Genes, Brain and Behavior (2014) **13:** 322–332 doi: 10.1111/gbb.12098

Effects of early-life exposure to Western diet and wheel access on metabolic syndrome profiles in mice bred for high voluntary exercise

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Experimental studies manipulating diet and exercise have shown varying effects on metabolic syndrome components in both humans and rodents. To examine the potential interactive effects of diet, exercise and genetic background, we studied mice from four replicate lines bred (52 generations) for high voluntary wheel running (HR lines) and four unselected control lines (C). At weaning, animals were housed for 60 days with or without wheels and fed either a standard chow or Western diet (WD, 42% kcal from fat). Four serial (three juvenile and one adult) blood samples were taken to measure fasting total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides and glucose. Western diet was obesogenic for all mice, even after accounting for the amount of wheel running and kilojoules consumed. Western diet significantly raised glucose as well as TC and HDL-C concentrations. At the level of individual variation (repeatability), there was a modest correlation ($r = 0.3–0.5$) of blood lipids over time, which was reduced with wheel access and/or WD. Neither genetic selection history nor wheel access had a statistically significant effect on blood lipids. However, HR and C mice had divergent ontogenetic trajectories for body mass and caloric intake. HR mice also had lower adiposity, an effect that was dependent on wheel access. The environmental factors of diet and wheel access had pronounced effects on body mass, food consumption and fasting glucose concentrations, interacting with each other and/or with genetic strain. These data underscore the importance (and often unpredictable nature) of genotype-by-environment and environment-by-environment interactions when studying body weight regulation.

Keywords: Artificial selection, diet, exercise, experimental evolution, genotype-by-environment interaction, repeatability, wheel running

Received 18 February 2013, revised 16 August 2013 and 16 October 2013, accepted for publication 21 October 2013

Obesity is a major public health issue. Among U.S. children and adolescents, the prevalence of obesity has increased threefold over the past few decades (Hedley *et al.* 2004). Alongside this trend, the constellation of metabolic risk factors has emerged in young people (Park *et al.* 2003). The increased prevalence of obesity and appearance of the metabolic syndrome in children and adolescents is indicative of elevated future risk of cardiovascular disease (CVD) and type II diabetes. One possible way to counteract these trends is through habitual physical activity, which is associated with many health-related benefits (e.g. Allen *et al.* 2001; Joyner & Green 2009; Mestek *et al.* 2010), including decreases in blood pressure, insulin resistance, blood lipids and adiposity, all of which are major risk factors for CVD morbidity and mortality (Must *et al.* 1999; U.S. Department of Health and Human Services 2008).

Although exercise *per se* has many benefits, high aerobic capacity (maximal oxygen consumption, VO_2max) may independently provide resistance to diet-induced obesity and associated comorbidities (Blair *et al.* 1995). In humans, aerobic capacity, as indexed by time to exhaustion on a treadmill, is inversely related to risk of metabolic syndrome and CVD mortality (Blair *et al.* 1995; Church 2010; LaMonte *et al.* 2005; Stevens *et al.* 2002). Although regular exercise training can enhance aerobic capacity, the response to training is under partial genetic control (Bouchard & Rankinen 2001), thus making it difficult to distinguish between health benefits derived from exercise *per se* vs. baseline aerobic capacity. Regardless of training, the same trend is seen when examining sedentary subjects with high and low physical fitness: high physically fit individuals who are sedentary show a nearly threefold decrease in all-cause mortality when compared with unfit individuals in both sexes, thus indicating that fitness level may be an important indicator of health outcomes independent of exercise (Blair *et al.* 2001).

In a replicated selection experiment using laboratory house mice (Careau *et al.* 2013; Swallow *et al.* 1998), breeding for high *voluntary* exercise on wheels has led to several correlated phenotypes, when compared with non-selected control (C) lines, that we hypothesized to be positive health

factors and to confer at least partial resistance to the adverse effects of an obesogenic diet and/or a sedentary conditions. These correlated responses to selection observed in the high runner (HR) lines include elevated cage activity in the absence of wheels (Malisch *et al.* 2009), innately low body fat (Nehrenberg *et al.* 2009; Swallow *et al.* 2005), increased VO_2max (Rezende *et al.* 2006) and running endurance measured during forced treadmill exercise (Meek *et al.* 2009), elevated muscle oxidative capacity (Bilodeau *et al.* 2009; Houle-Leroy *et al.* 2000), increased insulin-stimulated glucose uptake in isolated hindlimb muscle (Dumke *et al.* 2001) and increased upregulation of GLUT-4 transporter in response to 5 days of wheel access (Gomes *et al.* 2009).

In this study, we used the unique HR lines of mice and their non-selected counterparts to address three main questions. First, are the HR mice unusually resistant to some of the adverse effects of a Western diet (WD)? If so, then does this resistance occur even in the absence of voluntary exercise, i.e. if they are housed without wheels? Second, do diet and/or exercise interact with genetic background to produce differential responses in metabolic risk factors [circulating glucose, cholesterol and triglycerides (TGs)] and body fat? One reason this might occur is that male HR mice have been shown to respond behaviorally to WD (high in saturated fat plus added sucrose) by increasing total daily wheel running as much as 75%, whereas mice from the C lines showed no significant change (Meek *et al.* 2010). This genotype-by-environment interaction may directly or indirectly contribute to large differences in susceptibility to weight gain or diet-induced metabolic abnormalities. Third, do the experimental subjects – taken as a whole or as subgroups within genetic, diet and exercise categories – show some degree of consistency for individual differences in blood lipid profiles? As childhood obesity generally persists into adulthood, an essential line of research relates the ontogeny of obesity to genetic and environmental factors operating during critical periods of postnatal growth (Eisenmann 2003). Therefore, we sought to longitudinally study HR and C mice to determine if potential genetic and environmental influences altered the development of obesity and associated metabolic syndrome components.

Materials and methods

Experimental animals

All experiments were carried out in accordance with the guidelines of the NIH and institutional approval. Male mice from the 52nd generation of an artificial selection experiment for high voluntary wheel running were used for this experiment. Details of the experimental selection protocol have been described previously (Swallow *et al.* 1998), and major characteristics of these lines have been reviewed (Garland *et al.* 2011a,b; Rhodes *et al.* 2005). The original progenitors were outbred, genetically variable Hsd:ICR house mice (*Mus domesticus*). Four closed lines were bred for high voluntary running on wheels (HR lines) and four were bred without regard to wheel running (C lines). In each generation, mice are allowed access to wheels for 6 days when they reach 6–9 weeks of age. Within each HR-line family, the highest running male and female were chosen as breeders based upon their wheel revolutions on nights 5 and 6 of the 6-day trial.

For this study, 198 male mice from generation 52 were weaned at 21 days of age and then singly housed with access to Harlan Teklad Laboratory Rodent Diet (W)-8604 until they reached 24 days of age. Room temperature was maintained at approximately 73°F and photoperiod was 12:12, with lights on at 0700 Pacific Time.

Experimental groups

Mice were placed into experimental groups at an average of 24 ± 0.05 (SE) days of age (experimental day 1; see Fig. S1, Supporting Information for a graphical depiction of the timeline of experimental procedures). Half the mice received Wahman-type activity wheels (1.12 m circumference, 35.7 cm diameter, 10-cm-wide running surface) attached to standard cages ($27 \times 17 \times 12.5\,cm^3$), as used during routine wheel testing for the selection experiment, whereas the other half remained in standard cages without a wheel. Within each wheel or no-wheel group, mice were split by either standard diet (SD) [Harlan Teklad Rodent Diet (W) 8604, 14% kilojoules (kJ) from fat] or WD (Harlan Teklad TD.88137 WD, 42% kJ from fat with added sucrose), resulting in 24 mice per group.

Mice and food hoppers were weighed on experimental days 1, 8, 16, 30, 43 and 58 (age ~81 days old). Body length was measured at each time point from between the eyes (crown) to rump (base of tail) in non-anesthetized mice. Apparent food consumption was measured as the difference in hopper mass between two successive time points, after accounting for any obvious wastage (Koteja *et al.* 2003). Because the diets differ in mass-specific energy content for SD and WD, we converted food consumption from grams to energy intake, using 12.98 and 19.01 kJ/g, respectively.

For wheel-access animals, wheel revolutions were recorded in 1-min bins for approximately 23 h every day. From these records, we computed 3-day averages for wheel running (revolutions/day), the number of 1-min intervals with at least one revolution (min/day) and mean revolutions/min for the active intervals (rpm = revolutions/minutes active).

Blood samples

In a longitudinal study design, four blood samples were taken during the 8-week time course of the experiment, all during the diurnal phase (see Fig. S1) under similar conditions. All mice were fasted and restricted from wheel access for 4–6 h before blood sampling. The first blood sample was obtained before the start of the experiment when mice were 24 days old, the second at approximately 31 days of age, the third at approximately 39 days of age (approximate time of sexual maturation) and the last at the end of the experiment when mice averaged approximately 81 days of age. All four blood samples were 60 μl each, obtained from the submandibular vein using Goldenrod animal lancets (Medipoint, Inc., Mineola, NY, USA) and collected into heparinized microhematocrit tubes.

Thirty-five microliters from all blood samples were immediately divided into aliquots and used to determine total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), TGs and glucose in whole blood with an automated Cholestech LDX analyzer (Cholestech Corporation, Hayward, CA, USA) and cassettes (10-991). Values outside the normal operating range of the Cholestech machine were later determined in duplicate using Pointe Scientific (Canton, MI, USA; HDL-C) or Wako Diagnostics (Richmond, VA, USA; TG) commercially available kits calibrated to the same standards as the Cholestech machine. The only exception was for TC, which had a lower detection range of 100 mg/dl. A few mice from sample 1 received values of 100 mg/dl for TC, but no additional blood remained for verification so values of 100 mg/dl were analyzed. After the final blood sample, mice were sacrificed by decapitation and liver, heart ventricles, triceps surae muscles, retroperitoneal fat and epididymal fat pad masses (see Cinti 2005) were taken. We use the term abdominal fat to refer to the sum of both retroperitoneal and epididymal fat pad masses.

Statistical analyses

Analyses were performed using the Mixed Procedure in SAS 9.1.3 (SAS Institute, Cary, NC, USA) to apply analysis of covariance (ANCOVA) models with type III tests of fixed effects. Linetype (HR or C) and mini-muscle status (see next paragraph) were treated as fixed effects; line was nested within line type as a random

effect. Effects of linetype, diet, wheel access and the linetype × diet, linetype × wheel access, wheel access × diet and linetype × wheel access × diet interactions were tested relative to the variance among replicate lines, and degrees of freedom were always 1 and 6 (even though the total $n = 5–7$ per group) except in cases where repeated-measures analyses of variance were performed. In cases where residuals were not normally distributed data were log-transformed before analysis. Covariates depended on the trait analyzed and included age, body length or body mass, fasting time, time of day, wheel freeness and wheel running as revolutions/day.

The mini-muscle phenotype is currently found in two of the four HR lines and is characterized by a small-muscle phenotype, with an approximately 50% reduction in triceps surae, as well as whole-hindlimb muscle mass (Garland *et al.* 2002; Houle-Leroy *et al.* 2003). Pleiotropic effects of this Mendelian recessive allele include alterations in muscle fiber type composition (fewer type IIb fibers; Bilodeau *et al.* 2009), contractile properties (Syme *et al.* 2005) and a doubling of mass-specific aerobic capacity and hexokinase activity (Houle-Leroy *et al.* 2003). Because of the known differences in physiology, mini-muscle status was included as an independent variable in all analyses that involved the HR lines.

To examine the degree of individual consistency in the repeated measures of blood characteristics, we first computed residuals from regressions on appropriate covariates (e.g. age, fasting duration and bleed delay time), as indicated in the footnote of Table 1, using data for all mice. We then computed pairwise Pearson product–moment correlations (i.e. tracking coefficients) between the longitudinal measures. This was done for the entire set of mice, which is comparable to studies of human populations (Eisenmann *et al.* 2004), and also for subsets within genetic, diet and/or exercise categories.

Results

Body mass

After only 1 week, WD significantly increased body mass, and this effect remained significant throughout the experiment even when controlling for body length for both HR and C mice (Fig. 1a,b and Table S1). Wheel access significantly blunted the gain in body mass starting in week 4 (Fig. 1a,b; $F = 6.67$, $P < 0.05$) and continuing throughout the study. Wheel access had a much more appreciable effect on body mass for mice fed WD, as evident by the significant wheel access–diet interactions starting at week 2 and persisting throughout the study (Fig. 1c and Table S1). Inspection of the least-squares means shows that wheel access reduced body mass to a greater extent for mice on WD by reducing the gain in fat mass when compared with mice on SD.

Although the effect of WD on body mass was statistically significant for all weeks, the gain in body mass was progressive throughout the study (Fig. 1). This is further supported by a repeated-measures ANCOVA of body mass indicating a highly significant time–diet interaction ($F = 49.8$, $P < 0.001$, df = 5,30).

Body mass did not differ statistically between the linetypes (HR or C lines) for any weekly analysis alone (Fig. 1d). However, in the same repeated-measures ANCOVA, as noted above, there was a significant interaction between time and linetype ($F = 4.8$, $P = 0.003$, df = 5,30). This interaction remained significant even when body length and caloric intake were added as covariates ($F = 3.5$, $P = 0.036$, df = 5,30). The interaction implies that the trajectories of body mass throughout the experiment were different between the linetypes.

The prevention of body mass gain due to wheel running was accompanied by significantly lower abdominal fat pad mass and liver mass (Fig. 2 and Table S2; $F = 26.9$, $P = 0.024$; $F = 10.1$, $P = 0.019$, respectively). Wheel access increased ventricle mass, likely representing a physiologically adaptive training response ($F = 43.4$, $P < 0.001$). Western diet had a significant, positive effect on all organ masses (adjusted for body length), which remained significant even after adding kJ consumed as an additional covariate (data not shown). When controlling for caloric consumption, HR mice did not have significantly lower abdominal fat pad mass except when allowed access to wheels (significant linetype–wheel access interaction; $F = 6.1$, $P < 0.05$).

Caloric intake

Sedentary mice consumed substantially more kJ when on WD compared with standard chow (Fig. 3a,b and Table S3). This hyperphagia is likely driven by hedonic responses to WD rather than the slight differences in energy density between the diets. Interestingly, this differential effect was not observed in wheel-access mice, where (on average) no overall difference in caloric intake occurred on the WD beyond the second week (Fig. 3c). This finding is supported by the significant wheel access–diet interaction throughout the study with the exception of week 2 (Table S3).

Overall, wheel-access mice typically consumed more kJ ($F > 7.7$, $P < 0.03$ for all weeks). The HR mice consumed more kJ than C only when given access to wheels (data not shown), which can be attributed to the higher wheel-running activity of HR mice, because addition of wheel running as a covariate results in similar daily food consumption between the linetypes (Fig. 3d).

Plasma lipid and glucose profile

We found no statistically significant linetype or wheel access effects for any plasma metabolite, but did find effects of diet. Western diet significantly increased fasting glucose concentrations by the end of the experiment (Fig. 4a and Table S4; $F = 17.3$, $P = 0.006$). Repeated-measures ANCOVA (with age, fasting time and handling time as covariates) for glucose showed a significant time–diet interaction ($F = 6.2$, $P = 0.005$, df = 3,18), an effect particularly evident in sedentary mice at week 8 of the study (Fig. 4a). Fasting plasma TG concentrations were significantly decreased in response to WD at week 1 and week 8 (Fig. 4b; $F = 6.1$, $P < 0.05$; $F = 10.8$, $P = 0.017$, respectively).

Western diet also increased TC by the final blood sample (Fig. 4c). It had a large, positive influence on HDL-C at all time points, which showed a factorial increase greater than the observed increase in TC (Fig. 4d and Table S4). Concomitantly, WD decreased non-HDL-C [e.g. low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL); Table S4].

At the level of individual variation, and considering the entire sample of mice, plasma glucose concentrations were weakly positively correlated across sampling times, with only one of three correlations reaching statistical significance (Table 1). Plasma TGs showed a weak but statistically significant positive correlation between the first two samples. Total cholesterol was significantly correlated,

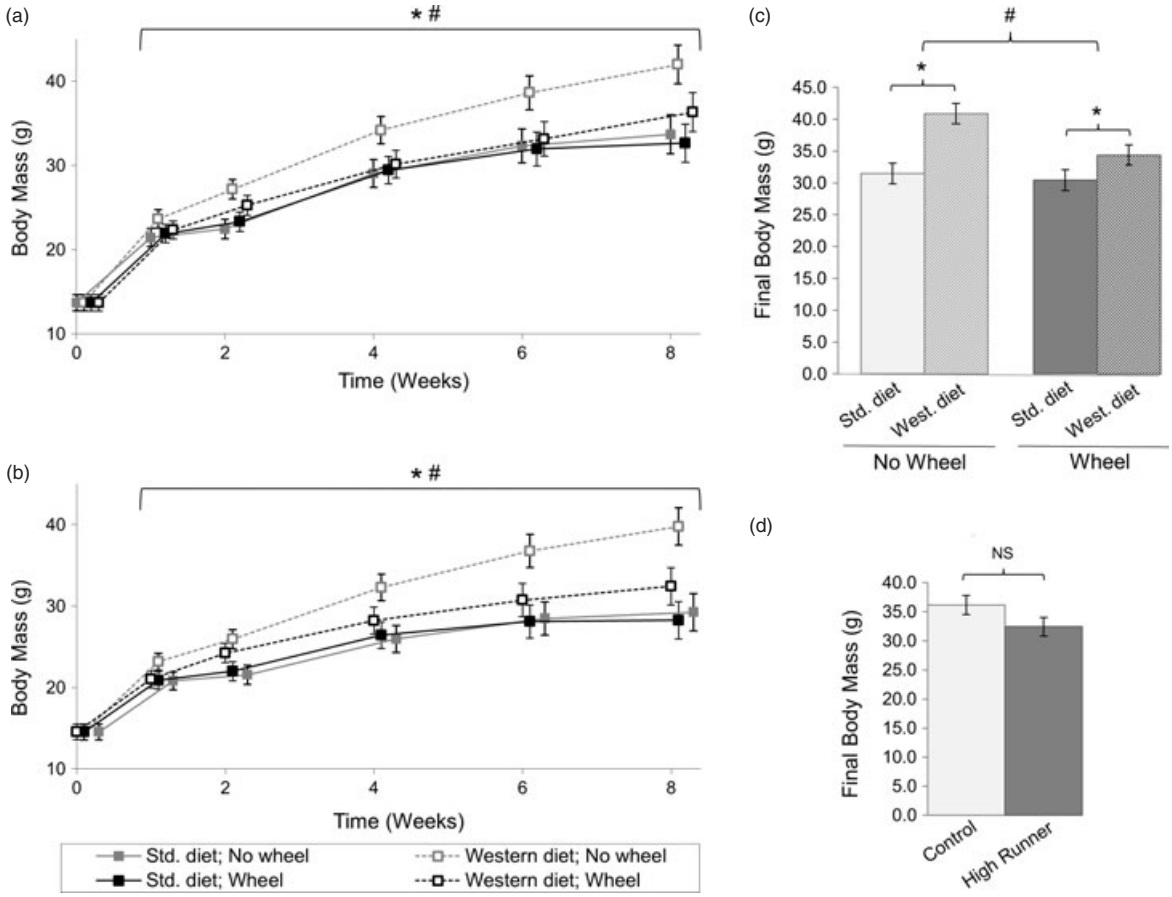

Figure 1: Least-squares means and standard errors for body mass. Results for control (a) and high runner (b) mice across the duration of the experimental treatments. At the start of week 0, mice were 24 days old, and separated into diet and wheel-access groups. Western diet and wheel access both significantly impacted body mass and also interacted starting at week 2, where mice fed WD gained much less mass when concomitantly allowed wheel access (c). Large mass differences did not exist between control and high runner genotypes when wheel running was accounted for (d). * indicates significance effect of diet and # indicates a significant diet-by-wheel access interaction. Total sample size was 5–7 per group. Degrees of freedom are 1 and 6 for effects of linetype (HR vs. C), wheel access, diet and their interactions.

although again weakly for two of three successive samples. High-density lipoprotein cholesterol had the strongest consistency across samples (all correlations were significant), with the correlations between samples 2 and 3 and between 3 and 6 moderately large ($r = 0.7$). For the ratio of TC/HDL-C, all cross-time correlations were positive and statistically significant (Table 1).

Given that we found no statistical effects of linetype on any plasma trait (Fig. 4), we analyzed the linetypes together but split analyses by diet and wheel access. For these four subgroups, TC/HDL-C shows a pattern of reduced individual consistency when environmental factors are added (Table 1). That is, sedentary mice on SD show the highest correlations between time points, those with both wheel access and WD (i.e. two environmental factors that might increase differences among individuals) show the lowest average correlations and those with either WD or wheel access show correlations of intermediate value. The trend observed with TC/HDL-C where environmental components decrease individual predictability holds true for the other blood metabolites as well (glucose, TC, HDL-C and to a lesser extent TG; data not shown).

Discussion

Human CVD and the metabolic syndrome are associated with low physical activity (sedentary lifestyle), particularly in concert with an obesogenic (e.g. Western) diet. Consequently, we expected that HR mice, which are genetically predisposed for high activity levels (both on wheels and in cages when wheels are not provided; Malisch *et al.* 2009), would better resist the detrimental effects of a high-fat WD. Although we did not find statistically significant differences between HR mice and their non-selectively bred controls for fasting lipid or glucose concentrations, HR mice had a

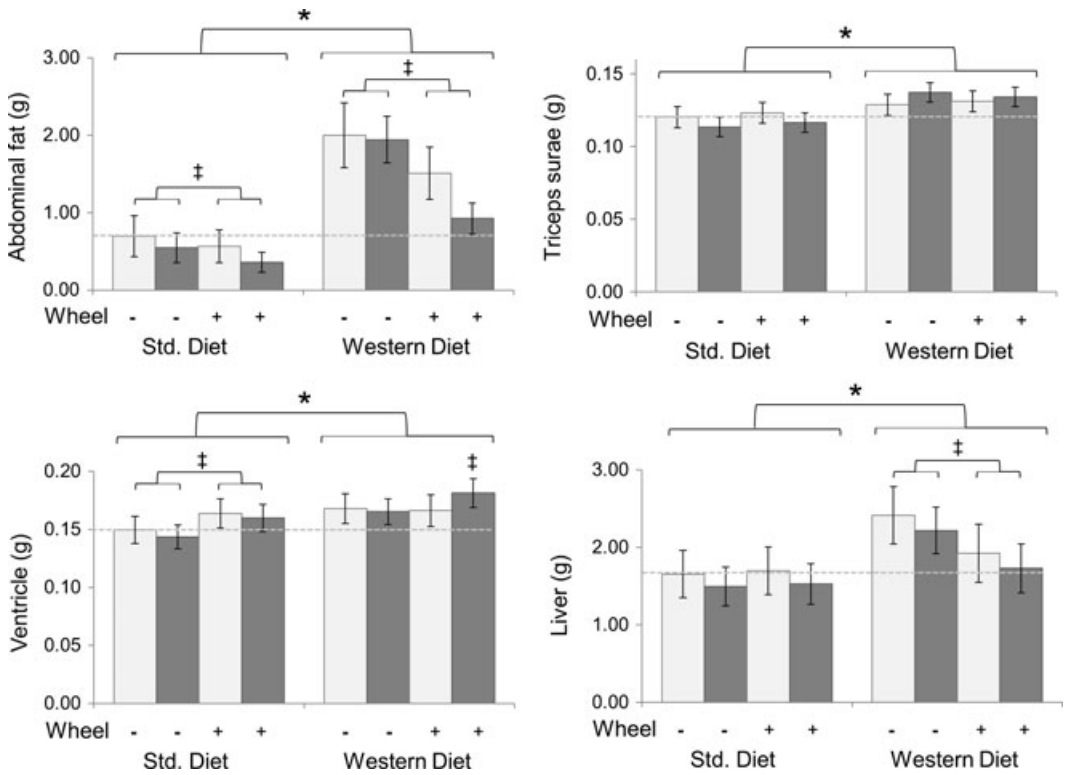

Figure 2: Least-squares means ± 95% CIs for organ masses for all eight groups, based on analyses that included $\log_{10}$ body length as a covariate. Abdominal fat is the combination of retroperitoneal + epididymal fat pad masses. White bars represent control mice and gray bars represent HR mice. * indicates significant ($P < 0.05$) effect of diet and ‡ indicates a significant effect of wheel access. For full statistical results, see Table S2. Total sample size was 5–7 per group with degrees of freedom of 1 and 6.

lower body mass trajectory over the 8-week study (Fig. 1), and lower fat mass, but only when they were allowed wheel access (environmentally dependent).

Eight weeks of a high-fat WD early in life is likely insufficient for the development of severe metabolic disorders in rodents that are neither genetically predisposed nor pharmacologically manipulated. Still, this time frame can illustrate the detrimental influence of early-life exposure to WD or a sedentary lifestyle on body mass, organ mass, food consumption and fasting glucose concentration, all of which show a significant adverse response by adulthood (Figs. 1–4). Furthermore, some physiological traits, such as body mass and food consumption, show significant differences in ontogenetic trajectories between HR and C mice (Figs. 1,3), as does the behavioral trait of wheel running (Fig. S2; see also Morgan *et al.* 2003).

In humans, it has been suggested that daily activity levels are regulated in such a way that beyond a certain 'set point' for activity (i.e. activitystat), such compensatory behaviors as increased food consumption and/or decreases in certain components of physical activity counteract weight loss and sometimes even result in weight gain (Sonneville & Gortmaker 2008; Wilkin *et al.* 2006). In our study, wheel access always had a positive effect on food consumption (converted to kJ/day for statistical analysis) after the first experimental week (Fig. 3). Despite the increase in food consumption caused by wheel access, voluntary exercise resulted in less gain in body mass over the experiment when compared with sedentary mice (Figs. 1,2). Wheel access prevented body mass gain the most in mice fed a WD. One important distinction to note, however, is that any activity measured in this study was purely voluntary and restricted to wheel running (i.e. voluntary cage activity was not assessed). In contrast, exercise intervention studies in which subjects are required (or forced) to exercise more may not truly represent 'voluntary' exercise and this may explain some of the human data showing an increase in body mass (and body fat) after participating in exercise regimes. As a consequence of forced exercise, different levels of psychological 'stress' (and the related consequences, e.g. increased cortisol) may come into play, much as they do for rodent studies that employ voluntary wheel running vs. forced exercise on a motorized treadmill (e.g. Brown *et al.* 2007; Fuss & Gass 2010; Girard & Garland 2002; Holmes *et al.* 2009). To provide efficient treatment of obesity, it is essential to understand the role of voluntary vs. forced exercise in weight loss, and what mechanisms underlie the compensatory responses that may occur in each paradigm.

Substituting a diet similar to our standard chow in place of WD can help reduce body mass; however, this is not

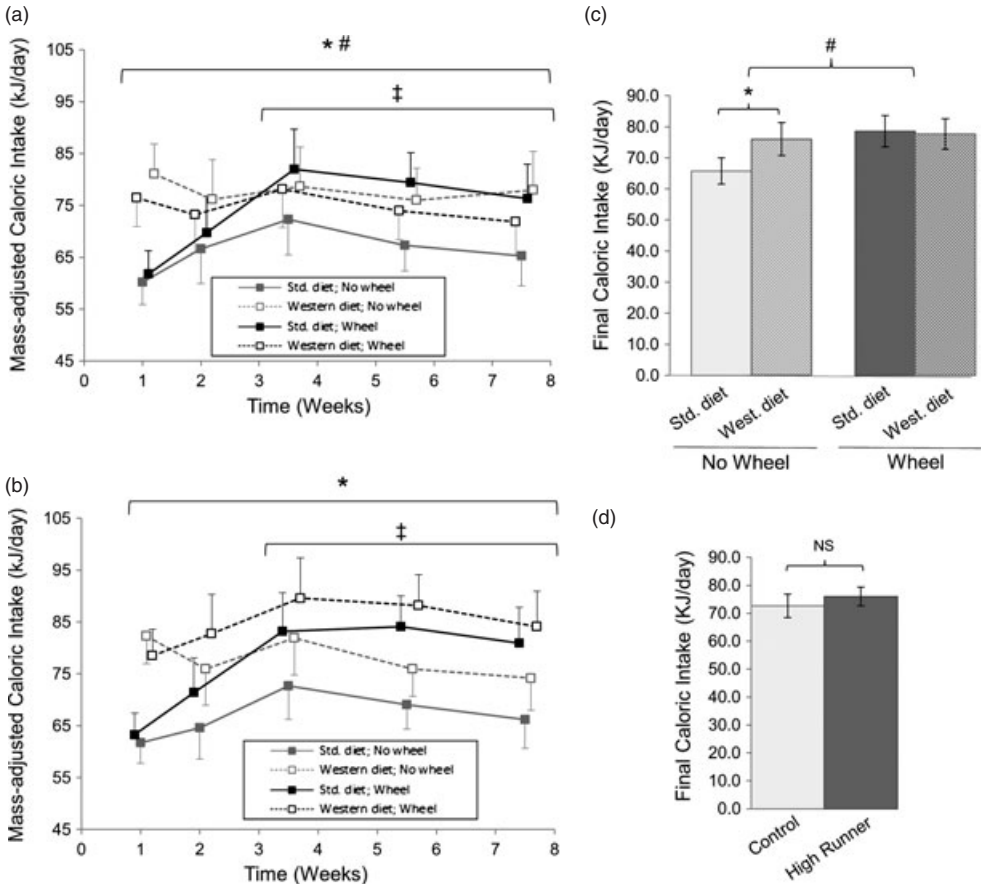

Figure 3: Body mass-adjusted caloric intake (ANCOVA) measured in kJ per day. Results for control (a) and high runner (b) mice through the duration of the study. Western diet and wheel access both significantly influenced caloric intake. Diet and wheel access significantly interacted such that sedentary mice on WD differentially ate more, but mice with wheels did not (c). Control and higher runner lines did not differ significantly in food intake when averaged across diet and wheel groups (d). * indicates significant effect of diet, ‡ marks a significant effect of wheel access and # indicates a significant diet-by-wheel access interaction. Total sample size was 5–7 per group. Degrees of freedom are 1 and 6 for linetype (HR vs. C), wheel access, diet and their interactions.

necessarily sufficient to restore normal body mass in mice throughout longitudinal experiments (Guo *et al.* 2009). In our study, the trajectories of body mass for the different diet groups were significantly different (time–diet interaction, $P < 0.0001$). The progression of body mass in Fig. 1 shows that mice fed SD did not simply have slower mass gain compared to mice eating WD. Rather, body mass appears to plateau during young adulthood with SD but continually trend upward in mice fed WD, suggesting a fundamental difference in body mass regulation caused by the difference in diet. Overweight and obese adolescent humans typically remain in their respective weight categories well into adulthood, thus indicating that developmental periods are crucial for managing healthy weights (Power *et al.* 1997). As recovery from long-term positive energy balance is exceedingly difficult (Crawford *et al.* 2000; Guo *et al.* 2009), the prevention of obesity during childhood may prove essential (Eisenmann 2003).

The effects of wheel access and WD were consistent throughout our study and the body mass trajectories of each group, while only modestly different during the juvenile period, resulted in substantial differences after only 8 weeks (age ~81 days old). By the end of the study, WD increased body mass by an average of 30% and wheel running prevented weight gain by an average of 13% when all groups were pooled (Fig. 1). A comparison of the most extreme groups, in terms of genetic and environmental factors that might affect body fat (control linetype, sedentary environment, WD vs. HR linetype, wheel access and SD), shows a 49% difference in body mass (42 vs. 28 g; Fig. 1) and a 454% difference in abdominal fat mass (2.0 vs. 0.36 g; Fig. 2). These dramatic differences emphasize the importance of understanding early-life exposures, longitudinal development and how genetic and environmental influences can significantly change the shape of physiological and behavioral ontogenies.

The interaction between wheel access and diet in the analysis of food consumption deserves further attention. Under sedentary conditions, mice fed WD ingested significantly more energy per day compared with mice fed standard

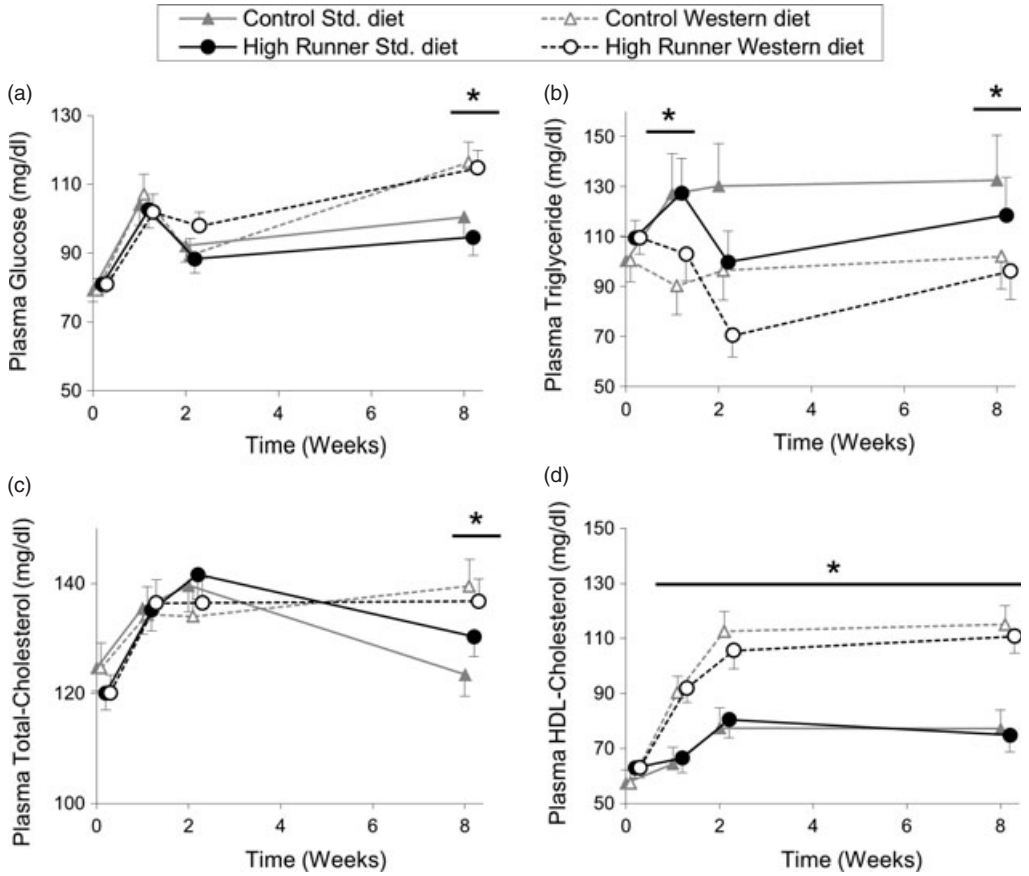

Figure 4: Results from nested ANCOVA in SAS Procedure Mixed for plasma lipid characteristics of sedentary mice only (no access to wheels). (Wheel access groups did not differ statistically from sedentary groups for any measure at any time point; Table S4.) Fasting time was a covariate in all analyses, handling time was a covariate for glucose analysis and age was an additional covariate for weeks 0, 1 and 2. Statistically significant differences ($P < 0.05$) only occurred with diet treatment, as indicated on the individual panels (*). Total sample size was 5–7 per group. Degrees of freedom are 1 and 6 for linetype (HR vs. C), wheel access, diet and their interactions.

chow (Fig. 3c). However, averaged across linetypes (HR vs. C), wheel-access mice demonstrated no change in food consumption when given a WD compared with standard chow (Fig. 3c). Stated differently, although wheel access significantly increased food consumption, a response likely driven by the higher energy demands of running animals, WD did not further increase caloric intake above and beyond that generated by wheel running. A failure to increase caloric intake on a high-fat diet is not necessarily uncharacteristic when comparing different studies or strains of animals (West & York 1998), but this result is in stark contrast with a distinctive increased intake of kJ from WD in sedentary conditions within this study. Because sedentary mice became hyperphagic on WD while wheel-access mice did not exhibit diet-dependent increases in food intake, the expression of hyperphagia is thus dependent on environmental context.

We do not know why the homeostatic mechanisms for control of body mass appear to have different 'baselines' in sedentary groups of mice when compared with those that are free to engage in voluntary exercise. The lower body mass in wheel-running groups is likely driven by increased energy demand by exercise, which is not completely matched by increases in caloric intake. Interestingly, WD does not additively increase food intake over the effects of wheel running (Fig. 3c) and this response likely accounts for the lower defended body masses in wheel-running mice fed WD compared to their sedentary counterparts (Fig. 1c). Why WD does not increase food consumption in wheel-access mice but does in sedentary animals is unclear, but perhaps the neurobiological reward for exercise is substituting for reward generated from eating a highly palatable diet. Consistent with this concept, wheel access can eliminate the preference for high-fat diet that exists in sedentary conditions in rats (Scarpace *et al.* 2010). In any case, hyperphagic behaviors will need to be interpreted through this environmentally dependent response.

The phenotypic response to a high-fat diet depends on diet composition (Dorfman *et al.* 2005), individual genetic background and additional environmental factors, such as room temperature, housing conditions or access to a wheel.

Table 1: Consistency of blood metabolite concentrations over time

			Correlations of different time points		
			0–1	1–2	2–8
All mice	Glucose	*r*	0.075	**0.231**	0.144
		P	0.368	0.007	0.071
		N	146	136	157
	Triglycerides	*r*	**0.154**	0.116	0.095
		P	0.049	0.146	0.221
		N	165	159	166
	TC	*r*	**0.187**	**0.217**	0.115
		P	0.022	0.010	0.149
		N	150	140	160
	HDL-C	*r*	**0.274**	**0.700**	**0.704**
		P	<0.001	<0.001	<0.001
		N	169	180	192
	TC/HDL-C	*r*	**0.329**	**0.600**	**0.452**
		P	<0.001	<0.001	<0.001
		N	138	141	157
Sedentary mice on standard diet	TC/HDL-C	*r*	**0.505**	**0.532**	**0.399**
		P	0.002	0.001	0.011
		N	35	35	40
Active mice on standard diet	TC/HDL-C	*r*	**0.524**	**0.377**	0.097
		P	0.002	0.022	0.557
		N	33	37	39
Sedentary mice on Western diet	TC/HDL-C	*r*	**0.420**	0.217	0.156
		P	0.012	0.217	0.349
		N	35	34	38
Active mice on Western diet	TC/HDL-C	*r*	0.205	0.282	0.281
		P	0.238	0.101	0.079
		N	35	35	40

Pearson product–moment correlations, two-tailed significance levels and sample sizes for residual plasma concentrations of glucose, triglycerides, total cholesterol, HDL-C and the ratio of total divided by HDL-C. Correlations are between blood samples from experimental weeks 0 and 1, 1 and 2 and 2 and 8. See Fig. 4 for group means. Correlations significant at $P < 0.05$ are in bold. Note that the critical value for statistical significance in the analysis of all mice ($r \sim 0.15$) is substantially lower than for analysis of the subsets ($r \sim 0.33$) because of the much smaller sample size in the latter.

In this study, WD led to an elevation of fasting plasma glucose in both HR and C mice. This increase likely represents a progression toward insulin resistance (not measured), with final glucose concentration of mice fed WD being the highest. Moreover, wheel access did not reduce the glucose levels even though wheel access nearly normalized body weight (Fig. 1c). Thus, the major contributing factor for changes in fasting glucose was primarily diet composition independent of body mass. This result suggests a meaningful non-weight-related glucose regulatory process that cannot be targeted without dietary intervention. These results are similar to some responses seen in humans, where despite having a normal body mass index some people exhibit the metabolic syndrome and are described as 'lean but metabolically unhealthy' (Durward *et al.* 2012).

Unlike fasting glucose concentrations, the diet effect for TC cannot be attributed to any adverse effects of the diet, but rather to the increase in HDL-C. Although TC also increases in humans when switched to a high-fat diet, the rise in cholesterol is due in large part to LDL-C increases. In contrast to humans, the increase of HDL-C observed in our study is a common response of mice when eating highly fatty diets (Albers *et al.* 1999; Mensink *et al.* 2003; Svenson *et al.* 2007). During caloric abundance or excess consumption of saturated lipids, many cellular factors are initially upregulated or downregulated in compensation for the dietary shift in lipid availability (Chan *et al.* 2008; Sparks *et al.* 2005). Perhaps, some of these changes account for the lack of differences in the metabolic syndrome phenotype among some strains of mice, even after long-term challenge with a high-fat diet. Svenson *et al.* (2007) studied males from 35 inbred strains, and 19 showed an appreciable increased HDL-C after 17 weeks on an atherogenic diet (15% dairy fat, 50% sucrose, 20% casein, 0.5% cholic acid and 1.0% cholesterol). In the same study, males from four strains showed a decrease in plasma TG after 17 weeks. For both sexes, the wide array of among-strain responses is mirrored in other traits composing the metabolic syndrome (Albers *et al.* 1999; Svenson *et al.* 2007) and is consistent with the results presented here.

WD in both genotypes of mice (HR and C) lowered fasting TG levels much like high-fat diet switches can do in short-term human interventions because of higher rates of lipid oxidation and lower rates of endogenous lipid release

(Schrauwen *et al.* 2000). Additionally, the genetic background of HRs did not confer any differential effect for TG or any blood metabolite. Interestingly, some mouse strains are known to be resistant to WD effects on blood metabolites (Svenson *et al.* 2007), but whatever genetic components are responsible for protective effects must not differ between HR and C animals.

Overall, the WD had pronounced detrimental effects at many levels of organismic function. The higher fasting glucose concentrations, increased fat mass and increased liver mass (which likely results from higher lipid storage) are suggestive of impaired energy homeostasis. Furthermore, in an animal model without HDL as the predominant cholesterol (e.g. the apoE knockout model; Ishibashi *et al.* 1994) the rise in TC would probably serve as an additional signal of metabolic impairment. Moreover, the correlation of the metabolic markers (Table 1) is a harbinger for future risk and suggests prevention may be an essential tool in curbing the current rise in obesity and associated metabolic diseases.

Several of our findings are relevant to understanding the complex interrelationships of genetics, energy intake, diet composition, voluntary exercise and energy balance. The effects of WD were nearly omnipresent, as almost every trait studied, at some time point, showed a significant response. Western diet was obesogenic, as it increased the mass of the abdominal fat and liver, even when adjusting for the increased caloric intake. This implies that either the diets are metabolized and/or absorbed differently or energy expenditure is affected by WD, a plausible outcome given the dramatic increase in wheel running exhibited by HR mice when eating WD (Fig. S2). Whether changes in insulation, basal metabolic rate, thermic effect of digestion or spontaneous physical activity in cages occur, we do not know, but the homeostatic regulation of body mass has changed. Access to wheels played a large role in determining body mass. Exercise prevented fat gain and helped to prevent hyperphagia on WD. Although 52 generations of selective breeding for voluntary exercise did not directly provide resistance to diet-induced obesity or other metabolic syndrome components, there are nonetheless important differences between HR mice and their non-selected control lines. However, these differences were only perceptible through interactions with diet or wheel access. These two environmental variables (diet and wheel access) also interacted to influence body mass and food consumption. These data underscore the relevance (and often unpredictable nature) of gene-by-environment and environment-by-environment interactions when studying the regulation of body mass. Finally, our demonstration of individual differences in blood lipid profiles that persist across time (Table 1; Fig. 4) bolsters the use of mouse models for longitudinal studies investigating the development of the metabolic syndrome phenotype.

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Acknowledgments

This work was supported by MSU Seed Grant to J.C.E. and NSF IOB-0543429 and IOS-1121273 to T.G. The authors declare no conflicts of interest.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1: Experimental design and sampling points for study animals. All mice were weaned at 21 days of age and placed into their respective diet and wheel groups at approximately 24 days old. Running data were recorded daily but analyzed in weekly or biweekly segments. Body mass, blood and food hopper mass were taken at the end of each 'Runday' segment at noon and the new segment started immediately thereafter. For example, Mass2 and blood sample 2 were measured on Runday 8 at noon followed by the start of Runday 9. The variable 'Ate' represents the total food consumption (grams) consumed during the indicated period. For analyses of food consumption, food mass was converted to kilojoules per day using the metabolizable energy for each diet (see *Materials and methods*).

Figure S2: Least-squares means and standard errors for wheel running for both C and HR mice on both diets averaged over the entire experiment. The HR mice ran substantially more than C mice when on standard diet, but this differential was greatly increased on Western diet (i.e. there was a highly significant linetype × diet interaction).

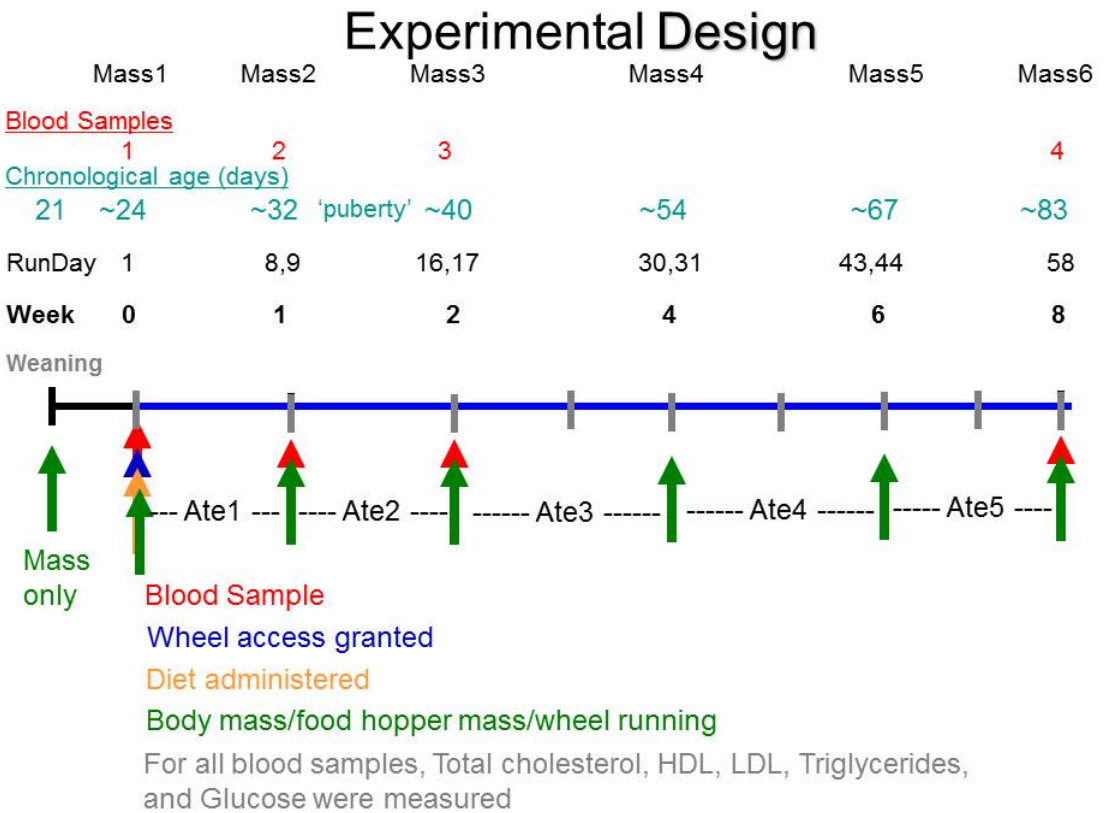
Table S1: *P* values from analysis of variance (ANOVA) or analysis of covariance (ANCOVA) for body mass.

Table S2: *P* values from ANCOVAs for organ masses with body length (nose-rump) and caloric intake as covariates.

Table S3: *F* and *P* values from ANCOVAs for log-transformed caloric intake (kJ/day).

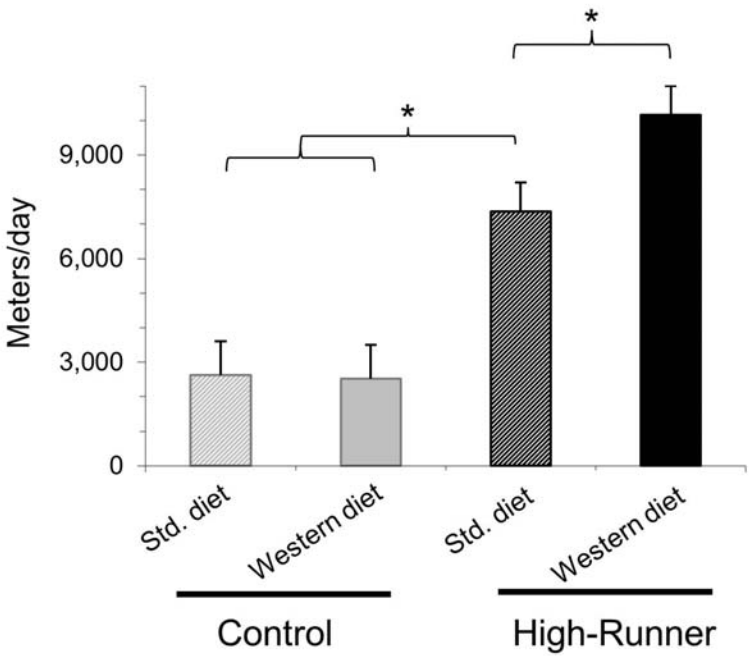
Table S4: *P* values from ANCOVA for fasting plasma metabolites.

Supplemental Information 1 Experimental design and sampling points.



Supplemental Information 1 Experimental design and sampling points for study animals. All mice were weaned at 21 days of age and placed into their respective diet and wheel groups at ~24 days old. Running data were recorded daily but analyzed in weekly or biweekly segments. Body mass, blood, and food hopper mass was taken at the end of each "Runday" segment at noon and the new segment started immediately thereafter. For example, Mass2 and blood sample 2 were measured on Runday 8 at noon followed by the start of Runday 9. The variable "Ate" represents the total food consumption (grams) consumed during the indicated period. For analyses of food consumption, food mass was converted to KJoules per day using the metabolizable energy for each diet (see Methods).

Supplemental Information 6 Wheel running



Supplemental Information 6 Least squares means and standard errors for wheel running for both C and HR mice on both diets averaged over the entire experiment. HR mice ran substantially more than C mice when on standard diet, but this differential was greatly increased on Western diet (i.e., there was a highly significant linetype X diet interaction).

Supplemental Information 2 *P* values from analysis of variance or analysis of covariance (ANCOVA) for body mass.

	Week 0		Week 1		Week 2		Week 4	Week 6
	(n = 194)		(n = 195)		(n = 198)		(n = 198)	(n = 197)
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Linetype (HR vs. C)	0.5070+	0.5483+	0.4816-	0.5388-	0.3817-	0.4640-	0.2044-	0.2176-
Wheel access (WhlAcc)			0.1298-	0.2463-	0.2615-	0.3100-	**0.0416-**	**0.0098-**
Diet			**0.0283+**	0.1102+	**0.0029+**	**0.0021+**	**0.0109+**	**0.0097+**
Mini-muscle	0.1440-	0.1284-	0.2972-	0.2857-	0.0582-	**0.0426-**	0.1066-	0.0714-
Linetype X WhlAcc			0.4916	0.5226	0.8888	0.9783	0.9834	0.8669
Linetype X Diet			0.9399	0.4241	0.9884	0.9137	0.5779	0.5129
Wheel access X Diet			0.0547	0.0634	**0.0342**	**0.0144**	**0.0038**	**0.0046**
Linetype X WhlAcc X Diet			0.8279	0.8616	0.7427	0.9913	0.9612	0.8313
Body length				**<0.0001+**		**<0.0001+**		
Age			0.0299+	0.2942+	0.4462+	0.5276+		

Supplemental Information 2 ... continued ...

		Week 8 (n = 197)		Week 8 (n = 197)	Week 8 (n = 198)	Week 8 (n = 197)
	P	*P*	*P*	(*NR covariate) *P*	([#]NR) *P*	([#]CR) *P*
Linetype (HR vs. C)	0.1715-	0.2371-	0.1739	0.2211-	0.5388-	0.3817-
Wheel access (WhlAcc)	**0.0096-**	**0.0243-**	**0.0090**	**0.0158-**	0.2463-	0.2615-
Diet	**0.0023+**	**0.0027+**	**0.0047**	**0.0021+**	0.1102+	**0.0029+**
Mini-muscle	0.1625-	0.2183-	0.2555	0.2051-	0.2857-	0.0582-
Linetype X WhlAcc	0.6990	0.3614	0.1816	0.3853	0.5226	0.8888
Linetype X Diet	0.6216	0.5915	0.6343	0.5789	0.4241	0.9884
Wheel access X Diet	**0.0183**	**0.0365**	**0.1140**	**0.0334**	0.0634	**0.0342**
Linetype X WhlAcc X Diet	0.6227	0.5152	0.1140	0.6668	0.8616	0.7427
Body length		**<0.0001+**	**0.2553**	**<0.0001+**		
Age			**<0.0001+**			
Log Caloric Intake			**0.0002**			

Supplemental Information 2 Results from nested ANOVA or ANCOVA with categorical factors of linetype (HR vs. C), absence or presence of wheel, standard or Western diet, and mini-muscle status, and covariate of body length measured from between the eyes (crown) to rump or from nose to rump (not anesthetized for either measure). Week 0 values are before mice were separated into diet and exercise groups. Week 4 and 6 body length was not measured. Age was a covariate in weeks 0, 1, and 2. + indicates direction HR > C, wheel access > sedentary, Western diet > standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (HR vs. C), wheel access, diet, and their interactions. For tests of mini muscle, d.f. are 1 and approximately 163. All *P* values are 2 tailed. Significant main effect values ($P < 0.05$) are in bold.

*Week 8 body mass was also analyzed with nose rump length as the covariate.

[#]Nose rump length and crown rump length measured at week 8 were analyzed separately as dependent variables.

Supplemental Information 3 *P* values from ANCOVAs for organ masses with body length (nose-rump) and caloric intake as covariates.

	log Fat	Triceps surae	log Ventri-cles	log Liver
	P	*P*	*P*	*P*
Linetype (HR vs. C)	0.217-	0.928-	0.915-	0.289-
Wheel access (WhlAcc)	**0.002-**	0.786+	**0.015**	**0.019-**
Diet	**<.001+**	**0.001+**	**0.002+**	**0.003+**
Mini-muscle	0.110+	**<.001-**	0.671+	0.981+
Linetype X WhlAcc	0.058	0.629	0.190	0.901
Linetype X Diet	0.611	**0.037**	0.151	0.919
Wheel access X Diet	0.239	0.483	0.198	**0.005**
Linetype X WhlAcc X Diet	0.527	0.736	0.260	0.935
Log Body length (NR)	**<.001+**	**<.001+**	**<.001+**	**<.001+**

Supplemental Information 3. Results are from analysis of covariance with categorical factors of linetype (HR vs. C), wheel access, diet, and mini-muscle status, and covariates of body length (nose to rump length, measured while anesthetized), and caloric intake N=198. Log values were analyzed except triceps surae, which had no transform. Fat is the combination of retroperitoneal + epididymal fat pad masses. + indicates direction of effect, including HR > C, wheel access > sedentary, Western diet > standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (HR vs. C), wheel access, diet, and their interactions. For tests of mini muscle, log body length, and log caloric intake degrees of freedom are 1 and 163. All *P* values are 2 tailed. Significant values ($P < 0.05$) for main effects are in bold. See Figure 2 for least square means for organ masses for all eight groups, based on the analyses that included $\log_{10}$ body length as a covariate. No statistical changes occur when caloric intake in included as an additional covariate (data not shown) except the linetype x wheel access interaction for abdominal fat mass becomes significant (p=0.039).

Supplemental Information 4 F and *P* values from ANCOVAs for log-transformed caloric intake (kJoule/day).

	Week 1 (n = 188)		Week 2 (n = 196)		Week 3 & 4 (n = 192)		Week 5 & 6 (n = 193)		Week 7 & 8 (n = 193)	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Linetype (HR vs. C)	0.58	0.4746+	0.34	0.5814+	1.44	0.2753+	6.43	**0.0444+**	2.61	0.1573+
Wheel access (WhlAcc)	0.86	0.3893-	7.69	**0.0323+**	3.41	**0.0029+**	39.89	**0.0007+**	16.12	**0.0070+**
Diet	180.40	**<0.0001+**	37.00	**0.0009+**	5.45	0.0583+	5.40	0.0592+	6.43	**0.0443+**
Mini-muscle	3.11	0.0798+	6.34	**0.0128+**	6.89	**0.0095+**	1.17	0.2817+	0.02	0.8953+
Linetype X WhlAcc	0.03	0.8618	6.80	**0.0403**	2.31	0.1797	8.04	**0.0298**	6.86	**0.0397**
Linetype X Diet	0.01	0.9195	2.69	0.1520	2.88	0.1407	1.59	0.2540	0.14	0.7253
Wheel access X Diet	11.84	**0.0138**	3.52	0.1096	6.04	**0.0492**	9.96	**0.0197**	10.07	**0.0192**
Linetype X WhlAcc X Diet	0.07	0.7935	1.88	0.2192	1.48	0.2696	3.84	0.0977	2.81	0.1444
Body length	239.28	**<0.0001+**	105.78	**<0.0001+**	9.13	**<0.0001+**	56.93	**<0.0001+**	34.38	**<0.0001+**
Age	0.01	0.9128-	1.09	0.2990-	2.55	0.1124				

Supplemental Information 4 Results from nested ANCOVA in SAS Procedure Mixed with categorical factors of linetype (HR vs. C), absence or presence of wheel, standard or Western diet, and mini-muscle status, and covariates of body mass (log transformed). + indicates direction HR > C, wheel access > sedentary, Western diet > standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (HR vs. C), wheel access, diet, and their interactions. For tests of mini muscle, log-transformed body mass, and wheel running, d.f. are 1 and approximately 158. All *P* values are 2 tailed. Significant main effect and interaction values ($P < 0.05$) are in bold.

Supplemental Information 5 *P* values from ANCOVA for fasting plasma metabolites

	Week 0	Week 1	Week 2	Week 8
	P	*P*	*P*	*P*
Glucose	(n = 175)	(n = 166)	(n = 166)	(n = 191)
Linetype (HR vs. C)	0.6780 [+]	0.7908 [-]	0.3686 [+]	0.7829 [-]
Mini-muscle	0.7626 [-]	0.2801 [+]	0.4366 [-]	0.3900 [-]
Wheel access		0.0605 [-]	0.4768 [+]	0.6077 [-]
Diet		0.5562 [+]	0.9245 [-]	**0.0059 [+]**
Linetype x WhlAcc		0.4886	0.2410	0.4456
Linetype x Diet		0.5689	0.1731	0.8074
WhlAcc x Diet		0.9964	0.1017	0.1163
Linetype x WhlAcc x Diet		0.6637	0.1315	0.6360
Triglycerides (Square root transform)	(n = 177)	(n = 185)	(n = 171)	(n = 188)
HR vs. C	0.3961 [+]	0.6480 [+]	0.2039 [-]	0.5758 [-]
Mini-muscle	0.3455 [+]	0.5482 [+]	0.4274 [+]	0.3295 [+]
Wheel access		0.6083 [-]	0.7208 [-]	0.2006 [-]
Diet		**0.0482 [-]**	0.0557 [-]	**0.0168** [-]
Linetype x WhlAcc		0.8113	0.1216	0.7565
Linetype x Diet		0.8543	0.7739	0.7730
WhlAcc x Diet		0.4374	0.1218	0.6629
Linetype x WhlAcc x Diet		0.2189	0.7691	0.4851
Cholesterol (Log)	(n = 176)	(n = 169)	(n = 167)	(n = 192)
HR vs. C	0.3718 [+]	0.8542 [-]	0.4396 [+]	0.4123 [+]

Mini-muscle	0.6579 [-]	0.4172 [-]	0.0575 [-]	0.1645 [-]
Wheel access		0.8606 [+]	0.5681 [-]	0.7161 [-]
Diet		0.6697 [+]	0.1786 [-]	**0.0469 [+]**
Linetype x WhlAcc		0.5628	0.7558	0.8057
Linetype x Diet		0.8139	0.9270	0.2320
WhlAcc x Diet		0.6847	0.9003	0.1524
Linetype x WhlAcc x Diet		0.5017	0.9833	0.6954
HDL-Cholesterol	(n = 183)	(n = 186)	(n = 195)	(n = 193)
HR vs. C	0.3438 [+]	0.9133 [-]	0.7595 [-]	0.4219 [-]
Mini-muscle	0.3155 [-]	0.7303 [-]	0.8740 [-]	0.6355 [+]
Wheel access		0.8506 [+]	0.3602 [+]	0.0558 [-]
Diet		**<0.0001 [+]**	**<0.0001 [+]**	<**0.0001** [+]
Linetype x WhlAcc		0.2995	0.7469	0.2083
Linetype x Diet		0.8920	0.4357	0.3084
WhlAcc x Diet		0.6309	0.1088	0.1203
Linetype x WhlAcc x Diet		0.9522	0.2346	0.5224
TC/HDL-Cholesterol	(n = 174)	(n = 164)	(n = 166)	(n = 185)
HR vs. C	0.2155 [-]	0.6956 [-]	0.9812 [+]	0.4466 [+]
Mini-muscle	0.2850 [+]	0.5937 [+]	0.6842 [-]	0.2944 [-]
Wheel access		0.5245 [-]	0.6408 [-]	0.2466 [+]
Diet		**0.0006 [-]**	**0.0006 [-]**	**0.0003 [-]**
Linetype x WhlAcc		0.6890	0.4857	0.3158
Linetype x Diet		0.5578	0.6477	0.9310
WhlAcc x Diet		0.3481	0.1684	0.6749
Linetype x WhlAcc x Diet		0.9239	0.4105	0.9090

Non HDL-Cholesterol (LDL+VLVL) (n = 175)		(n = 167)	(n = 167)	(n = 188)
HR vs. C	0.1819 [-]	0.7640 [-]	0.7082 [+]	0.2551 [+]
Mini-muscle	0.4654 [+]	0.4205 [-]	0.4224 [-]	0.5290 [-]
Wheel access		0.8513 [+]	0.3356 [-]	0.0929 [+]
Diet		**0.0005 [-]**	**<0.0001 [-]**	**<0.0001 [-]**
Linetype x WhlAcc		0.8393	0.8639	0.3783
Linetype x Diet		0.8509	0.5794	0.5827
WhlAcc x Diet		0.3745	0.2109	0.5550
Linetype x WhlAcc x Diet		0.9753	0.6290	0.8200

Supplemental Information 5 Results from nested ANCOVA in SAS Procedure Mixed. Fasting plasma metabolites analyzed by linetype and different environmental groups. Non HDL-C was not directly measured but calculated from (total cholesterol – HDL-C). Fasting time was a covariate in all analyses, handling time was a covariate for glucose analysis, and age was an additional covariate for weeks 0, 1, and 2 (results not shown). + indicates direction HR>C, + mini>HR normal muscle, wheel access group > sedentary, high fat > standard chow. Degrees of freedom are 1 and 6 for all tests except mini. All *P* values are 2 tailed. Significant values ($P < 0.05$) are in bold. See Figure 4 in manuscript and Supplemental Information 4 for means.