Leptin Levels and Body Composition of Mice Selectively Bred for High Voluntary Locomotor Activity

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ABSTRACT

Selective breeding produced four replicate lines of high-runner (HR) mice that run on wheels for approximately 2.7 times more revolutions per day than four unselected control lines. Previous studies found that HR mice of both sexes have lower body fat (isotope dilution at 15 wk of age) and that males (females not studied) have smaller retroperitoneal fat pads (17 wk). HR mice also exhibit elevated plasma corticosterone and insulin-stimulated glucose uptake by some hindlimb muscles but apparently do not differ in circulating insulin or glucose levels (males at 18 wk). Given their lower body fat and higher activity levels, we hypothesized that HR mice would have lower circulating leptin levels than controls. Female mice were given wheel access for 6 d at 7 wk of age, as part of the routine wheel testing for the selective breeding protocol, and then were killed after one additional week without wheels to reduce possible acute effects of activity on leptin. As hypothesized, serum leptin levels were significantly lower in HR mice. ANCOVA indicated that leptin was strongly positively correlated with both total body fat (measured by ether extraction) and body mass change from weaning, but HR mice still had significantly lower adjusted leptin levels (ANCOVA). Within HR lines but not within control lines, individual variation in leptin levels was negatively correlated with amount or speed of wheel running measured a week before being killed. Growth from weaning to euthanasia and body dry mass were lower in HR mice than in controls, but absolute dry masses of the ventricles, liver, gut, and uterus plus ovaries did not significantly differ, nor did percentage of the total dry mass as fat. HR mice offer a novel model for studying the causes and consequences of physiologically relevant variations in serum leptin.

Introduction

Natural selection and sexual selection typically act most strongly on phenotypes at relatively high levels of biological organization, including life-history components, behavior, and organismal performance abilities (e.g., see Fig. 1 in Garland and Kelly 2006). The evolutionary response to selection on such complex phenotypes will necessarily involve changes in multifarious lower-level (subordinate) traits (Swallow and Garland 2005), potentially including phenotypic plasticity at various levels (Garland and Kelly 2006). Endocrine axes may play a crucial role in the evolution of complex traits because they can affect and potentially integrate numerous aspects of development, life history, physiology, neurobiology, and behavior (e.g., see Zera and Harshman 2001; Sinervo and Calsbeek 2003; Ketterson et al. 2005). Nevertheless, relatively few vertebrate selection experiments have examined correlated responses in endocrine traits. In one striking example, Bünger et al. (1999) reported that the correlated response of circulating leptin levels to bidirectional selection for fatness in mice (60–340-fold) was much greater than the direct response (five- or sixfold).

Leptin, the 16-kDa protein hormone product of the ob (Lep) gene first discovered in 1994 (Zhang et al. 1994), plays an important role in regulation of body mass, energy balance, and possibly activity level (Bouassida et al. 2006). Ob/ob mice, which carry a spontaneous mutation in the leptin gene and lack the leptin gene product, are characterized by hyperphagia and morbid obesity, as well as reduced physical activity. Adipose tissue is the major site of leptin production. Leptin circulates in plasma at concentrations proportional to the number and size of adipocytes in mice (Frederich et al. 1995), rats (Roberts et al. 2002), cattle (Geary et al. 2001), and humans (Considine et al. 1996). Leptin messenger RNA (mRNA) expression in adipose tissue is also proportional to fat cell size in mice (Maffei et al. 1995), steers (Yang et al. 2003), and humans (Considine et al. 1996). Exogenous administration of leptin to ob/ob mice caused an increase in activity levels, along with a rapid reduction in food intake and body fat (Pelleymounter et al. 1995), leading to early speculation that leptin functioned primarily as

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a lipostatic signal to the central nervous system (CNS) to inhibit food intake as adipose mass increased (Halaas et al. 1995).

Recent evidence demonstrates that the role of leptin is substantially more complex than was originally appreciated (e.g., see Ahima et al. 2000; Popovic and Dunlas 2005; Bouassida et al. 2006; Karsenty 2006). Leptin expression has been found in several nonadipose tissues (including stomach, muscle, and placenta), and leptin receptors have been identified in both the CNS and such peripheral tissues as skeletal muscle, liver, pancreas, and adipose tissues (Muoio and Lynis Dohm 2002). Leptin is now known to affect a wide range of processes, including food intake, locomotor activity (Ahima et al. 1999), conception and lactation (Malik et al. 2001), glucose utilization (Harris 1998), bone formation (Elefteriou et al. 2004; Karsenty 2006), stress responses (Salzmann et al. 2004), and immune function (Siegmund et al. 2004).

We argue that most existing models for the study of voluntary exercise, obesity, and physiologically relevant variations in leptin are limited and that the relevance of existing models for understanding the evolutionary role of leptin is even more tenuous. Leptin replacement or addition inadequately simulates changes in leptin levels that are associated with exercise, obesity, or disease because it often induces leptin levels outside the physiologically relevant range of variation. Recombinant leptin is available for several model species, including mice (Barrachina et al. 1997), rats (Park et al. 2001; Crowley et al. 2004), dogs (Iwase et al. 2000), pigs (Ramsay et al. 1998), and cows (Raver et al. 2000), and can be administered to mimic high plasma levels associated with obesity. However, the temporal pattern of leptin secretion is difficult to replicate. In vivo, leptin is secreted in pulses, peaking at night in both mice and humans (Licinio et al. 1998; Jeong et al. 2004). In women, changes in plasma leptin are related to levels of luteinizing hormone and are associated with variations in estradiol (Licinio et al. 1998). A model with physiologically relevant variation and pulsatility in leptin levels in the absence of gene and receptor mutations would be an important addition to the study of hormone function in whole organisms.

We suggest that the classical genetic technique of selective breeding can provide good models to study both the physiological and the evolutionary roles of leptin. For example, as noted above, Bünger et al. (1999) reported that lines of mice selectively bred for either high or low body fat also showed large differences in circulating leptin levels in comparison with each other and with a control line that showed intermediate leptin levels. They also noted (p. 43) a major discrepancy between their results and the known effects of administering exogenous leptin: "administration of exogenous leptin to increase circulating levels leads to a reduction in obesity; whereas our results suggest that effective selection for leanness would be by selecting to decrease levels of endogenous circulating leptin!"

The purposes of this study were to determine whether circulating leptin levels have been altered in mice selectively bred for high voluntary wheel-running activity and, secondarily, to consider the utility of these lines to serve as a novel model for study of the physiological effects of leptin. Thirty-one generations of artificial selection for voluntary running on wheels has produced four replicate lines of high-runner (HR) mice that run about 2.7 times more revolutions per day than the four replicate control (unselected) lines (Swallow et al. 1998; Garland 2003; Rhodes et al. 2005). The HR lines exhibit a variety of apparent adaptations for sustained aerobic exercise, including elevated maximal oxygen consumption during forced treadmill exercise (Swallow et al. 1998; Rezende et al. 2006*a*, 2006*b*), increased insulin-stimulated glucose uptake by some hindlimb muscles (Dumke et al. 2001), more-symmetrical hindlimb bones (Garland and Freeman 2005), larger femoral heads, and thicker hindlimb bones (Kelly et al. 2006). HR mice also consume more food, even when housed without wheel access (Swallow et al. 2001), and have reduced body mass (Swallow et al. 1999, 2001; Dumke et al. 2001), less body fat (by isotope dilution; Swallow et al. 2001), and lower relative retroperitoneal fat pad weight (by dissection in males; Dumke et al. 2001), compared with controls. In addition, HR mice exhibit elevated plasma corticosterone levels (Girard and Garland 2002; Malisch et al. 2007). The elevated corticosterone levels may represent an adaptation that helps support sustained locomotor activity, via mobilization of energy reserves, but it may also be causally related to the lower growth rates and smaller body sizes of HR mice (Girard and Garland 2002; Malisch et al. 2007).

The foregoing observations, in combination with the observation that leptin has been shown to have an overall inhibitory effect on the hypothalamic-pituitary-adrenal (HPA) axis (Ahima et al. 2000), suggest that HR mice might also differ from controls in leptin expression. Endogenous leptin levels are closely and positively correlated with body mass index (a ratio of mass to the square of body length) in humans (Franks et al. 2003; Yildiz et al. 2004) and in mice, rats, and hamsters (Maffei et al. 1995; Bünger et al. 1999). Studies in other rodents suggest that changes in leptin levels can precede changes in body mass or food intake. In laboratory-maintained woodchucks, serum leptin levels initially increased during periods of body mass gain in late spring and reached peak values in summer during the initial decline in body mass, before decreasing to basal values in winter concomitantly with body weight (Concannon et al. 2001). In Siberian hamsters, reductions in white adipose tissue during short days coincided with reductions in leptin mRNA expression in adipocytes, but food intake did not change (Demas 2002). Exogenous administration of leptin in mice generally causes reductions in food intake and body weight, but the response may be altered by method of leptin dosing, strain, and housing (Bowen et al. 2003). In ob/ob mice, administration of leptin decreases fat mass and increases locomotor activity (Ahima et al. 1999). Body fat in rat pups was also negatively related to leptin dose delivered via osmotic pumps (Eiden et al. 2001).

In this study, we compared circulating leptin levels, total body fat, body mass and growth rates, and organ masses in female mice from the HR and control lines. Although other predictions are possible, because of their lower body fat and higher voluntary activity levels, we hypothesized that HR mice would have lower circulating leptin levels than controls.

Material and Methods

Animals

We studied female Hsd:ICR mice (*Mus domesticus*) from generation 31 of an artificial-selection experiment for high voluntary wheel-running behavior (Swallow et al. 2001). Four replicate HR lines have been maintained by within-family selective breeding for high wheel running (total distance) on days 5 and 6 of a 6-d exposure to wheels. Four control lines are maintained by breeding without respect to wheel running. In this study, we used females because they run more than males in both HR and control lines (Swallow et al. 1998; Garland 2003; Koteja et al. 2003) and because females were used in a previous study of plasma corticosterone levels (Girard and Garland 2002). Six nonsiblings from each of the eight lines (four HR, four control) were randomly chosen as subjects ($n = 48$ in total). All animals came from litters born within 3 d (March 18–20, 2002), and all were weaned at 21 d as part of the normal husbandry routine. At weaning, pups were toe-clipped for individual identification, weighed, and housed in groups of four by sex until measurement of wheel running. The lighting schedule was 12L : 12D, with lights on at 0530 hours CST. All animals had continuous ad lib. access to water and food (Harlan Teklad Rodent Diet [W] 8604: crude protein [min.] 24.0%, crude fat [min.] 4.0%, crude fiber [max.] 4.5%). Days of the study refer to the average age of the mice (in days).

Wheel Access and Postwheel Housing

As part of the routine selection protocol (see Swallow et al. 1998), we placed the study females individually in cages with attached running wheels (1.12-m circumference; Lafayette Instruments, Lafayette, IN) at 49 d of age (range = 48–50 d). The study females were measured in the same batch and room. Voluntary wheel running was monitored for 6 d as previously described (Swallow et al. 1998). Wheel revolutions were recorded in 1-min bins by attached photocell counters interfaced with a personal computer through customized software (San Diego Instruments, San Diego, CA). Data collected for each 24-h period included the total number of clockwise and counterclockwise revolutions (revolutions), the number of 1-min intervals with any revolutions (intervals), the average speed (revolutions/intervals), and the maximum number of revolutions for any 1-min interval (maximum speed). Because the trait of selection is the average total revolutions on days 5 and 6 of the 6-d trial, wheel-running traits presented here are the average of days 5 and 6.

Mice were removed after 6 d of wheel access (day 55) and housed singly in regular shoebox cages with side-mounted food hoppers (day 1 of individual shoebox housing). Each mouse was given a weighed portion of food sufficient to allow ad lib. consumption. Water was also available ad lib. At noon on the seventh day of individual shoebox housing (day 61), food hoppers were reweighed to allow estimation of apparent food consumption for the week (Koteja et al. 2003). Mice were not handled, and the food hoppers were replaced. The week of home cage housing following wheel access was intended to reduce any acute effects of exercise on leptin levels induced by the great difference in running activity between the linetypes (e.g., see Leal-Cerro et al. 1998 on effects of marathon running in humans; Garland and Kelly 2006).

Blood Sampling and Body Composition

Mice were euthanized between 1930 and 2156 hours on the seventh day of individual shoebox housing at an average age of 61 d. Beginning at 2 h after lights off, mice were weighed and then anesthetized with isoflurane (Isoflo, Abbott Laboratories, Abbott Park, IL) to cessation of breathing. Body length from nose tip to base of tail was measured to the closest millimeter with a ruler. Mice were killed by terminal exsanguination through a deep incision of the left jugular vein. Blood was collected directly from the jugular vein into a nonheparinized 5-mL syringe and transferred to a chilled centrifuge tube. Serum was separated from whole blood by centrifugation and stored at −80°C until hormone assays. Given this experimental design, we were not able to study possible alterations in the circadian pattern of leptin concentration (e.g., see Jeong et al. 2004), and it is also possible that single-point samples would be inadequate to characterize HR-versus-control differences in "average" daily leptin levels. Be that as it may, we find statistically significant correlates of individual variation in leptin levels as well as significant differences between the HR and C lines (see "Results"), which suggests that our samples are adequate for the goals of this study.

Food hoppers were weighed a third time to estimate apparent food consumption in the hours before euthanasia. Apparent food consumption was calculated for the week (total food in g from 1200 hours on day 55 to 1200 hours on day 61) and for the hours before sampling (total food in grams from 1200 hours on day 61 to time of euthanasia/hours elapsed; consumption rate then standardized to 8 h), with adjustments for initial and final water content of food.

On the day after euthanasia, mice that had been refrigerated were weighed and dissected, and the wet masses of various organs were recorded: uterus and ovaries (combined), liver, ventricles of the heart (atria were discarded), stomach, small intestine, cecum, and large intestine. The stomach, cecum, and

Table 1: Wheel running measured in female mice from lines genetically selected for high voluntary wheel running (high-runner [HR]) and from nonselected control lines

Trait	HR	Control	Transformation	P_{cov}[a]	$P_{linetype}$
Revolutions/d	13,782 ± 4,310	4,760 ± 1,761	$x^{.75}$	.4577	<.0001
1-min intervals/d[b]	518 ± 116	477 ± 137	$x^{.5}$	.6123	.4915
Average rpm	26.80 ± 7.14	9.83 ± 2.17	$x^{.75}$	.4577	<.0001
Maximum revolutions in 1 min	41.27 ± 8.73	20.42 ± 3.11	None	.1337	<.0001

Note. Simple means ± SD for $n = 24$ mice per linetype for average wheel-running traits on days 5 and 6 of a 6-d exposure to wheels. Significance of the linetype effect (HR vs. control; df = 1, 6; see "Material and Methods") was derived from a nested ANCOVA model with wheel freeness included as a covariate and traits transformed as indicated. *P* values are for two-tailed tests.

[a] Significance level for wheel freeness (square root transformed) as a covariate.

[b] Number of 1-min intervals in which revolutions were >0.

intestines were dissected and rinsed clean of contents before weighing. Skin and fur were not removed from the carcass. Organs were dried to constant mass in a 60°C oven, and a dry mass was recorded separately for the organ-free body (carcass including skin and fur), uterus and ovaries (combined), liver, ventricles, stomach, and intestines and cecum (combined). All the organs, except the liver, were combined with the carcass for fat extraction. Fat from the liver was extracted separately. Fat was extracted using petroleum ether (Goldfische apparatus; Labconco, Kansas City, MO), as in Kristan (2002); fat content for each sample was computed by subtraction as (dry mass before extraction minus dry mass after extraction). Liver fat was expressed as an absolute mass and as a percentage of liver dry mass. Absolute body fat equaled the total fat mass of the carcass and organs plus liver fat mass, and the total percentage fat was calculated relative to the total dry mass of the body (i.e., carcass and organs plus liver).

Hormone Analysis

Leptin was assayed in triplicate 100-μL samples of serum using the mouse leptin RIA kit produced by Linco Research (catalog no. ML-82K, St. Charles, MO). The limit of sensitivity of the assay was 0.2 ng/mL at 90% binding. All samples were included in a single assay; percent binding was 53.4%, with intra-assay coefficients of variation of 1.9% and 2.4% for the low- and high-quality control standards, respectively.

Statistical Analysis

Data were analyzed in SAS for Windows (ver. 8.2; SAS, Cary, NC) using nested ANCOVA to compare HR and control linetypes. As in previous analyses of these lines of mice, we applied a mixed model (SAS procedure "Mixed") with linetype as a fixed effect and replicate lines nested within linetype as a random effect. Because, for some traits, it appeared that HR and control lines might differ in variability, we also allowed for different among-line variances for the two linetypes. Covariates were included as appropriate for various dependent variables. The effect of linetype was tested over the effect of line (df = 1, 6). Data were transformed as necessary to improve normality of residuals and linearity of relations with covariates. Serum leptin levels (ng/mL) were $\log_{10}$ transformed for all analyses. A significance level of $P = 0.05$ was used. Although we hypothesized that leptin concentration would be lower in HR mice than in controls, for simplicity two-tailed tests were used for this and for all other traits. Potential outliers were examined using the test described by Cook and Weisberg (1999). No data met the strict criteria for exclusion at $P = 0.05$, although analyses were repeated with the omission of marginally significant outliers. In no case was the outcome of an analysis substantially altered by exclusion of one or more outliers; therefore, results reported below are for the full data set ($n = 24$ mice per linetype). In preliminary analyses, neither time of day at euthanasia nor the (z-transform of time)^2 were related with leptin levels in any model ($P > 0.6$), and so they were not included in the analyses reported here.

Results

Wheel Running

Similar to previous reports (Koteja et al. 2001), HR females ran about 2.9 times more revolutions per day than controls on days 5 and 6 of the 6-d wheel access trial (days 54 and 55; Table 1). This increase in average total revolutions per day was caused mainly by an increase in running speed: average and maximum (1-min) running speeds were 2.7 and 2.0 times higher in HR than in controls, respectively, whereas the number of 1-min intervals with wheel activity did not differ significantly (Table 1; see also Fig. 1, *bottom*). Wheel-running data for the 48 leptin females were similar to those recorded for generation 31 as a whole (data not shown) and to those obtained for most generations since generation 16 (Koteja et al. 2001, 2003).

Leptin

As hypothesized, serum leptin levels (ng/mL; $\log_{10}$ transformed for analyses) were significantly lower in HR lines than in con-

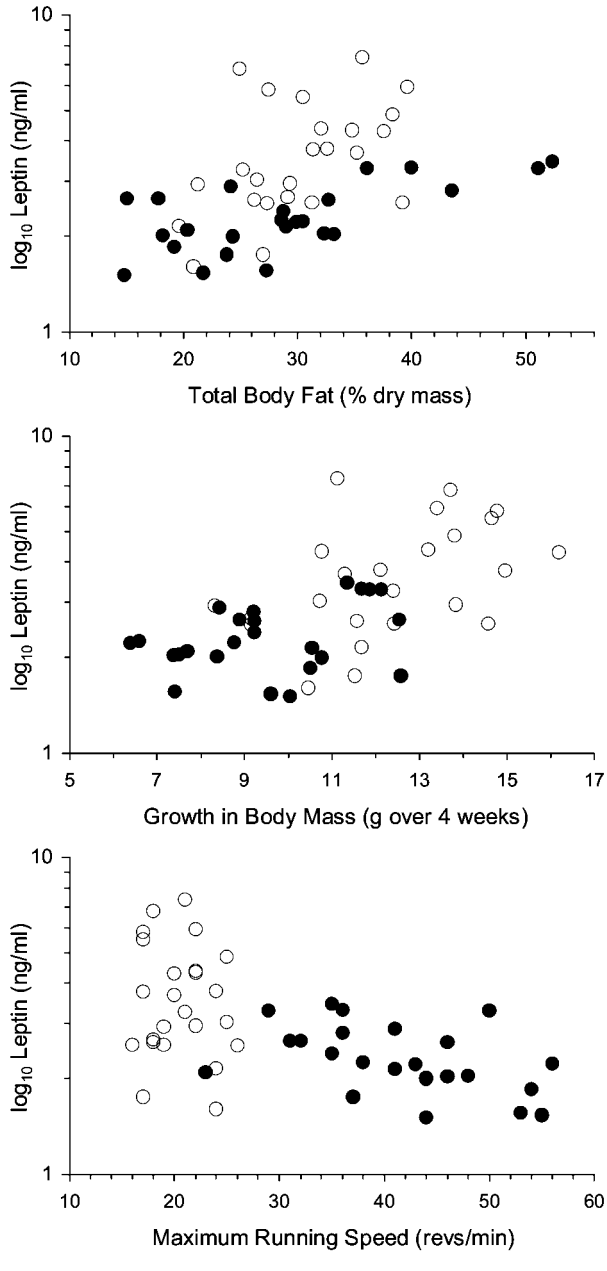

Figure 1. At the level of individual variation within linetypes, circulating leptin concentration is significantly positively correlated with both percent body fat (*top*) and the change in body mass between weaning and euthanasia (*middle*) and shows a trend for a negative correlation with maximum voluntary running speed on wheels (*bottom*). See "Results" for results of ANCOVA models comparing high-runner (*filled circles*) and control (*open circles*) lines of mice while incorporating the various covariates.

trols (Table 2; two-tailed $P = 0.0059$). Leptin levels ranged from 1.5 to 3.5 ng/mL in HR and from 1.6 to 7.3 ng/mL in controls. As would be expected, percent total body fat (as a percentage of total dry mass) was a positive predictor of leptin levels ($\log_{10}$ transformed) in both linetypes (Fig. 1, *top*), yet the linetype difference became even more statistically significant in a model including fat content as a covariate (percent total fat $P = 0.0004$; linetype $P = 0.0036$). Leptin levels were also significantly predicted by total dry mass (total dry mass $P = 0.0002$; linetype $P = 0.0179$), which would be expected, given the high correlation between total body fat and total dry mass noted above. Leptin levels were not related to liver fat in either absolute (liver fat [g] $P = 0.3183$; linetype $P = 0.0055$) or relative (liver fat [%] $P = 0.9406$; linetype $P = 0.0064$) measures.

Leptin level ($\log_{10}$ transformed) was significantly positively related to the change in body mass from weaning to euthanasia (Fig. 1, *middle*) when it was included as the only covariate in an ANCOVA (mass change $P = 0.0060$; linetype $P = 0.0796$). When percent total body fat was included as an additional covariate, all three factors were significant predictors of leptin level (percent body fat $P = 0.0015$; mass change $P = 0.0245$; linetype $P = 0.0415$). We adopted this ANCOVA model predicting leptin levels from percent body fat, mass change from weaning to euthanasia, and linetype as our basic model to explore the statistical significance of further candidate variables.

Leptin levels were significantly positively related to whole-animal food consumption (FC) over the week before euthanasia (FC week $P = 0.0009$; linetype $P = 0.0033$) but not if body mass was included in the model (body mass $P = 0.1721$; FC week $P = 0.0653$; linetype $P = 0.0293$). The FC in the previous week was still a significant positive predictor of leptin levels even when percent body fat was included in the model (percent body fat $P = 0.0161$; FC week $P = 0.0472$; linetype $P = 0.0033$) but not if body mass at euthanasia was added to the model (body mass $P = 0.5559$; percent body fat $P = 0.0380$; FC week $P = 0.1516$; linetype $P = 0.0180$). Leptin levels were not significantly positively related to FC during the hours before euthanasia (FC hours $P = 0.3868$; linetype $P = 0.0055$), including when percent body fat was in the model (percent body fat $P = 0.0004$; FC hours $P = 0.3149$; linetype $P = 0.0038$) and when body mass was added to the model (body mass $P = 0.2707$; percent body fat $P = 0.0216$; FC hours $P = 0.8354$; linetype $P = 0.0320$).

None of the wheel-running traits was a significant predictor of leptin levels when used as the single covariate, and the linetype effect remained statistically significant only in the model including number of 1-min intervals (intervals $P = 0.6414$, linetype $P = 0.0087$; total revolutions $P = 0.1051$, linetype $P = 0.2664$; mean speed $P = 0.1632$, linetype $P = 0.3175$). The negative relation between leptin levels and maximum running speed approached significance in the nested ANCOVA (maximum speed $P = 0.0866$, linetype $P = 0.4273$; Fig. 1, *bottom*). Wheel freeness (an inverse measure of rotational resistance) was never a significant predictor in the simple analyses of wheel running (see above and Table 1) and so was not included as an additional covariate in these models.

We also tested for possible relationships between leptin levels

Table 2: Serum leptin levels, organ wet and dry masses, and fat content in high-runner (HR) and control lines of mice

Trait	HR	Control	Transformation	P_{cov}[a]	$P_{linetype}$
Leptin concentration (ng/mL)	2.356 ± .580	3.797 ± 1.566	Log_{10}		.0059
Organ masses (g):					
Carcass, wet	18.47 ± 1.35	20.89 ± 2.205	Log_{10}		.0171
Carcass, dry	5.806 ± 1.065	6.601 ± .983	Log_{10}		.0405
Carcass, dry, length adjusted			Log_{10}	.2292	.1322
Liver, wet	1.200 ± .156	1.305 ± .194	Log_{10}		.2314
Liver, wet, mass adjusted			Log_{10}	.0001	.4576
Liver, dry	.350 ± .044	.376 ± .047	x^{33}		.2863
Liver, dry, length adjusted			None	.0036	.7671
Heart, wet	.107 ± .009	.110 ± .012	None		.5981
Heart, wet, mass adjusted			x^2	.0006	.3648
Heart, dry	.026 ± .002	.027 ± .003	None		.5185
Heart, dry, length adjusted			None	.1062	.9017
Stomach (St), wet	.202 ± .024	.231 ± .029	x^2		.0137
St, wet, mass adjusted			x^2	.0599	.0730
Small intestine (SI), wet	.705 ± .140	.794 ± .154	None		.1647
SI, wet, mass adjusted			None	.0926	.5227
Large intestine (LI), wet	.167 ± .027	.178 ± .028	None		.3374
LI, wet, mass adjusted			None	.0005	.4994
Cecum (C), wet	.139 ± .036	.157 ± .033	Log_{10}		.0975
C, wet, mass adjusted			None	.0012	.7889
Whole digestive tract (St + SI + LI + C), wet	1.214 ± .170	1.360 ± .195	None		.0893
Whole digestive tract, wet, mass adjusted			None	.0065	.4775
Whole digestive tract, dry	.397 ± .088	.438 ± .074	None		.2299
Whole digestive tract, dry, length adjusted			None	.1304	.5761
Uterus + ovaries, wet	.066 ± .038	.073 ± .043	Log_{10}		.4556
Uterus + ovaries, wet, mass adjusted			Log_{10}	.0430	.7397
Uterus + ovaries, dry	.018 ± .007	.019 ± .007	Log_{10}		.4939
Uterus + ovaries, dry, length adjusted			Log_{10}	.0136	.6653
Body mass, dry	6.596 ± 1.165	7.460 ± 1.081	Log_{10}		.0492
Body mass, dry, length adjusted			Log_{10}	.1893	.1589
Liver fat	.010 ± .004	.011 ± .004	None		.5742
Liver fat/dry liver (%)	2.772 ± 1.061	2.841 ± 1.047	None		.8690
Total body fat (g)	1.869 ± .994	2.146 ± .699	Log_{10}		.2224
Body fat/dry body mass (%)	28.94 ± 10.22	30.12 ± 5.78	x^5		.6073

Note. Simple means ± SD for $n = 24$ female mice per linetype. Significance of the linetype (HR vs. control) effect shown was derived from a nested ANOVA or, for length- or mass-adjusted values, a nested ANCOVA; the significance of body length or log_{10} (body mass) at euthanasia on day 61 is indicated when covariates were included in P_{cov}. When necessary, traits were transformed for analysis as noted. *P* values are for two-tailed hypotheses.

[a] Partial regression coefficients for body length and body mass were always positive.

and wheel running by adding a wheel-running trait as an additional covariate to a basic ANCOVA model including effects of linetype, percent body fat, and mass change from weaning to euthanasia (see above). No measure of wheel running was a significant predictor in these models (revolutions $P = 0.3189$, intervals $P = 0.7284$, mean speed $P = 0.1771$, maximum speed $P = 0.1065$), and significance of the effects of percent total fat ($P < 0.003$) and mass change ($P < 0.06$) was similar to values for simple models without wheel-running traits. When control lines were analyzed separately (with line as a fixed effect), again no measure of wheel running was a significant predictor of log leptin levels. However, when the HR lines were analyzed, we found negative relations with revolutions ($P = 0.0711$), mean speed ($P = 0.0223$), and maximum speed ($P = 0.0064$) but not with intervals ($P = 0.6052$).

Body Mass and Composition

HR and control lines did not differ significantly in body mass at weaning (21 d of age); however, as expected from previous

studies, HR mice were significantly smaller upon introduction to wheels at 49 d (Table 3). Growth over the 4 wk from weaning (day 21) to the beginning of wheel access (day 49), calculated as the net change in mass, was 21% lower in HR mice than in controls ($P = 0.0013$). During the 6-d wheel access trial, change in body mass was not significantly different from 0 in either HR or control mice (one-sample t-test, $P > 0.2$ in both cases) and also did not differ between linetypes (Table 3). HR mice maintained a significantly lower body mass than controls when removed from wheels (day 55; Table 3). Mass change during the final week of solitary home cage housing (from day 55 to day 61) was not significantly different from 0 in either linetype (one-sample t-test, $P > 0.3$ in both cases) and also did not differ between linetypes (Table 3).

At euthanasia (61 d), body mass in HR mice was 11% lower than in controls. HR mice were also significantly smaller than the controls, as measured by body length at euthanasia: average lengths were 3 mm, or about 3%, lower in HR. Adding body length as a covariate in an ANCOVA eliminated the statistical significance of the linetype difference in body mass at euthanasia (Table 3).

Total body dry mass was significantly lower in HR mice than in controls (Table 2). On an absolute basis, wet stomach mass was smaller in HR lines ($P = 0.0137$). Other than that, no organ mass (wet or dry) was significantly different between HR and control lines either on an absolute basis or in ANCOVA with body mass or length as the covariate (Table 2).

Total body fat/dry body mass averaged 28.9% in HR mice (range = 13.7%–47.1%) and 30.1% in controls (range = 18.5%–37.8%), a difference that was not statistically significant (two-tailed $P = 0.6073$; Table 2). Fat content of the liver and total body fat, expressed either as absolute mass or as a relative percentage, also did not differ between linetypes. Not surprisingly, total dry mass and total body fat (%) were significantly correlated within linetypes (HR: Pearson's $r = 0.949$, $P < 0.0001$; control: $r = 0.894$, $P < 0.0001$). Absolute total body fat was not correlated with liver fat in controls ($r = 0.338$, $P = 0.1063$) but was positively correlated with liver fat in HR mice ($r = 0.478$, $P = 0.0177$).

Because females were sampled at an age corresponding to development of reproductive maturity, mass of the uterus plus ovaries was used as an indicator of reproductive status. Although neither absolute dry mass nor body mass-adjusted values differed between linetypes, within-linetype relations of uterus-plus-ovaries mass with body fat did differ. Dry mass of uterus plus ovaries was negatively correlated with total body fat (%) in HR mice ($r = -0.4609$, $P = 0.0234$) and positively correlated with body fat (%) in controls ($r = 0.4065$, $P = 0.0487$).

Discussion

General Considerations

Consistent with our hypothesis, serum leptin levels were significantly lower (−38%, two-tailed $P = 0.0059$) in females from replicate lines of mice that had been selectively bred for high voluntary wheel running (HR lines), as compared with their nonselected control lines (but see Vaanholt et al. 2007 on males at 10 and 18 mo of age). HR mice also tended to have less total body fat (see Table 2), but the difference was not statistically significant, unlike the results of our earlier studies (Dumke et al. 2001; Swallow et al. 2001). As demonstrated in previous studies of mice, rats, humans, and other mammals (e.g., Bünger et al. 1999; Ajov et al. 2004; Geary 2004; Overton

Table 3: Body mass, length, and food consumption in high-runner (HR) and control lines of mice

Trait, age	HR	Control	Transformation	P_{cov}	$P_{linetype}$
Body mass at weaning, 21 d (g)	11.32 ± 1.75	11.18 ± 1.91	x^2		.8737
Body mass on wheels, 49 d (g)	22.07 ± 1.59	24.84 ± 2.08	x^2		.0040
Body mass off wheels, 55 d (g)	22.19 ± 1.80	24.08 ± 2.20	None		.0396
Body mass at euthanasia, 61 d (g)	20.85 ± 1.48	23.50 ± 2.44	x^2		.0223
Body mass at euthanasia, 61 d (g), length adjusted			x^2	.0004	.1144
Body length, 61 d (mm)	96.79 ± 3.01	99.96 ± 2.63	x^5		.0236
Body mass change, 21–49 d (g)	10.76 ± .27	13.67 ± .44	None		.0013
Body mass change, 49–55 d (g)	.12 ± 1.28	−.76 ± 1.36	None		.1915
Body mass change, 55–61 d (g)	−1.34 ± 1.61	−.58 ± 2.18	None		.3912
Food consumption, 55–61 d (g/wk)	35.66 ± 3.11	35.52 ± 4.56	Log_{10}		.8843
Food consumption, 55–61 d (g/wk), mass adjusted			Log_{10}	.0001	.8664
Food consumption, 61 d (g/8 h)	1.38 ± .67	1.46 ± .55	x^5		.6908
Food consumption, 61 d (g/8 h), mass adjusted			None	.0014	.6795

Note. Simple means ± SD for $n = 24$ female mice per linetype. Significance of the linetype effect (HR vs. control) was derived from a nested ANOVA or, for length- or mass-adjusted values, a nested ANCOVA. The significance of body length or $\log_{10}$ (body mass) at euthanasia, when included, is also indicated (P_{cov}). Traits were transformed for analysis as noted. P values are for two-tailed tests.

and Williams 2004), leptin levels were positively correlated with total body fat within both the HR and control lines (Fig. 1, *top*). However, the lower leptin levels of HR mice were not entirely explained by their lower body fat levels. Rather, HR mice still have significantly lower serum leptin levels than controls even after correction for differences in total body fat, and the difference in leptin levels could not be explained by variation in FC either on the day of sampling or during the week before sampling. Leptin levels were positively related to the amount or average speed of wheel running recorded a week before sampling in HR lines but not in control lines.

Our result that genetic selection for high locomotor activity leads to a reduction in circulating leptin levels, partly independent of the amount of body fat, seems inconsistent with results for ob/ob mice, which were reported to exhibit hypoactivity that was ameliorated by leptin administration (Pelleymounter et al. 1995). However, that study measured activity in a 15-min test in small cages, which may bear little relation to daily activity levels or energy expenditure in either mice or humans. For example, daily wheel-running activity in mice is generally unrelated to behavior in a standard short-term open-field test (Bronikowski et al. 2001 and references therein). On the other hand, mice from our HR lines also exhibit elevated activity in ordinary housing cages without wheel access (Rhodes et al. 2005; J. A. Malisch and T. Garland Jr., unpublished data). Our results are consistent with a human population study in which plasma leptin and activity levels were negatively correlated in both sexes (Franks et al. 2003).

We found that serum leptin levels were significantly positively correlated with FC in the week before leptin sampling even when percent body fat was included in the ANCOVA model (percent body fat $P = 0.0161$; FC week $P = 0.0472$; linetype $P = 0.0033$) but not if body mass was also included in the model (body mass $P = 0.5559$; percent body fat $P = 0.0380$; FC week $P = 0.1516$; linetype $P = 0.0180$). The positive relationship between leptin and FC is the opposite of what one would expect based on the previously accepted view that leptin functions primarily as a lipostatic signal to the CNS to inhibit food intake (e.g., Halaas et al. 1995; see "Introduction"). Importantly, the positive correlation between FC and circulating leptin levels was apparent in both HR and control mice, although it was slightly weaker in the former (Pearson's $r = 0.351$, $P = 0.0926$ and $r = 0.513$, $P = 0.0104$, respectively). In any case, this positive relationship is another example of how population-level studies of "normal" animals (including human beings) may yield results quite different from those that employ animals pathologically deficient in leptin function (see also Franks et al. 2003).

Taken together, our results suggest that selective breeding for high locomotor activity has altered the relations among body fat, circulating leptin levels, FC, and locomotor activity. In principle, this correlated response to selection could be caused by various changes in the leptin pathway, including alterations in the average size or number of adipose cells, leptin secretion rate, leptin clearance rate, amount of binding protein, and sensitivity of receptors. Further studies will be required to discriminate among such possibilities.

Whatever the underlying mechanism, alteration of leptin physiology might be related to other changes that have been observed in the HR lines, including their smaller body size (see also Malisch et al. 2007 regarding the possible relation with corticosterone levels) and reduced body fat compared with controls. The linetypes diverged in body mass during the weeks after weaning, when HR growth rates were only 79% of those of controls. This reduced growth rate may reflect energy intake rates insufficient to match the additional energy expenditures in growth and activity. In calorie-restricted mice, leptin administration (via daily intraperitoneal injections) preserved longitudinal growth by correcting the growth deficiency in the tibia of semistarved mice (Gat-Yablonski et al. 2004). In exercising HR mice, reduced leptin levels thus may play a role in their reduced linear dimensions. HR females also demonstrate chronically elevated corticosterone levels when housed both with and without wheels (Girard and Garland 2002; Malisch et al. 2007), and corticosterone-leptin interactions have been demonstrated in both rats (Yildiz et al. 2004; Coppola et al. 2005) and mice (Jeong et al. 2004). Although administration of glucocorticoids via osmotic pumps results in elevated leptin synthesis (Ahima and Osei 2004) and peripheral administration of corticotrophin-releasing hormone (CRH) restores normal leptin expression in CRH-deficient mice (Jeong et al. 2004), the relation of the two hormones is likely complex and nonlinear. In any case, our previous finding that HR lines have elevated circulating corticosterone levels lends support to the hypothesis proposed by Jacobson (2005) that leptin and the HPA axis may work together as a metabolic-neuroendocrine feedback loop that controls fat storage.

HR Mice as a Novel Model for Studying the Physiology of Leptin

Although the general evolutionary role of leptin has received virtually no attention, its role in obesity is under intense scrutiny. Mouse and rat models used in leptin studies include the ob/ob mouse, the obese C57bl/6J mouse (Lin et al. 2000*a*, 2000*b*), the diabetic db/db mouse, the obese spontaneously hypertensive rat, and the Zucker fatty rat. The most widely used animal model, the ob/ob mouse, represents the extreme case of a total absence of leptin because it is homozygous for the leptin gene (Lep) mutation. Ob/ob mice do respond to exogenous leptin administration, and leptin treatment can reverse the symptoms associated with obesity in these animals. Diabetic db/db mice have a mutation in the gene coding for the transmembrane leptin receptor (LEPR): these mice are hy-

perphagic and morbidly obese and do not respond to leptin administration (Chen et al. 1996; Lee et al. 1996). Obese, spontaneously hypertensive rats (SHROB or Koletsky) lack all cell surface LEPRs. SHROB rats have very high plasma leptin levels but appear to be resistant to both endogenous and exogenous leptin (Wu-Peng et al. 1997). Zucker fatty (fa/fa) rats also exhibit a mutation in LEPR resulting in a reduction in receptor expression and leptin binding. In contrast to db/db mice and obese hypertensive rats, partial functioning of leptin receptors allows fa/fa rats to respond to high doses of exogenous leptin (Cusin et al. 1996).

Most existing animal models used to study leptin function shown some striking differences with human beings. For example, leptin gene and receptor mutations appear to be rare in human beings (Ahima and Osei 2004). A deletion mutation has been identified in members of a Pakistani family (Montague et al. 1997), and a missense mutation has been identified in a Turkish family (Ozata et al. 1999; Rau et al. 1999). Members of a French family have a substitution mutation in the gene coding LEPR, leading to a nonfunctional mutant receptor lacking transmembrane and intracellular domains (Clement et al. 1998). Although affected humans in all three families suffer from hyperphagia and morbid obesity, leptin gene and receptor mutations clearly are not involved in the overwhelming majority of human obesity cases (Ahima and Osei 2004). Moreover, although studies of the relation between leptin and physical activity in human populations are rare (see recent review by Popovic and Dunlas [2005]), Franks et al. (2003) found that plasma leptin and physical activity energy expenditure (ratio of total energy, expenditure to basal metabolism rate, computed on a daily basis) were negatively correlated in both sexes. This differs from the apparent relation in ob/ob mice, which have been reported to exhibit hypoactivity that is normalized (increased) by leptin treatment (Pelleymounter et al. 1995) but is consistent with our finding that HR mice have reduced leptin levels.

In conclusion, HR mice clearly show promise as a model for studying the causes and consequences of physiologically relevant variation in circulating leptin levels, and a number of areas for future research are apparent. For example, it will be important to examine relations between circulating leptin levels and other physiological and behavioral traits in both sexes (see also Vaanholt et al. 2007) because sex differences (including interactive effects between sex and housing conditions) have been documented for a variety of traits that may be interrelated with leptin function, including amount and average speed of wheel running, body mass, food consumption, and maximal oxygen consumption (e.g., Swallow et al. 1999, 2001; Rezende et al. 2006*b*). Simultaneous measurement of leptin levels in mice that never had wheel access and quantification of differences in home cage activity could elucidate the relations of leptin and activity levels per se. Finally, it would also be important to conduct longitudinal studies in which leptin is administered to HR mice to increase levels to those of the control lines.

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Literature Cited

Ahima R.S., J. Kelly, J.K. Elmquist, and J.S. Flier. 1999. Distinct physiologic and neuronal responses to decreased leptin and mild hyperleptinemia. Endocrinology 140:4923–4931.

Ahima R.S. and S.Y. Osei. 2004. Leptin signaling. Physiol Behav 81:223–241.

Ahima R.S., C.B. Saper, J.S. Flier, and J.K. Elmquist. 2000. Leptin regulation of neuroendocrine systems. Front Neuroendocrinol 21:263–307.

Ajov M.M., D. Lamosova, and M. Zeman. 2004. Role of leptin in farm animals. J Vet Med 51:157–166.

Barrachina M.D., V. Martinez, J.Y. Wei, and Y. Tache. 1997. Leptin-induced decrease in food intake is not associated with changes in gastric emptying in lean mice. Am J Physiol 272: R1007–R1011.

Bouassida A., D. Zalleg, S. Bouassida, M. Zaouali, Y. Feki, A. Zbidi, and Z. Tabka. 2006. Leptin, its implication in physical exercise and training: a short review. J Sports Sci Med 5: 172–181.

Bowen H., T.D. Mitchell, and R.B. Harris. 2003. Method of leptin dosing, strain, and group housing influence leptin sensitivity in high-fat-fed weanling mice. Am J Physiol 284:R87–R100.

Bronikowski A.M., P.A. Carter, J.S. Swallow, I.A. Girard, J.S. Rhodes, and T. Garland Jr. 2001. Open-field behavior of house mice selectively bred for high voluntary wheel-running. Behav Genet 31:309–316.

Bünger L., M. Nicolson, and W.G. Hill. 1999. Leptin levels in lines of mice developed by long-term divergent selection on fat content. Genet Res 73:37–44.

Chen H., O. Charlat, L.A. Tartaglia, E.A. Woolf, X. Weng, S.J. Ellis, N.D. Lakey, et al. 1996. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell 84:491–495.

Clement K., C. Vaisse, N. Lahlou, S. Cabrol, V. Pelloux, D. Cassuto, M. Gourmelen, et al. 1998. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392:398–401.

Concannon P., K. Levac, R. Rawson, B. Tennant, and A. Ben-

sadoun. 2001. Seasonal changes in serum leptin, food intake, and body weight in photoentrained woodchucks. Am J Physiol 281:R951–R959.

Considine R.V., M.K. Sinha, M.L. Heiman, A. Kriauciunas, T.W. Stephens, M.R. Nyce, J.P. Ohannesian, C.C. Marco, L.J. McKee, and T.L. Bauer. 1996. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334:292–295.

Cook R.D. and S. Weisberg. 1999. Applied Regression Including Computing and Graphics. Wiley, New York.

Coppola A., R. Meli, and S. Diano. 2005. Inverse shift in circulating corticosterone and leptin levels elevates hypothalamic deiodinase type 2 in fasted rats. Endocrinology 146: 2827–2833.

Crowley W.R., G. Ramoz, R. Torto, and S.P. Kalra. 2004. Role of leptin in orexigenic neuropeptide expression during lactation in rats. J Neuroendocrinol 16:637–644.

Cusin I., F. Rohner-Jeanrenaud, A. Stricker-Krongrad, and B. Jeanrenaud. 1996. The weight-reducing effect of an intracerebroventricular bolus injection of leptin in genetically obese fa/fa rats: reduced sensitivity compared with lean animals. Diabetes 45:1446–1450.

Demas G.E. 2002. Splenic denervation blocks leptin-induced enhancement of humoral immunity in Siberian hamsters (*Phodopus sungorus*). Neuroendocrinology 76:178–184.

Dumke C.L., J.S. Rhodes, T. Garland Jr., E. Maslowski, J.G. Swallow, A.C. Wetter, and G.D. Cartee. 2001. Genetic selection of mice for high voluntary wheel running: effect on skeletal muscle glucose uptake. J Appl Physiol 91:1289–1297.

Eiden S., G. Preibisch, and I. Schmidt. 2001. Leptin responsiveness of juvenile rats: proof of leptin function within the physiological range. J Physiol 530:131–139.

Elefteriou F., S. Takeda, K. Ebihara, J. Magre, N. Patano, C.A. Kim, Y. Ogawa, et al. 2004. Serum leptin level is a regulator of bone mass. Proc Natl Acad Sci USA 101:3258–3263.

Franks P.W., I.S. Farooqi, J. Luan, M.Y. Wong, I. Halsall, S. O'Rahilly, and N.J. Wareham. 2003. Does physical activity energy expenditure explain the between-individual variation in plasma leptin concentrations after adjusting for differences in body composition? J Clin Endocrinol Metab 88:3258–3263.

Frederich R.C., A. Hamann, S. Anderson, B. Lollmann, B.B. Lowell, and J.S. Flier. 1995. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med 1:1311–1314.

Garland T., Jr. 2003. Selection experiments: an underutilized tool in biomechanics and organismal biology. Pp. 23–56 in V.L. Bels, J.P. Gasc, and A. Casinos, eds., Vertebrate Biomechanics and Evolution. BIOS Scientific, Oxford.

Garland T., Jr., and P.A. Freeman. 2005. Selective breeding for high endurance running increases hindlimb symmetry. Evolution 59:1851–1854.

Garland T., Jr., and S.A. Kelly. 2006. Phenotypic plasticity and experimental evolution. J Exp Biol 209:2234–2261.

Gat-Yablonski G., T. Ben-Ari, B. Shtaif, O. Potievsky, O. Moran, R. Eshet, G. Maor, Y. Segev, and M. Phillip. 2004. Leptin reverses the inhibitory effect of caloric restriction on longitudinal growth. Endocrinology 145:343–350.

Geary N. 2004. Endocrine controls of eating: CCK, leptin, and ghrelin. Physiol Behav 81:719–733.

Geary T.W., E.L. McFadin-Buff, M.D. MacNeil, R.N. Funston, R.E. Short, E.E. Grings, and D. Keisler. 2001. A possible role of the leptin assay in assessing carcass fat and composition in beef cattle. J Anim Sci 79(suppl. 2):125.

Girard I. and T. Garland Jr. 2002. Plasma corticosterone response to acute and chronic voluntary exercise in female house mice. J Appl Physiol 92:1553–1561.

Halaas J.L., K.S. Gajiwala, M. Maffei, S.L. Cohen, B.T. Chait, D. Rabinowitz, R.L. Lallone, S.K. Burley, and J.M. Friedman. 1995. Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269:543–546.

Harris R.B. 1998. Acute and chronic effects of leptin on glucose utilization in lean mice. Biochem Biophys Res Commun 245: 502–509.

Iwase M., K. Kimura, N. Sasaki, R. Komagome, K. Ishioka, M. Morimatsu, T. Murakami, and M. Saito. 2000. Canine leptin: cDNA cloning, expression and activity of recombinant protein. Res Vet Sci 68:109–114.

Jacobson L. 2005. Hypothalamic-pituitary-adrenocortical axis regulation. Endocrinol Metab Clin N Am 34:271–292.

Jeong K.H., S. Sakihara, E.P. Widmaier, and J.A. Majzoub. 2004. Impaired leptin expression and abnormal response to fasting in corticotropin-releasing hormone-deficient mice. Endocrinology 145:3174–3181.

Karsenty G. 2006. Convergence between bone and energy homeostases: leptin regulation of bone mass. Cell Metab 4:341–348.

Kelly S.A., P.P. Czech, J.T. Wight, K.M. Blank, and T. Garland Jr. 2006. Experimental evolution and phenotypic plasticity of hindlimb bones in high-activity house mice. J Morphol 267:360–374.

Ketterson E.D., V. Nolan Jr., and M. Sandell. 2005. Testosterone in females: mediator of adaptive traits, constraint on the evolution of sexual dimorphism, or both? Am Nat 166(suppl.):S85–S98.

Koteja P., P.A. Carter, J.G. Swallow, and T. Garland Jr. 2003. Food wasting by house mice: variation among individuals, families, and genetic lines. Physiol Behav 80:375–383.

Koteja P., J.G. Swallow, P.A. Carter, and T. Garland Jr. 2001. Maximum cold-induced food consumption in mice selected for high locomotor activity: implications for the evolution of endotherm energy budgets. J Exp Biol 204:1177–1190.

Kristan D.M. 2002. Maternal and direct effects of the intestinal nematode, *Heligmosomoides polygyrus*, on offspring growth and susceptibility to infection. J Exp Biol 205:3967–3977.

Leal-Cerro A., P.P. Garcia-Luna, R. Astorga, J. Parejo, R. Peino, C. Dieguez, and F.F. Casanueva. 1998. Serum leptin levels in male marathon athletes before and after the marathon run. J Clin Endocrinol Metab 83:2376–2379.

Lee G.-H., R. Proenca, J.M. Montez, K.M. Carroll, J.G. Darvishzadeh, J.I. Lee, and J.M. Friedman. 1996. Abnormal splicing of the leptin receptor in *diabetic* mice. Nature 379:632–635.

Licinio J., A.B. Negrao, C. Mantzoros, V. Kaklamani, M.L. Wong, P.B. Bongiorno, A. Mulla, et al. 1998. Synchronicity of frequently sampled, 24-h concentrations of circulating leptin, luteinizing hormone, and estradiol in healthy women. Proc Natl Acad Sci USA 95:2541–2546.

Lin S., L.H. Storlien, and X.-F. Huang. 2000*a*. Leptin receptor, NPY, POMC mRNA expression in the diet-induced obese mouse brain. Brain Res 875:89–95.

Lin S., T.C. Thomas, L.H. Storlien, and X.F. Huang. 2000*b*. Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. Int J Obes 24:639–646.

Maffei M., J. Halaas, E. Ravussin, R.E. Pratley, G.H. Lee, Y. Zhang, H. Fei, et al. 1995. Leptin levels in human and rodent: measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. Nat Med 1:1155–1161.

Malik N.M., N.D. Carter, J.F. Murray, R.J. Scaramuzzi, C.A. Wilson, and M.J. Stock. 2001. Leptin requirement for conception, implantation, and gestation in the mouse. Endocrinology 142:5198–5202.

Malisch J.L., W. Saltzman, F.R. Gomes, E.L. Rezende, D.R. Jeske, and T. Garland Jr. 2007. Baseline and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. Physiol Biochem Zool 80: 146–156.

Montague C.T., I.S. Farooqi, J.P. Whitehead, M.A. Soos, H. Rau, N.J. Wareham, C.P. Sewter, et al. 1997. Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387:903–908.

Muoio D.M. and G. Lynis Dohm. 2002. Peripheral metabolic actions of leptin. Best Pract Res Clin Endocrinol Metab 16: 653–666.

Overton J.M. and T.D. Williams. 2004. Behavioral and physiologic responses to calorie restriction in mice. Physiol Behav 81:749–754.

Ozata M., I.C. Ozdemir, and J. Licinio. 1999. Human leptin deficiency caused by a missense mutation: multiple endocrine defects, decreased sympathetic tone, and immune system dysfunction indicate new targets for leptin action, greater central than peripheral resistance to the effects of leptin, and spontaneous correction of leptin-mediated defects. J Clin Endocrinol Metab 84:3686–3695.

Park J.H., H.H. Lee, S.Y. Na, S.K. Ju, Y.J. Lee, M.K. Lee, and K.L. Kim. 2001. Recombinant expression of biologically active rat leptin in *Escherichia coli*. Protein Expr Purif 22:60–69.

Pelleymounter M.A., M.J. Cullen, M.B. Baker, R. Hecht, D. Winters, T. Boone, and F. Collins. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269:540–543.

Popovic V. and L.H. Dunlas. 2005. Leptin TRH and ghrelin: Influence on energy homeostasis at rest and during exercise. Horm Metab Res 37:533–537.

Ramsay T.G., X. Yan, and C. Morrison. 1998. The obesity gene in swine: sequence and expression of porcine leptin. J Anim Sci 76:484–490.

Rau H., B.J. Reaves, S. O'Rahilly, and J.P. Whitehead. 1999. Truncated human leptin (delta133) associated with extreme obesity undergoes proteasomal degradation after defective intracellular transport. Endocrinology 140:1718–1723.

Raver N., E.E. Gussakovsky, D.H. Keisler, R. Krishna, J. Mistry, and A. Gertler. 2000. Preparation of recombinant bovine, porcine, and porcine W4R/R5K leptins and comparison of their activity and immunoreactivity with ovine, chicken, and human leptins. Protein Expr Purif 19:30–40.

Rezende E.L., F.R. Gomes, J.L. Malisch, M.A. Chappell, and T. Garland Jr. 2006*a*. Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. J Appl Physiol 101:477–485.

Rezende E.L., S.K. Kelly, F.R. Gomes, M.A. Chappell, and T. Garland Jr. 2006*b*. Effects of size, sex, and voluntary running speeds on costs of locomotion in lines of laboratory mice selectively bred for high wheel-running activity. Physiol Biochem Zool 79:83–99.

Rhodes J.S., S.C. Gammie, and T. Garland Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integr Comp Biol 45:438–455.

Roberts C.K., J.J. Berger, and R.J. Barnard. 2002. Long-term effects of diet on leptin, energy intake, and activity in a model of diet-induced obesity. J Appl Physiol 93:887–893.

Salzmann C., M. Otis, H. Long, C. Roberge, N. Gallo-Payet, and C.D. Walker. 2004. Inhibition of steroidogenic response to adrenocorticotropin by leptin: implications for the adrenal response to maternal separation in neonatal rats. Endocrinology 145:1810–1822.

Siegmund B., J.A. Sennello, J. Jones-Carson, F. Gamboni-Robertson, H.A. Lehr, A. Batra, I. Fedke, M. Zeitz, and G. Fantuzzi. 2004. Leptin receptor expression on T lymphocytes modulates chronic intestinal inflammation in mice. Gut 53: 965–972.

Sinervo B. and R. Calsbeek. 2003. Physiological epistasis, ontogenetic conflict and natural selection on physiology and life history. Integr Comp Biol 43:419–430.

Swallow J.G., P.A. Carter, and T. Garland Jr. 1998. Artificial selection for increased wheel-running behavior in house mice. Behav Genet 28:227–237.

Swallow J.G. and T. Garland Jr. 2005. Selection experiments as a tool in evolutionary and comparative physiology: insights

into complex traits: an introduction to the symposium. Integr Comp Biol 45:387–390.

Swallow J.G., P. Koteja, P.A. Carter, and T. Garland Jr. 1999. Artificial selection for increased wheel-running activity in house mice results in decreased body mass at maturity. J Exp Biol 202:2513–2520.

———. 2001. Food consumption and body composition in mice selected for high wheel-running activity. J Comp Physiol 171:651–659.

Vaanholt L.M., P. Meerlo, T. Garland Jr., G.H. Visser, and G. van Dijk. 2007. Plasma adiponectin is increased in mice selectively bred for high wheel-running activity, but not by wheel running per sé. Horm Metab Res 39:377–383.

Wu-Peng X.S., S.C. Chua, N. Okada, S.M. Liu, M. Nicolson, and R.L. Leibel. 1997. Phenotype of the obese Koletsky (f) rat due to Tyr763Stop mutation in the extracellular domain of the leptin receptor (Lepr): evidence for deficient plasma-to-CSF transport of leptin in both the Zucker and Koletsky obese rat. Diabetes 46:513–518.

Yang S.H., T. Matsui, H. Kawachi, T. Yamada, N. Nakanishi, and H. Yano. 2003. Fat depot-specific differences in leptin mRNA expression and its relation to adipocyte size in steers. Anim Sci J 74:17–21.

Yildiz B.O., M.A. Suchard, M.L. Wong, S.M. McCann, and J. Licinio. 2004. Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. Proc Natl Acad Sci USA 101:10434–10439.

Zera A.J. and L.G. Harshman. 2001. The physiology of life history trade-offs. Annu Rev Ecol Syst 32:95–127.

Zhang Y., R. Proenca, M. Maffei, M. Barone, L. Leopold, and J.M. Friedman. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372:425–432.