample was collected immediately thereafter. Samples were obtained within the same window of time (1600–1800 hours), with individual mice sampled in the same order, and using the same methods as were used for baseline samples. Blood was obtained within 2 min of termination of restraint; mice were then weighed, and stage of estrus was again scored for females. Approximately 2 wk after the restraint procedure, mice were killed, weighed, and measured for nose-rump length, and adrenal glands were excised. The left adrenal gland was cleaned of fat and weighed to the nearest 0.1 mg (the right adrenal was removed and fixed for possible future analysis).

Restraint Stressor

Mice were restrained in a clear acrylic tube (internal diameter 26 mm, length 150 mm) with ample 6-mm diameter ventilation holes. Escape from the tube was prevented by 1/4-in hardware cloth affixed to one end and by a three-holed rubber stopper inserted into the opposite end. Mice were able to move forward and backward within the tube but were unable to turn around. To minimize exposure to room vibration, visual stimulation, and olfactory stimulation, the restraint tube was placed on foam rubber inside a clean, opaque, standard housing cage. Mice were observed continuously and remained awake during the entire restraint procedure.

Mice were restrained for 40 min. In most other studies, restraint duration has varied between 2.5 min and 24 h (see Glavin et al. 1994 for review). The duration of 40 min was chosen based on a previous study (Coleman et al. 1998) that examined the time course (up to 60 min) of the CORT response to forced treadmill running, a stressor with both physical and psychological components, in mice from the same strain as was used for the founder population of our HR and C animals. Coleman et al. (1998) found that CORT concentrations increased gradually over the first 30 min and reached maximal concentrations in both males and females after approximately 40 min of forced running.

Corticosterone Assay

Blood samples were assayed for CORT at the National Primate Research Center at the University of Wisconsin–Madison. In brief, steroids were extracted from plasma with 7.5 mL ether in two washes. The fraction containing CORT was eluted with 50% ethyl acetate in isooctane from a celite microcolumn with a 1 : 1 ethylene glycol to propylene glycol solid phase. Total CORT concentration was determined in duplicate aliquots by radioimmunoassay as described by Girard and Garland (2002), using trace from American Radiolabeled Chemicals (St. Louis) and monoclonal antibody B3-163 from Esoterix Endocrinology (Calabasas Hills, CA). Antibody cross-reactivities were 4% for desoxycorticosterone, 1% for 5β-pregnanedione, 0.6% for progesterone, 0.4% for cortisol, 0.4% for 5α-pregnanedione, and $\leq$0.2% for all other steroids.

The CORT assay was fully validated for use with mouse plasma. Serial dilutions of a mouse plasma pool (10–0.156 μL, $N = 7$) yielded a displacement curve parallel to that obtained with cortisol standards (Sigma, St. Louis). The mean recovery of CORT standards added to 20 μL of a 1 : 20 dilution of mouse plasma was 107.85% ± 3.43% (±SEM; $N = 8$). Assay sensitivity was 5.2 ng/mL, and inter- and intra-assay coefficients of variation of a plasma pool assayed in quadruplicate in each assay were 10.05% and 4.89%, respectively.

Statistical Analysis

For all analyses, plasma CORT concentrations were square root transformed to stabilize variances among groups and to improve normality of residuals. Analyses that included all animals employed a two-way mixed-model nested ANCOVA using SAS PROC MIXED (SAS Institute, Cary, NC). The primary grouping factors were linetype (HR vs. C) and sex (male vs. female), both fixed effects. Replicate lines were a random effect nested within linetype, and family was nested within line. Degrees of freedom for testing the linetype effect were always 1 and 6. The sex effect and the sex × linetype interaction were tested over the sex × line-within-linetype interaction, again with 1 and 6 df. The age of the animal, latency from initial disturbance of animal to termination of the blood sampling procedure (bleed delay time, in s), time of day, and (z-transformed time of day)^2 were used as covariates in the model. For analysis of adrenal mass, we also ran a model with log body mass as a covariate.

To test for differences among the replicate lines within linetypes, we considered the sexes separately to avoid complications stemming from family as an additional nested random effect. Line differences were assessed by comparing the differences in

ln of restricted likelihoods for models with and without line in the model: twice the difference in ln likelihoods asymptotically follows a χ^2 distribution with 1 df. For females, stage of estrus was not included because (1) it was not statistically significant and (2) as an additional random effect, it complicates analysis of line effect. To compute adjusted line means, we also used SAS PROC MIXED but excluded linetype from the model and specified line as a fixed effect, again separately by sex.

Statistical significance was judged at $P < 0.05$. All P values presented are two-tailed.

Results

Combined Analyses of Males and Females

Baseline CORT. As expected, females had significantly higher baseline plasma CORT concentrations than males (Fig. 1; Table 1). In addition, HR mice had significantly higher baseline CORT concentrations than C mice regardless of sex (the sex × linetype interaction was never significant; Fig. 1; Table 1). Baseline plasma CORT concentrations were approximately twice as high in HR males and females as in C males and females, respectively (Fig. 1). When Hct was included as a covariate in the foregoing analysis, it was a marginally significant ($P = 0.0877$) positive predictor of baseline CORT concentration, but the significance levels of other factors and covariates were little changed (e.g., $P_{sex} = 0.0026$, $P_{selection} = 0.0397$). Similarly, when left adrenal mass was included as a covariate, it was never a significant predictor of baseline CORT concentrations, and the significance levels of other factors and covariates were only minimally changed (e.g., $P_{sex} = 0.0228$, $P_{selection} = 0.0455$).

CORT response to restraint. Plasma CORT concentrations increased dramatically after restraint (Fig. 1). As with baseline concentrations, females had significantly higher CORT concentrations than males (Table 1). Postrestraint CORT concentrations did not, however, differ significantly between HR and C mice in either sex (Table 1). When Hct was included as a covariate in the foregoing analysis, it had a significant ($P = 0.0396$) positive association with postrestraint CORT concentration, but significance levels of other factors and covariates showed little change (e.g., $P_{sex} = 0.0005$, $P_{selection} = 0.8681$). When these analyses were repeated with left adrenal mass as a covariate, it was never a significant predictor of stress-induced CORT concentrations, and the significance levels of other factors and covariates were only minimally changed (e.g., $P_{sex} = 0.0018$, $P_{selection} = 0.9548$).

Ratio of and difference between postrestraint and baseline CORT concentrations. To further investigate the relative response to stress, we performed two analyses. First, we analyzed the ratio of postrestraint to baseline CORT concentrations. This ratio was highly positively skewed, as were residuals from the nested ANCOVA, and log transformation still yielded highly skewed residuals; therefore, we used a rank transformation, which yielded residuals that were approximately normally distributed. The rank-transformed ratio did not differ between males and females, but it was significantly lower for HR mice than for C mice (Table 1). Second, we analyzed the difference between postrestraint and baseline CORT concentrations. This difference was significantly greater in females than in males ($P = 0.0192$; Table 1) and tended to be greater in C than in HR mice ($P = 0.0785$). As shown in Table 2, adjusted mean ($\pm$SE) differences for HR males and females were 164.3 $\pm$ 32.0 and 256.3 $\pm$ 32.2 ng/mL, respectively, and corresponding values for C males and females were 220.9 $\pm$ 32.6 and 336.6 $\pm$ 32.1 ng/mL, respectively. Adrenal mass was never significant when added as a covariate in these analyses, and the significance levels of other factors and covariates were little changed.

Body size and adrenal mass. As reported previously (e.g., Swallow et al. 1999), males were heavier than females, and C mice

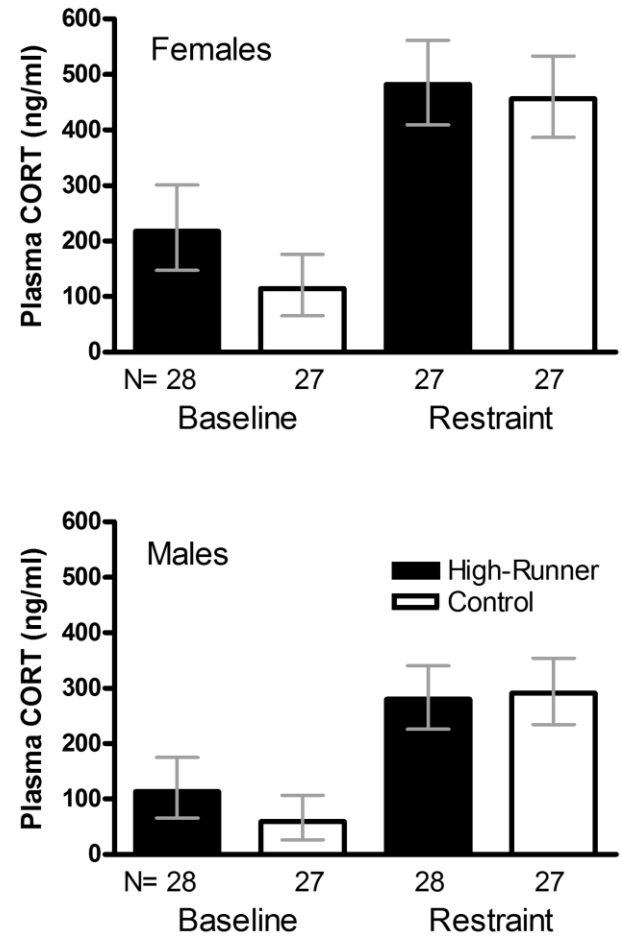

Figure 1. Baseline and stress-induced plasma corticosterone (CORT) concentrations of high-runner and control male and female mice. Bars represent adjusted least squares means (from two-way nested ANCOVAs, as shown in Table 1), back transformed from the square root scale, along with back-transformed 95% confidence intervals. Table 2 presents least squares means before back transformation.

Table 1: Significance levels from two-way nested ANCOVA models analyzing effects of linetype and sex and from separate one-way ANCOVAs comparing the eight lines, split by sex

Trait	Transformation	N	Sex	Linetype	Sex × Linetype	Female Line χ^2	Female Line P	Male Line χ^2	Male Line P
Degrees of freedom			1, 6	1, 6	1, 6		1		1
Body mass	Log	107	**.0002**+	**.0019**−	.2934	.35	.5523	4.09	**.0432**
Body mass with log nose-rump length as a covariate[a]	Log	107	**.0081**+	**.0177**−	.5485	4.27	**.0387**	6.45	**.0111**
Nose-rump length	Log	107	**.0010**+	**.0254**−	.5189	1.12	.2902	7.27	**.0070**
Left adrenal gland mass	Log	107	**<.0001**−	.7160+	.8961	2.33	.1271	2.17	.1408
Left adrenal gland mass with log body mass as a covariate[b]	Log	107	**<.0001**−	.7551+	.8180	3.35	.0671	.71	.3980
Baseline CORT	Square root	110	**.0034**−	**.0373**+	.4732	4.35	**.0370**	.00	.9545
Restraint CORT	Square root	109	**.0004**−	.8658+	.5195	.22	.6386	5.59	**.0180**
Restraint/baseline ratio	Rank	109	.4336+	**.0248**−	.6724	2.67	.1020	.17	.6832
Restraint − baseline	None	109	**.0192**−	.0785−	.7237	.84	.3604	2.18	.1396
Hematocrit at baseline CORT	None	106	.7435+	.2416+	.2686	.00	~1	1.37	.4424
Hematocrit at restraint CORT	None	109	.5490−	.9356−	.2439	.59	.4424	.00	~1

Note. Significance levels given are P values; boldface indicates $P < 0.05$. All analyses were implemented in SAS PROC MIXED. For two-way ANCOVAs, signs following P values indicate direction of effect: for sex, "+" indicates males > females; for linetype, "+" indicates selected high-runner lines > control lines. Additional covariates included in analyses of body mass, length, baseline and restraint corticosterone (CORT), and hematocrit (age, time of day, and [z-transformed time of day]^2) were never statistically significant and so are not shown. For baseline and postrestraint CORT and hematocrit, bleed delay time was also included as a covariate and was not significant. For the ratio of and difference between CORT values, only age was used as a covariate. For body mass, length, and adrenal mass, age was also used as a covariate.

[a] Nose-rump length (log transformed) was an additional covariate in this model and had a highly significant ($P < 0.0001$) positive effect.

[b] Body mass (log transformed) was an additional covariate in this model and had a nonsignificant ($P = 0.1366$) positive effect.

were heavier than HR mice; a similar pattern was found for nose-rump length (Table 1). Body mass differences between sexes and linetypes remained significant when nose-rump length was included as a covariate; in other words, males were heavier for a given body length than females, and similarly, C mice were heavier for their length than HR mice (the sex × linetype interaction was not significant; Table 1). Regardless of whether body mass was included as a covariate, females had larger left adrenal glands (mass was not determined for the right adrenal gland) than males; however, adrenal mass did not differ significantly between HR and C mice in either sex (Table 1).

Hematocrit. Hematocrit did not vary significantly in relation to sex, linetype, or the sex × linetype interaction (Tables 1, 2). This result is consistent with a previous report for mice from generation 14 that were housed without wheel access (Swallow et al. 2005).

Line Differences and Estrus Effects

Stage of estrus and plasma CORT. Only three animals were in the fifth stage of estrus (postestrus; Rugh 1968) on the day of baseline blood sampling; therefore, mice in stage 5 were not included in the analysis of estrous-cycle effects. As expected from previous studies (Montano et al. 1991; Girard and Garland 2002), baseline plasma CORT concentrations of both C and HR females were highest during stage 3 of estrus, early estrus; however, stage of estrus was not a significant predictor of baseline plasma CORT concentrations in the analysis of females ($P = 0.6468$). Plasma CORT concentrations after restraint stress were very similar across all stages of the estrous cycle regardless of linetype ($P = 0.9645$).

Line effects and correlations of line means. In females, as shown in Table 2, the line effect was significant for square-root-transformed baseline CORT ($P = 0.0370$) but not for square-root-transformed postrestraint CORT concentration ($P = 0.6386$) in models that included age, bleed delay time, time of day, and (z-transformed time of day)^2 as covariates (stage of estrus was not included in the model because it was not significant). In males, line effects were reversed, being nonsignificant for baseline values ($P = 0.9545$) but significant for postrestraint values ($P = 0.0180$). For log-transformed body mass, the line effect was significant for males ($P = 0.0432$) but not for females ($P = 0.5523$) in models that included age, time of day, and (z-transformed time of day)^2 as covariates. Line effects were significant for both females and males when body length was included as an additional covariate (Table 2). Males (but not females) also showed a highly significant ($P = 0.0070$) line effect for body length. Neither left adrenal mass nor Hct varied in relation to line in either sex (Table 2).

Analysis of least squares (adjusted) line means indicated sig-

Table 2: Least squares (adjusted) means (±SE) corresponding to two-way nested ANCOVA models implemented in SAS PROC MIXED, as presented in Table 1

Trait	Transformation	Female Control	Female High Runner	Male Control	Male High Runner
Body mass (g)	Log	1.474 ± .0111	1.397 ± .0110	1.548 ± .0110	1.485 ± .0112
Body mass with log nose-rump length as a covariate (g)	Log	1.478 ± .0076	1.445 ± .0081	1.504 ± .0081	1.478 ± .0076
Nose-rump length (cm)	Log	1.036 ± .0039	1.020 ± .0039	1.053 ± .0039	1.040 ± .0039
Left adrenal gland mass (g)	Log	−2.324 ± .0246	−2.333 ± .0245	−2.612 ± .0254	−2.626 ± .0250
Left adrenal gland mass with log body mass as a covariate (g)	Log	−2.322 ± .0268	−2.306 ± .0321	−2.639 ± .0329	−2.632 ± .0273
Baseline CORT (ng/mL)	Square root	10.70 ± 1.055	14.75 ± 1.065	7.73 ± 1.064	10.67 ± 1.053
Restraint CORT (ng/mL)	Square root	21.38 ± .701	21.96 ± .709	17.07 ± .719	16.74 ± .703
Restraint/baseline ratio	Rank	64.9 ± 7.36	40.9 ± 7.39	72.1 ± 7.46	43.3 ± 7.35
Restraint − baseline (ng/mL)	None	336.6 ± 32.09	256.3 ± 32.23	220.9 ± 32.56	164.3 ± 32.09
Hct at baseline CORT (% RBC)	None	.482 ± .0043	.482 ± .0043	.478 ± .0044	.489 ± .0043
Hct at restraint CORT (% RBC)	None	.501 ± .0045	.496 ± .0046	.494 ± .0046	.498 ± .0045

Note. CORT = corticosterone; Hct = hematocrit; RBC = red blood cells.

nificant negative relations between baseline CORT concentrations and both body mass and body length in both sexes (Fig. 2). In all cases, ANCOVA indicated no significant differences in the relations between the four HR lines and the four C lines, so a single Pearson correlation was computed for all eight data points combined.

Discussion

Results of this study confirm a previous finding of elevated baseline CORT concentrations in female mice from replicate lines that had been selectively bred for high voluntary wheel running but were housed without wheels (Girard and Garland 2002). Additionally, for the first time, we have extended these findings to males. In spite of the pronounced (twofold) elevation in baseline CORT concentrations in both sexes, HR mice did not exhibit elevated plasma CORT concentrations after a restraint stressor, regardless of sex. We also found that females had significantly higher baseline and postrestraint CORT concentrations and significantly higher adrenal masses than males, regardless of linetype (HR or C). Replicate lines within linetype showed significant differences in body mass, length, baseline CORT concentrations, and postrestraint CORT concentrations in one or both sexes. Finally, by correlating the eight line means, we found a significant negative relation between baseline CORT concentrations and two measures of body size (mass and length) in both sexes (Fig. 2), which we interpret as an evolutionary cost (trade-off) of elevated CORT levels.

Linetype and Baseline CORT Levels

The twofold difference in baseline CORT concentration between HR and C lines is similar in magnitude to the upper limit of natural variation seen among individuals within species and, we believe, is large enough that it might have important physiological and/or behavioral consequences. In humans, for example, nearly twofold elevations in baseline glucocorticoid (cortisol) concentrations are commonly found in individuals with major depressive disorder, as compared with healthy controls, and these increased cortisol levels are thought to play a significant role in the pathogenesis of depressive symptoms (Gold et al. 1986; Holsboer 2000). In surveys of multiple species, the ratio of baseline glucocorticoid levels in subordinate and dominant individuals ranged from 0.45 to 1.54 in primates (Abbott et al. 2003) and from 0.68 to 2.53 in a phylogenetically diverse sample of wild mammals and birds (Goymann and Wingfield 2004). In these studies, elevated baseline glucocorticoid levels in both dominants and subordinates were found to correlate with social stress or increased allostatic load. Among populations of the lizard *Sceloporus occidentalis*, Dunlap and Wingfield (1995) reported differences in baseline corticosterone levels that approached threefold (but were largely statistically indistinguishable). Two populations of house sparrows exhibited baseline CORT differences on the order of two- to threefold that were claimed to be adaptive in the context of parasite load (Martin et al. 2005). On the other hand, Coe et al. (1992) reported differences in "basal" values among primate species that were up to 10-fold, mainly because many Ceboidea show exceptionally high values. Taken together, the foregoing studies suggest that a twofold difference in baseline CORT levels could be biologically important.

We tentatively interpret the elevated CORT concentrations in HR mice as an adaptation to support higher physical activity, given the known role of CORT in mobilizing energy reserves during sustained locomotion (Ingle 1934; Tharp 1975; Terjung

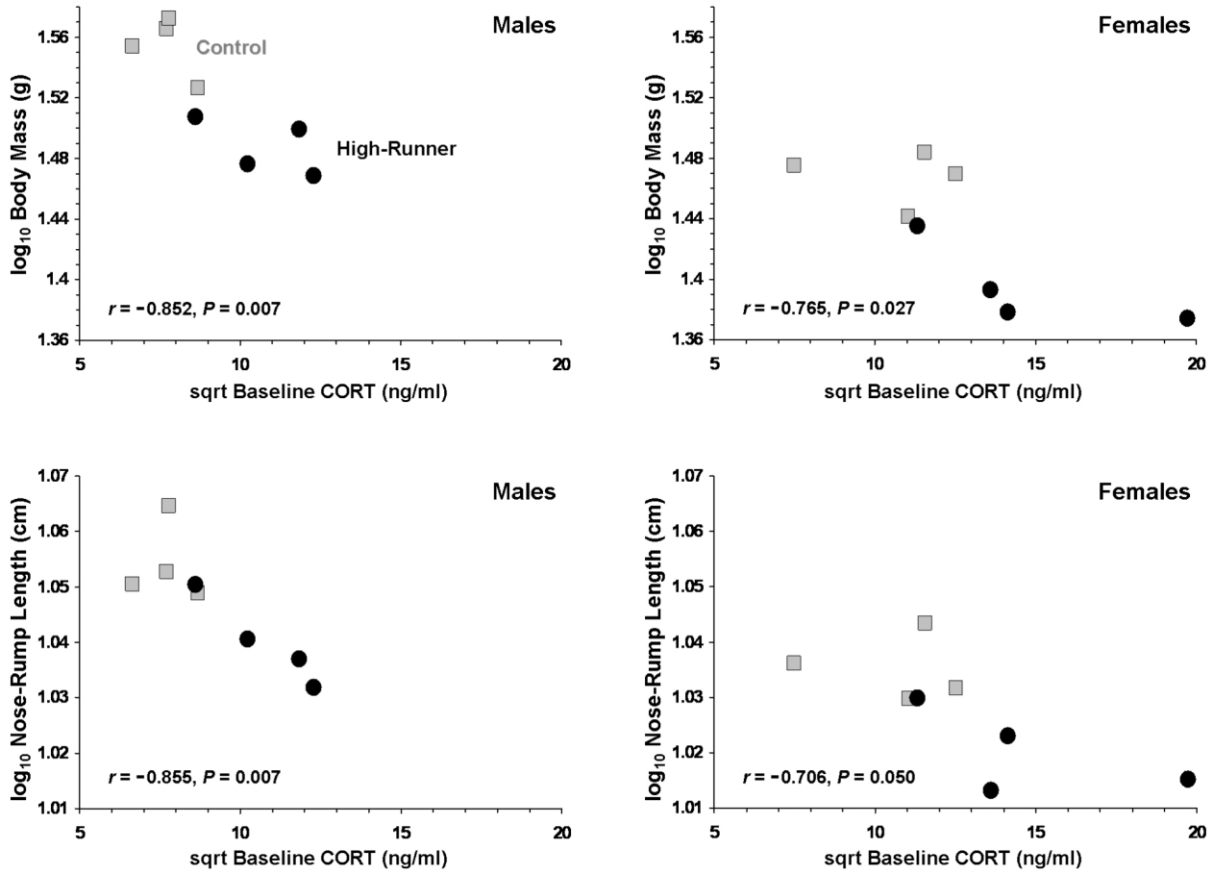

Figure 2. Negative relations between baseline plasma CORT concentrations and both body mass and length, based on least squares (adjusted) means for each of the eight lines (see "Material and Methods"). ANCOVA showed no statistical differences in the relations between high-runner (*circles*) and control lines (*squares*), so simple Pearson product-moment correlation coefficients and associated *P* values for two-tailed tests are shown.

1979; Wasserman and Halseth 1998). During prolonged locomotor activity, the energetic demands of skeletal muscle are met by glucose and free fatty acids (Terjung 1979; Wasserman and Halseth 1998; Wilmore and Costill 1999). CORT promotes lipolysis and proteolysis and stimulates gluconeogenesis. The combined effect of these actions is an increase in blood glucose and free fatty acids, which are available for uptake at the muscles (Tharp 1975).

In addition to effects on energy metabolism, CORT may directly affect wheel-running behavior. In rats, for example, adrenalectomy has been shown to abolish or greatly reduce wheel running (Richter 1936; Moberg and Clark 1976), and subsequent replacement of CORT or administration of the synthetic glucocorticoid dexamethasone (DEX) reinstates the behavior (Kendall 1970; Moberg and Clark 1976). Supraphysiological doses of DEX increase wheel running above normal levels in adrenalectomized rats (Pedersen-Bjergaard and Tonnesen 1954; Kendall 1970), and in intact rats, pharmacological elevation of CORT concentrations has been shown to increase locomotor behavior in both acute and chronic administration protocols (Wolkowitz 1994; Sandi et al. 1996). These studies suggest that CORT may be necessary for wheel-running behavior and that increased CORT concentrations promote increased activity (see also Rhodes et al. 2005).

Alternatively, the increase in baseline CORT concentrations observed in HR mice may be a nonadaptive or possibly even maladaptive by-product of the selection regime; our finding that line differences in baseline CORT concentration negatively predict body mass (Fig. 2; see also Girard and Garland 2002) suggests a maladaptive effect (see below). Additional studies are needed to characterize the functional significance and consequences of elevated baseline CORT in HR mice, to determine whether "countermeasures" (such as increased corticosterone-binding globulin concentrations or decreased corticosteroid receptor numbers) have evolved in parallel with increased baseline CORT concentrations, and to identify possible changes in upstream control by the brain and pituitary.

Because CORT concentrations increase acutely with physical

activity, our findings could reflect an acute response to increased home-cage activity, even in the absence of wheels, and some evidence does indeed suggest increased home-cage activity in HR mice as measured over a 24-h period (see discussion in Rhodes et al. 2005). We obtained blood samples in late afternoon, however, during the typical inactive period for mice, and most individuals appeared to be asleep immediately before blood sampling. Furthermore, wheel running by HR and C lines shows a similar time of onset, with peak running between 1800 hours and midnight for both linetypes, suggesting that HR and C lines do not differ in their circadian pattern of activity (Girard et al. 2001; Girard and Garland 2002).

Linetype and Postrestraint CORT Concentrations

Postrestraint CORT concentrations reported here are comparable to those reported in other studies of mice (Hennessy et al. 1977; Hennessy 1991; Montano et al. 1991; Droste et al. 2003) and are similar to the highest concentrations observed during voluntary wheel running (Girard and Garland 2002) and forced treadmill running (Coleman et al. 1998), suggesting that restraint for 40 min elicited the maximum stress response. CORT concentrations did not differ between HR and C mice after restraint, however, which suggests that selection for high activity level has not enhanced the stress response or increased adrenocortical steroidogenic capacity. To the contrary, one measure of relative stress responsiveness, the ratio of postrestraint to baseline CORT concentrations, was significantly lower in HR than in C mice (Tables 1, 2; twofold for HR mice and fourfold for C mice), which may indicate functionally *reduced* stress responsiveness in HR mice. However, we evaluated CORT at only one time point after restraint, so we cannot rule out the possibility that the time course of the stress response or the net integrated response over some shorter or longer time period differs between HR and C mice.

Independent evolution of baseline and stress-induced cortisol (the main glucocorticoid in fish) concentrations has also been reported for rainbow trout divergently selected for high or low poststressor (3-h confinement) cortisol concentrations (Øverli et al. 2005). Poststress cortisol concentrations for all three postselection generations differed significantly between lines; however, baseline cortisol did not differ (Øverli et al. 2005). In a comparison among populations of one lizard species, Dunlap and Wingfield (1995) also reported a dissociation between baseline corticosterone concentrations and those observed after a 60-min stress test.

Linetype and Adrenal Gland Mass

Although adrenal gland mass is at best a crude indicator of HPA activity, increases in adrenal size after increased physical activity have been demonstrated in mammals, including humans, rats, and mice (Ingle 1934; Riss et al. 1959; Kjaer 1992; Droste et al. 2003). In mice, 4 wk of wheel access led to a significant increase in both baseline CORT concentrations and right adrenal gland mass (Droste et al. 2003). In contrast, we found no difference in body mass–corrected adrenal size between HR and C mice despite a twofold difference in baseline CORT concentrations. Similarly, a study of generation-14 mice in this experiment found no difference between HR and C lines and no effect of 8 wk of voluntary wheel access (Swallow et al. 2005).

Sex Differences in CORT Concentrations and Adrenal Gland Mass

Females had higher baseline CORT concentrations, higher stress-induced CORT concentrations, and heavier adrenal glands than males (Tables 1, 2; Fig. 1), consistent with previous findings in mice, rats, and most other mammals (Reeder and Kramer 2005). Female-biased sex differences have been studied extensively in the rat and can be explained partially by the known involvement of gonadal steroids (testosterone and estrogen) in the regulation of CORT secretion and metabolic clearance rate (Kitay 1963; Kitay et al. 1971; Handa et al. 1994). Androgens generally exert a suppressive effect on the HPA axis, whereas estrogens promote HPA activity via numerous mechanisms, including increasing secretion of adrenocorticotropic hormone and CORT, elevating circulating concentrations of corticosteroid-binding globulin, and reducing central negative-feedback sensitivity (Sandberg and Slaunwhite 1959; Handa et al. 1994).

Sex and Linetype Differences in Body Size and Shape

At generation 14, both male and female HR mice were reported to have significantly lower body mass than C mice—12.9% lower for females and 14.3% lower for males (Swallow et al. 1999). Reduced body size in HR mice, as measured by nose-rump length, has also been reported (Kelly et al. 2006). Results from our study corroborate previous findings for both body mass and length. Notably, HR mice are also significantly smaller in body mass even when body length is used as a covariate, indicating that HR mice are "thinner" than C mice (Kelly et al. 2006; this study). With the exception of the adrenal glands, we did not measure any other components of body size; however, previous studies have found differences in several components of body size, including lower body fat and reduced hindlimb muscle mass in HR lines (Dumke et al. 2001; Swallow et al. 2001; Garland et al. 2002).

Line Effects and Line Correlations

Significant variation among replicate lines was found for a number of traits, including two measures of body size and both baseline and postrestraint plasma CORT concentrations in one

or both sexes (Table 1). As reviewed elsewhere (Garland 2003), such differences may reflect random genetic drift (possibly involving different mutations) and/or different adaptive mechanisms (for other examples in these mice, see Swallow et al. 1998, 2001; Garland et al. 2002; Gammie et al. 2003). Further analysis of the line means revealed a significant negative relation between baseline CORT and body size (Fig. 2), which can be taken to suggest that CORT suppresses growth. This interpretation is consistent with the finding from generation-21 females where an inverse relation was observed for individual variation in nighttime CORT and growth (measured as body mass change) over 4 wk (Girard and Garland 2002). Implant studies in rats (Johnson et al. 2006) and birds (Romero et al. 2005) have demonstrated that elevation of CORT to high but physiologically relevant levels inhibits body mass increase and feather growth, as compared with results in control animals. One possible explanation is that CORT has been shown to have suppressive effects on the neuroregulation of growth hormone (reviewed in Giustina and Veldhuis 1998). However, it should be kept in mind that our results are descriptive and may represent two independent traits that both responded to selection on wheel running and may not be causally related. In any case, a reduction in body size, both between HR and C lines and among replicate lines, may constitute a cost of elevated CORT, one that trades off with any potential benefit with respect to facilitating elevated locomotor activity.

Concluding Remarks

Our results may provide insight concerning physiological evolution of animals in the wild. Selection experiments allow us to see evolution in action and the potential future of coadapted traits under specific selection regimes (Gibbs 1999; Garland 2003). They can also be the source of novel hypotheses regarding evolution in the wild (see, e.g., Gibbs 1999; Garland and Freeman 2005). Based on our finding that selection for high activity level has led to a correlated increase in baseline glucocorticoid concentrations in house mice, we propose the novel hypothesis that wild species of mammals that vary in daily activity levels will exhibit correlated variation in baseline CORT concentrations. This hypothesis could be tested by an interspecific comparative approach (e.g., Coe et al. 1992; Abbott et al. 2003; Goymann and Wingfield 2004). We predict a positive correlation between baseline CORT concentrations and activity (e.g., home range size or daily movement distance) among species of mammals.

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