UNIVERSITY OF CALIFORNIA
RIVERSIDE

Physiological Underpinnings of High Voluntary Exercise in Selectively Bred Mice: Effects of Western Diet

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Evolution, Ecology, and Organismal Biology

by

Thomas Harris Meek IV

December 2011

Dissertation Committee:
Dr. Theodore Garland, Jr., Chairperson
Dr. Mark A. Chappell
Dr. Richard A. Cardullo

The Dissertation of Thomas Harris Meek IV is approved:

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Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

I would like to thank my dissertation committee: Dr. Theodore Garland, Jr. (chair), Dr. Mark Chappell, and Dr. Richard Cardullo. You have all contributed to my growth as a scientist, and any success I may find in this field will certainly be due, in no small way, to your mentorship. Thank you especially to Ted for always requiring the most rigorous scientific approach and for holding me to the highest standard.

Thank you to my fellow members of the Garland lab: Jessica Malisch, Scott Kelly, Rob Hannon, Chris Oufiero, Gabriel Gartner, Brooke Keeney, Wendy Acosta, Gerald Claghorn, Kevin Middleton, Heidi Schultz, and Vincent Careau. I have benefited greatly from our conversations over the years. You have all unselfishly helped me grow as a scientist, a leader, and a person. To Mart, Ann, Trynke, Arne, Chris, Kimmy, Bre, and Zack, I will always value our friendship.

Thank you to both my mother and father. To my mom, for never wavering in her belief that her children could do anything they wanted. For raising us to be curious, and giving us a gift that can never be taken away - an education. To my father who worked tirelessly so his children could have everything they needed. And for teaching us there are no rules to life. We can make the best or the worst of it. You both have served as a source of reason and inspiration.

I am also grateful to my sisters Laura and Kathy, and brother Jeff. You have always been compassionate and loving. I knew I could count on you for anything, and I

still can. You have offered me different points of view and taught me to be a better observer of the human condition.

Of course, there is the girl with flowers in her hair. Brea, I feel as though I have discovered the world with you. You have sacrificed as much as anyone and I owe you endless gratitude. You stood by me through many challenges, always patient, always kind. Finally, I can only say, I could not have taken a single step of this journey if it were not for your support, encouragement, and love.

Chapter 1 of this dissertation has been published in the *Journal of Experimental Biology* and is adapted with permission from The Company of Biologists, Ltd (full citation below):

Meek TH, Lonquich BP, Hannon RM, Garland Jr T. Endurance capacity of mice selectively bred for high voluntary wheel running. *J Exp Biol* 212: 2908–2917, 2009.

Chapter 3 of this dissertation has been published in the *International Journal of Obesity* and is adapted with permission from Nature Publishing Group (full citation below):

Meek TH, Eisenmann JC, Garland T Jr. Western diet increases wheel running in mice selectively bred for high voluntary wheel running. *Int J Obesity* 34: 960-9, 2010.

ABSTRACT OF THE DISSERTATION

Physiological Underpinnings of High Voluntary Exercise in Selectively Bred Mice: Effects of Western Diet

by

Thomas Harris Meek IV

Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, December 2011
Dr. Theodore Garland, Jr., Chairperson

Obesity is a growing health problem worldwide, particularly within the United States. The percent of both overweight and obese Americans (~2/3 combined as of surveys completed in 1999-2004) has continued to increase over the past two decades. Exercise is a cornerstone for the treatment of obesity, and understanding the regulation of energy balance in response to exercise is essential for the prevention of obesity and the metabolic syndrome.

Using lines of house mice that have been selectively bred for high voluntary wheel running (high runner [HR]) and their non-selected control lines, I have characterized changes in both ability to exercise and propensity to exercise, both of which, at least in humans, can independently impact mortality rates. The increased endurance capacity observed in the four replicate high runner lines of mice provides evidence of a genetic correlation between the ability and propensity for aerobic exercise. However, this genetic advantage does not appear to confer resistance to diet-induced

obesity or key components of the metabolic syndrome. But the elevated running behavior of HR mice does reduce susceptibility to diet induced obesity. In an 8-wek study, I found that circulating blood lipids are not affected by wheel running in either high runner or control mice, but gain of fat mass is greatly reduced by wheel access, even when animals are eating a Western diet, high in fat and with added sucrose.

I also found an unprecedented genotype-by-environment interaction. High runner mice showed a remarkable increase in wheel running when given Western diet, whereas wheel running of control mice was unaffected. Based on results from skeletal muscle enzyme assays, molecular levels of 5' adenosine monophosphate-activated protein kinase (AMPK) phosphorylation, the increase in running is likely related more to central nervous system fatigue or motivation rather than the availability of lipid substrates as an energy source.

In conclusion, my research provides further evidence of important genetic differences between the high runner lines of mice and their non-selected controls, further elucidating how athletic performance evolves. My results also demonstrate the importance of genotype-by-environmental interactions, which are likely to account for a substantial fraction of the differences among individuals for many complex phenotypes, including energy balance, obesity, and voluntary exercise. Understanding these interactions will likely prove essential for stopping and reversing the current trends in human obesity and many other complex human diseases.

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Dissertation Introduction

Obesity is a growing health problem worldwide, particularly within the United States. The percent of both overweight and obese Americans (65.7% combined) (Hedley et al., 2004) has continued to increase over the past two decades (Ogden et al., 2004). The prevalence of obesity in children has followed a similar trend, and was recently estimated at 31.5%, having risen 2.3-3.3 fold over the past 25 years (CDC, Health Statistics, National Center for Health Statistics, 2006). Consistent with the obesity epidemic is an increase in the prevalence of the metabolic syndrome, defined by the National Institute of Health (NHLBI, 1998) as the presence of three of the following five metabolic irregularities: obesity, elevated blood pressure, high resting glucose levels, high triglycerides concentrations, and low levels of high density lipoprotein cholesterol (HDL). The metabolic syndrome is considered a major risk factor for polycystic ovarian disease, nonalcoholic fatty liver disease, neurodegeneration (Biddinger et al., 2006), Type 2 diabetes (Helmrich et al., 1991), coronary heart disease, stroke (NHLBI, 1998), and cancer (Grundy and Barnett, 1990. The high rates of childhood obesity and the health consequences related to metabolic syndrome may lead to a decrease in life expectancy of future generations (Olshansky et al., 2005).

Understanding the regulation of energy balance is essential for the prevention of obesity and the metabolic syndrome. Energy balance is a complex phenomenon that encompasses many systems. The processes involved in monitoring and maintaining energy balance are affected by genes, environmental factors, and gene X environment interactions. These processes (including those of the endocrine, neural, skeletal,

muscular, digestive, and immune systems) must coordinate both short- and long-term energy balance. For example, epinephrine, insulin, and glucagon regulate blood glucose and the rate of fatty acid synthesis on a short-term (hourly) scale. Such hormones as adiponectin, leptin, ghrellin, tumor necrosis factor-α (TNF-α), and interluken-6 have recently been found to have long-term effects in a wide range of tissues (McMurray and Hackney, 2005). All of these hormones are interact with the central nervous system, and they have a variety of functions, some of which are associated with satiety, hunger, and metabolism (McMurray and Hackney, 2005). The diversity of the systems involved in regulating energy balance -- and the intricacies within each system -- demand an integrative approach for a better understanding of energy balance (Garland et al., 2011).

Ontogeny of Metabolic Disease

Since 1980, the obesity rate for adults has doubled and the rate for children and adolescents has tripled (ages 6-19) (Hedley et al., 2004). Allison et al. (1999) estimated the annual deaths due to causes related to obesity in the U.S. as 300,000. This number will likely increase as childhood obesity levels persist into adulthood (Olshansky et al., 2005), indicating that developmental periods may be crucial for managing healthy weights (Braddon et al., 1986; Yang et al., 2006). Moreover, changes in population health due to long-term illnesses are mirrored by concomitant economic changes, which result in an increasing burden being placed on the health care system (Wang and Dietz, 2002).

Given the upward trend in childhood obesity, the next generation is going to be more physically unfit and greater numbers of individuals will be overweight or obese

during adulthood, if corrective action is not taken (Ogden et al., 2004; CDC, Health Statistics, National Center for Health Statistics, 2006). Based on data from 1999-2004, prevalence of obesity and overweight individuals in the United States are 15% and 40% respectively, in 16-19 year olds (Ogden et al., 2002). Thus, research focusing on the causes and consequences of childhood and adolescent obesity and the emergence of the metabolic syndrome is extremely important.

Although several studies have examined childhood obesity and the biomarkers of adult obesity, they have mostly been cross-sectional in nature and have not considered the ontogeny (during childhood or adulthood) of the metabolic syndrome. Single time point cross-sectional studies are limited because they do not consider the progression of the disease. Knowing what individuals are at risk before they show clear symptoms would provide a time period when prevention rather than treatment could be employed, which may be the key to reversing the current trends in obesity. Due to the time-consuming nature of longitudinal studies in human beings, the changes in the biomarkers and the independent and combined roles of genetics and environment on the development of the metabolic syndrome remain obscure. Additionally, it is essential to measure blood characteristics associated with the metabolic syndrome, instead of using body mass as the only indicator. Body mass, typically converted to the body mass index (BMI), is an often used measure of excess body fat in humans. BMI is one clear risk factor in the metabolic syndrome, but fat mass and risk factors are not necessarily the same. A substantial number of heavy individuals are classified with a low cardiovascular risk factor, and

many lean people are at high risk of a cardiovascular event (Wildman et al., 2004; Lee et al., 2005a; Eisenmann, 2007).

Role of Exercise

Exercise is one factor affecting energy balance (Garland et al., 2011) and is a cornerstone for the treatment of obesity (Thorburn and Proietto, 2000). Exercise is associated with many health-related benefits, and many functions have been shown to improve in response to exercise training (e.g., Rodnick et al., 1989; Swallow et al., 1998b; Zhan et al., 1999; Houle-Leroy et al., 2000; Allen et al., 2001; Rose et al., 2007). The metabolic alterations associated with exercise have been linked to decreases in fat mass, low density lipoprotein (LDL) (Adult Treatment Panel III), blood pressure (Must et al., 1999), insulin resistance, and an increase in high density lipoprotein (HDL) (Adult Treatment Panel III). Thus, physical activity helps correct every component of the metabolic syndrome. Exercise also improves many of the deleterious effects obesity has on the respiratory system, including increased work of breathing, reductions in lung volume, airflow obstruction, hypoxemia, and dyspnea (Shore, 2011). In addition, exercise-induced metabolic alterations have been associated with the prevention of Type II diabetes melititis (Helmrich et al., 1991), heart disease, stroke (NHLBI, 1998), cancer (Grundy and Barnett, 1990), and depression (Lobstein et al., 1983). Understanding what genetic and environmental factors account for individual and population differences in voluntary exercise is important for stopping and reversing the current trends in human obesity and associated comorbidities.

Many studies which aim to investigate energy imbalance and obesity using rodent models examine animals confined to their home cages (Svenson et al., 2007). Although these studies have contributed much information on the underpinnings of the metabolic syndrome, physical inactivity caused by confinement in a relatively small cage predisposes the animals to increased risk of multiple chronic diseases and may not serve as the most appropriate reference group (Booth and Lees, 2007).

Voluntary wheel running can help maintain energy balance and prevent obesity, non-alcoholic fatty liver disease, and type 2 diabetes in obese rodent models (Shima et al., 1993, Bi et al., 2005, Morris et al., 2008, Rector et al., 2008, Simoncic et al., 2008, Haskell-Luevano et al., 2009). These genetic or environmental-dependent responses suggest the lack of an appropriate outlet for natural, self-rewarding behavior, such as wheel running, is an experimental design "flaw" that can lead to misinterpretation of results and/or to the presence of inappropriate phenotypes. When not interpreted in the context of a built, atypical environment for a given genome (e.g., a cage for a mouse), conclusions can be tenuous and inconsistent. In other words, the mechanisms for weight gain in a mouse restricted to a sedentary environment may be distinct from those of a mouse who chooses to be inactive even when it has access to a wheel.

A similar scenario has recently been observed in human populations. Adults who are homozygous for the *fat mass and obesity-associated* (FTO) allele (rs9939609 AA) weigh about 3 kilograms more, on average, and have a 1.67-fold increased odds of developing obesity compared to non-carriers (Frayling et al., 2007). This increase weight and rate of obesity are either not observed or greatly attenuated in homozygous

individuals who meet the daily physical activity recommendations (Andreasen et al., 2008, Ruiz et al., 2010; for other fat-mass related polymorphsims, see Rampersaud et al., 2008, Vimaleswaran et al., 2009). Therefore, FTOs predisposition towards obesity only appears to exist when-physical activity is insufficient (Booth et al., in press).

Evolution and Energy Balance

The various processes and systems involved in maintaining energy balance can be altered over evolutionary time, as well as modified during an organism's life through phenotypic plasticity (Garland and Kelly, 2006; Piersma and Van Gils, 2011). For example, in human beings, the so-called "thrifty genotype" refers to a condition, often found in small, indigenous, isolated populations (e.g., Pima Indians; Papua New Guinea islanders), where exposure to conventional western food sources and life styles has been minimal or completely absent. Within these populations, digestive efficiency and nutrient absorption are very high, and the excess nutrients are stored and well "protected" within adipose tissue, traits that have enabled individuals to survive when resources were scarce (Neel, 1962). The combination of the thrifty genotype and the western diet, which is typically composed of high carbohydrates and high saturated/trans fats, results in elevated rates of obesity and health-related problems (Neel, 1962; Neel et al., 1998; Booth et al., 2002a; 2002b). These populations, such as the Pima Indians, when exposed to western foods appear to lack any compensatory mechanisms to offset the dramatic increase in caloric intake (Ravussin et al., 1994; Pratley, 1998). Mammals, such as the spiny mouse (*Acomys cahirinus*) and Egyptian sand rat (*Psammomys obesus*), have also been reported to exhibit the thrifty genotype. These animals show no detected type II

diabetes in wild populations, but once in captivity with *ad libitum* food they express the disease (Wendorf and Goldfine, 1991).

Exercise, Lipids, and Fatigue

Although exercise has many health-related benefits, the ability to sustain any type of exercise depends on many factors, including prior training, cellular macronutrient availability, intensity, and duration of activity. The limitation(s) to endurance exercise performance depend on these and other factors, but the depletion of energy substrates is a commonly considered cause of fatigue. The ingesting, loading or utilization of particular dietary macronutrients is important in maintaining muscular activity and delaying fatigue during exercise. The alteration in the rate of use or balance of carbohydrates and lipids, often through training or diet, can lead to increased performance. For instance, Simi et al. (1991) found high-fat diet acts in an additive fashion with endurance training, leading to increased VO_{2max} (maximal oxygen consumption during forced exercise) and submaximal endurance in rats. High-fat diet can serve to increase the duration of moderate physical activity by sparing glycogen, the depletion of which is considered to be a causal factor in fatigue (Hickson et al., 1977).

Whole-organism energy balance is ultimately determined by the cumulative activity of cells. Within each cell, energy is constantly being used and re-directed into various pathways. In 1988, AMP-Kinase (AMPK) was first identified and has since been called the cellular fuel gage (Hardie et al., 1989; Carling, 2004; Hardie, 2008). AMPK or a homologue has been found in all eukaryotic species so far examined (Carling, 2004). One way AMPK is activated is by low levels of the cellular AMP/ATP ratio, which

reflect low cellular energy levels. The priming of AMPK by cellular AMP allows for serine/threonine kinase 11 (LKB1) or calcium/calmodulin kinase to phosphorylate AMPK, resulting in activation of energy producing processes and the cessation of energy-consuming processes throughout the cell (Hardie et al., 2003; Rutter et al., 2003; Carling 2004; Hardie and Sakamoto 2006).

One of the major characteristics of AMPK is its ability to coordinate the regulation of fatty acid synthesis and oxidation. An immediate downstream target of AMPK is acetyl-CoA carboxylase (ACC). ACC catalyzes the irreversible carboxylation of acetyl-CoA to malonyl-CoA. An increase in AMPK-P levels is caused by low levels of cellular energy (high AMP/ATP ratio) and results in the phosphorylation of ACC, which inhibits enzyme activity (Hardie et al., 2003; Lee et al., 2005b). Low ACC activity then leads to decreases in malonyl-CoA levels (the first committed step of fatty acid synthesis), which, when present, inhibits carnitine palmitoyltransferase-1 (CPT-1), the rate limiting step in fatty acid oxidation (Hardie et al., 2003; Rutter et al., 2003; Carling 2004; Lee et al., 2005b). CPT-1 is found in the outer walls of mitochondria and binds acyl-CoA with carnitine, forming acyl-carnitine, which is then transported into the mitochondrial matrix via CPT-II. Through AMPK phosphorylation there is an inhibitory effect of ACC, resulting in decreased levels of malyonly-CoA and an increase in mitochondrial β-oxidation (Hardie and Sakamoto, 2006; Treebak and Wojtaszewski, 2008). Thus, the regulation of energy balance by AMPK is effective because it simultaneously coordinates many cellular pathways involved in both energy production and use.

AMPK was first thought to only respond to direct cellular signals acting as a second messenger. More recently, hormones such as leptin and adiponectin have been shown to stimulate AMPK (Yamauchi et al., 2002; Minokoshi et al., 2002; Minokoshi et al., 2004; Lafontan and Viguerie, 2006; Marette, 2008), while resistin has been shown to inhibit it (Banerjee et al., 2004). These hormones have also been implicated in the regulation of energy balance (McMurray and Hackney, 2005). The role these processes play in determining fatigue and the training response to exercise (Narkar et al., 2008) may potentially influence body weight, body composition, and the presence of certain disease states related to lipotoxicity.

Model of Study

Much of our knowledge about the metabolic syndrome comes from studies using rodents (Peters et al., 2007). For instance, the specific roles of such hormones as leptin started to become apparent with the discovery of the mutation of the obese gene in mice (Castracane and Henson, 2006). More recently, the involvement of the skeletal system in energy metabolism has come into focus through the use of knock-out mice, as Lee et al. (2007) report the skeletal system as a source of whole-body energy regulation through its influences on the endocrine system. Similar studies with mice have elucidated the importance of muscle myokines (Pedersen, 2011). Laboratory mice have been shown to be good models for addressing human health concerns because they mimic much of the genetic and phenotypic variation present in human populations (Svenson et al., 2007). Specifically, the mouse is not only a very well-studied organism, but it shares many of the diseases humans suffer, including diabetes, atherosclerosis, heart disease, cancer,

anemia, hypertension, obesity, asthma, and many others (Peters et al., 2007). Much of our knowledge about the metabolic syndrome comes from studies using rodents (Peters et al., 2007).

The animals used in this dissertation are mice (*Mus domesticus*) from an ongoing artificial selection experiment for high voluntary wheel running (Swallow et al., 1998a; Garland, 2003; Rhodes et al., 2005; Middleton et al., 2008; Swallow et al., 2009). The original population was the outbred ICR strain from Harlan-Sprague-Dawley (Indianapolis, Indiana, USA). After two generations of random mating by Dr. Garland and colleagues, mice were randomly separated into eight closed lines. Four lines have been selected for high voluntary wheel running (HR lines) and the other four lines have been bred without regard to running, thus serving as controls (C lines). To determine breeders, approximately 600 mice each generation are housed (at 6-8 weeks of age) in standard cages with a 7.7-cm-diameter hole and a 5.5-cm-long stainless steel tube connecting the housing cage to a large (rat-sized) running wheel (1.12 m circumference). Therefore, mice may voluntarily choose to stay in their cage or run in the wheel. All revolutions are recorded using photocell counters linked to an automated computer system. High runner-line mice with the highest mean distance run on nights five and six of the six-day test are picked as breeders for the next generation. The control lines are also given wheel access to quantify running, but breeders are chosen randomly from within families. In all cases, sibling mating is disallowed. Food and water are always available *ad libitum*, and photoperiod is 12L:12D.

As of August 2011, these 8 lines have been studied for almost 65 generations. By generation 16, the HR mice ran ~170% more than C mice; interestingly, no further increases in running by the HR lines appear to have occurred, despite continued selective breeding. The difference between HR and C lines occurs primarily through an increase in the speed of wheel running in the HR lines, especially for females (Koteja et al., 1999; Garland et al., 2011a). The changes in wheel running have been associated with correlated changes at multiple levels of biological organization. At the whole-organism level, the HR lines have an increased maximal oxygen consumption during forced treadmill exercise (Swallow et al., 1998b; Rezende et al., 2006a,b; Kolb et al., 2010) and a reduced body mass (Swallow et al., 1999), while being significantly leaner despite eating more than their control counterparts (Swallow et al., 2001). Behaviorally, the HR lines differ in certain aspects of open-field locomotion (Bronikowski et al., 2001), thermoregulatory nest-building (Carter et al., 2000), and predatory aggression (Gammie et al., 2003).

Examination of sub-organismal traits reveals significant increases in kidney mass, hematocrit, hemoglobin levels, and heart ventricle mass in one or both sexes from the HR lines (Swallow et al., 2005; S. A. Kelly and T. Garland, Jr., unpub. data). When provided chronic wheel access, HR line females demonstrated greater plasticity than C line females in some organ masses (Swallow et al., 2005) and in citrate synthase and hexokinase activities in mixed hind limb muscles (Houle-Leroy et al., 2000). Alterations in the skeleton have also been observed: larger femoral heads, thicker tibiafibulas, and more symmetrical hind limb bones (Garland and Freeman, 2005; Kelly et al., 2006).

Additionally, the HR lines have an increased insulin-stimulated glucose uptake in extensor digitorum muscle (Dumke et al., 2001) and an increase in GLUT-4 transporter in gastrocnemius muscle (greater plasticity after five days of access to wheels: Gomes et al., 2009).

Several hormonal differences have been observed in HR and C mice measured at rest. HR lines of mice have higher corticosterone levels (Malisch, et al., 2007), lower circulating leptin levels even after accounting for their lower body fat (Girard et al., 2007), and higher circulating adiponectin levels (Vaanholt et al., 2007b; see also Vaanholt et al., 2008). These hormones are known to be important in the regulation of energy balance (McMurray and Hackney, 2005), and may also affect activity levels directly.

Within two of the four HR lines, a recessive Mendelian trait called the mini-muscle phenotype has increased in frequency and results in a 50% mass reduction in the triceps surae muscle (gastrocnemius, soleus, plantaris) (Garland et al., 2002), more specifically the medial and lateral gastrocnemius (Syme et al., 2005). The mini-muscle individuals exhibit twice the oxidative capacity per gram of tissue in mixed hindlimb muscle as compared with wild-type animals (Houle-Leroy et al., 2003). Additionally, mini-muscle mice have elevated heat shock protein (HSP72) expression in muscle (Belter et al., 2004), increased glycogen content in gastrocnemius (Gomes et al., 2009), altered myosin heavy chain composition (fewer type IIb fibers) (Guderley et al., 2005), a reduced force per cross sectional area in gastrocnemius (Syme et al., 2005), and elevated whole-

animal VO_2max in hypoxia (Rezende et al., 2006a). These traits could lead to differences in cellular fuel balance, energy regulation, and obesity phenotypes.

With regard to the motivational component of the HR phenotype, previous studies on the neurobiology of HR mice have implicated the dopaminergic (Rhodes et al., 2001; Rhodes and Garland, 2003; Li et al., 2004; Rhodes et al., 2005) and endocannabinoid (Keeney et al., 2008) systems as important components of the increased wheel-running capability, at least for females. This inherent motivational component, along with the performance-associated components (e.g., morphology, physiology, biochemistry), creates a complex phenotype comprising both motivation and ability (see also Garland et al., 2011b).

Although the dopaminergic system is altered in HR mice, other pharmacological studies have not demonstrated any changes in the serotinergic (Rhodes et al., 2001) or the opioidergic (Li et al., 2004) systems. In regards to the dopaminergic changes, Rhodes and Garland (2003) found that HR mice have a reduced D1 receptor functionality. Rhodes and Garland (2003, their table 1) gave mice three doses of the D1 receptor antagonist SCH-23390. Although wheel running in both HR and C lines was depressed following the administration of this antagonist, when compared to C mice, HR mice were less sensitive to the effects of both the middle and high doses. This differential response was not observed with the D2 receptor antagonist raclopride at any dose.

Using immunocytochemical quantification of the transcription factor cFos, which assays neural activation, Rhodes et al. (2003) examined brain activation after wheel running was prevented. The removal of wheel access resulted in differential activation of

some the brain areas between the linetypes, included the caudate-putamen complex, lateral hypothalamus, prefrontal cortex (PFC), medial frontal cortex (MFC), nucleus accumbens (NA), piriform cortex, and sensory cortex. Many of these areas (e.g., MFC, PFC, NA) have been previously implicated in reward-stimulated behaviors (Kelley and Berridge, 2002), and Rhodes et al. (2003) concluded that these brain regions may underlie increased motivation for exercise in the HR lines (see also Rhodes and Kawecki, 2009).

Many of the evolved differences in the HR lines of mice could contribute to different susceptibilities to obesity and metabolic syndrome components, including increased voluntary wheel running, decreased body fat, altered dopamine functioning in reward centers of the brain (Rhodes et al., 2005; Geiger et al., 2008; Keeney et al., 2008), and altered circulating hormone levels. Considerable effort has been expended to create animal models with a propensity to develop obesity, the metabolic syndrome, and/or cardiovascular disease. Much less effort has been aimed at developing models resistant to adverse effects of a sedentary lifestyle or a high-fat diet. The HR and control lines of mice may allow for an integrative view of how metabolic syndrome resistance exists in a polygenic model. Animals resistant to obesity and the metabolic syndrome could better our understanding of what mechanisms contribute to this phenotype. The selective breeding that produced high runner mice is no doubt different than the selection human populations have experienced. But, identifying gene X environmental interactions in human populations is difficult because environments often cannot be controlled. Furthermore, it is essential to understand the interacting role of genes and environment.

The director of the National Institutes of Health, Francis Collins, wrote in *Science* (Schwartz and Collins, 2007),

> "…the best opportunity to reduce risk in genetically susceptible people for the foreseeable future will not be to re-engineer their genes, but to modify their environment. We need to understand how genetic factors and environmental exposures interact in individuals to alter normal biological function and to affect the risk of disease development."

Using genetically HR lines of mice provides a useful way to uncover these interactions and perhaps eventually identify candidate genes involved in increased activity, which may offer insight into what genetic mechanisms are operating in human populations to produce predisposition or resistance to obesity (Kelly et al., 2010a,b, 2011).

Dissertation Research Summary

My first chapter showed that endurance, a measure of aerobic ability, was significantly elevated in mice from the HR lines. Chapter 2 investigated whether superior aerobic ability conferred decreased susceptibility to obesity, and factors associated metabolic syndrome. Western diet influenced nearly every trait studied and was obesogenic. Access to wheels played a large role in energy balance, as evidenced by lineytpe-dependent effects on caloric intake, body fat, and wheel running. Exercise prevented fat mass gain and helped to prevent hyperphagia particularly, in Western-diet groups. Linetype did not directly affect any health associated metabolic marker (TG, TC, HDL, glucose). Some differences were only perceptible through interactive effects with diet or wheel access. These data underscore the relevance (and often unpredictable

nature) of gene-by-environment or environment-by-environment interactions when studying body weight regulation.

Since generation 16, wheel running in the HR mice has plateaued, and further increases in wheel running, despite continued selection, have not occurred. The reason for this plateau is unclear, but could involve counterpoising natural selection in the laboratory, genetic correlations with other traits under selection (e.g., aspects of the life history), diminished additive genetic variance for wheel-running traits, or physiological factors involved with reward or performance ability. Knowing HR mice have reduced fat mass, and stores of liver and muscle glycogen remaining during and after wheel running (Gomes et al., 2009), in chapter 3 I administered Western diet to determine if lipids could increase wheel running or if HR mice were resistant to the diet's adverse effects.

Western diet did increase wheel running in HR mice, but the reason for the increase is unclear. The stimulatory effect of Western diet on wheel running in HR mice could improve maximal performance, although in chapter 4 maximum mass-specific enzymatic rates of triceps surae muscle were not differentially higher in HR mice compared to C in Western diet groups. Perhaps low grade flux of fatty acids has increased in HR mice without change to maximum ability, or perhaps Western diet directly affect motivation. Lipids are known to have direct and indirect effects on the brain. Leptin and/or adiponectin, both released from adipocytes, help coordinate whole-body energy balance, of which locomotion can be a major component (Garland et al., 2011b). As compared with mice from the non-HR control lines, mice from the HR lines are known to have changes in dopamine and endocannabinoid signaling, as well as low

leptin and high adiponectin even after taking into account differences in body adiposity (see above). All these changes may be involved in the unique response HR mice had to high fat diet.

In chapter 5, I found that administration of recombinant mouse leptin increased wheel running only in mice from HR lines. Several lines of evidence suggests a linkage between voluntary running, diet, and leptin. Unlike all pharmacological agents thus far tested, Western diet (WD) substantially increases wheel running in our HR mice, and leptin concentrations increased nearly 5-fold after only 18 days on WD. Additionally, acute leptin injections also increased wheel running in HR mice. However, we did not observe a time X diet interaction in analysis of HR mice, so it is difficult to conclude leptin injections differentially affected wheel running in the different diet groups. Given the nature of our findings, more data are needed to resolve the discrepancy and establish if HR mice on WD respond the same as standard-fed HR mice. The use of a leptin antagonist would help clarify the issue, and provide evidence as to what, if any, leptin-independent effects WD has.

Results of my dissertation research offer insight into the physiological correlates and processes underlying high levels of locomotor activity. The present results make clear that genetic selection history has a large impact on such complex traits as locomotor behavior, susceptibility to weight gain, and possibly central nervous system reward generated from exercise or eating. A better understanding of these traits is imperative for addressing obesity and the development of the metabolic syndrome. Moreover, our results emphasize the importance of considering gene-by-environment interactions when

studying the limits to sustained, relatively high-speed, voluntary locomotion. Finally, results of this work may be relevant to understanding how animals in nature respond to changes in the selective regime that affects locomotor behavior (Feder et al., 2010).

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Chapter 1

Endurance capacity of mice selectively bred for high voluntary wheel running

Thomas H. Meek[1], Brian P. Lonquich[1], Robert M. Hannon[1], and Theodore Garland Jr.[1]

[1]University of California, Riverside, Riverside, CA 92521, USA

Abstract

Mice from four lines bred for high voluntary wheel activity run ~3-fold more revolutions/day and have elevated maximal oxygen consumption during forced treadmill exercise, as compared with four unselected control (C) lines. We hypothesized that these High Runner (HR) lines would have greater treadmill endurance-running capacity. Ninety six mice from generation 49 were familiarized with running on a motorized treadmill for 3 days. On days 4 and 5, mice were given an incremental-speed test (starting at 20 m/min, increased 1.5 m/min every 2 min) and endurance was measured as total time or distance run to exhaustion. Blood samples were taken to measure glucose and lactate at rest during the photophase, during peak nightly wheel running, and immediately following the second endurance test. Individual differences in endurance time were highly repeatable between days ($r = 0.79$), and mice tended to run longer on the second day (paired t-test, $P < 0.0001$). Blood glucose following the treadmill test was low for all animals (~53 mg/dl) and lactate was high (~6.5 mM/l), suggesting that exhaustion occurred. The HR lines had significantly higher endurance than C (1-tailed $P < 0.05$), whether or not body mass was used as a covariate in the analysis. The relation between line means for wheel running and treadmill endurance differed between the sexes, reinforcing previous studies that indicate sex-specific responses to selective breeding. HR mice appear to have higher endurance capacity than reported in the literature for inbred strains of mice or transgenics intended to enhance endurance.

Introduction

Locomotion is vital for such activities as obtaining food, escaping from predators, and locating or courting mates (e.g., see Djawdan and Garland, 1988; Garland et al., 1988; Irschick and Garland, 2001; Irschick and Le Galliard, 2008; Le Galliard and Ferriere, 2008). One aspect of locomotion that is receiving increasing attention is the correlates or determinants of individual or interspecific variation in voluntary activity levels (e.g., see Thorburn and Proietto, 2000; Miles et al., 2007). Both within and among species, variation in activity levels may reflect underlying differences in "motivation" for being active as well as physical abilities to engage in locomotion of particular intensities or for certain durations. Beyond this, activity levels may be influenced by the external environment, including physical (e.g., temperature), ecological (e.g., food availability, predator abundance), and societal factors.

Studies examining the relation between endurance capacity and voluntary activity level provide mixed results. For example, among species of lizards, daily movement distance measured in the field is positively correlated with treadmill endurance capacity (Garland, 1999). Within one population of the lizard *Lacerta vivipara*, individuals with a low endurance at birth tended to have reduced activity in the field, reduced growth rate, and high parasite load, but experienced relatively low predation risk as assessed by tail losses, whereas individuals with high endurance had high activity and growth rates, low parasite load, and high incidence of broken tails (Clobert et al., 2000). Studies of individual variation in rats (Lambert et al., 1996) and mice (Friedman et al., 1992) found no statistically significant correlation between voluntary wheel running and treadmill

endurance or maximal oxygen consumption ($\dot{V}O_2max$). Among six inbred mouse strains, Lerman et al. (2002) found a positive but statistically non-significant correlation between treadmill endurance and voluntary wheel-running speed. Lightfoot et al. (2004) compared wheel running and treadmill endurance (Lightfoot et al., 2001) of 10 inbred mouse strains and concluded that "the two strain distribution patterns are not concordant and thus implicate different genetic contributions to these two phenotypes." Conversely, two lines of rats differentially selected for high and low treadmill endurance show respective high and low voluntary wheel running (Waters et al., 2008).

We have used an experimental evolution approach (e.g., Garland and Kelly, 2006; Garland and Rose, 2009) with laboratory house mice to study the correlated evolution of motivation and ability during selective breeding for high daily activity levels, as measured by voluntary wheel-running behaviour on days 5 and 6 of a 6-day period of wheel access (Swallow et al., 1998a). Individuals from the four replicate High Runner (HR) lines run approximately three times as many revolutions per day as compared with those from four non-selected Control (C) lines (e.g., Girard et al., 2001; Garland, 2003; Keeney et al., 2008; Gomes et al., 2009), and also exhibit elevated home-cage activity when housed without wheel access (Malisch et al., 2008, 2009). Previous studies have shown behavioural and neurobiological differences between the HR and C lines that appear to indicate altered motivation for wheel running in the HR lines (e.g., see Koteja et al., 1999b; Rhodes et al., 2003; Rhodes et al., 2005; Belke and Garland, 2007).

With respect to performance abilities, mice from the HR lines exhibit higher

maximal oxygen consumption when measured during forced treadmill exercise ($\dot{V}O_2max$: Swallow et al., 1998b [males from generation 10, +6% on a mass-adjusted basis]; Rezende et al., 2006b [males from generation 32, +18%]; Rezende et al., 2006a [females from generation 36, +13%]) and differences in various sub-organismal traits potentially associated with exercise performance, including increased hindlimb bone symmetry and larger femoral heads (Garland and Freeman, 2005; Kelly et al., 2006), increased insulin-stimulated glucose uptake by isolated extensor digitorum longus muscle (Dumke et al., 2001), and more GLUT4 in the gastrocnemius after five days of wheel access (Gomes et al., 2009). In addition, blood hemoglobin concentration was a positive predictor of $\dot{V}O_2max$ in the HR lines, but a negative predictor in the C lines (Rezende et al., 2006c). However, direct measurements of endurance-running ability have not yet been performed.

The purpose of the present study was to test the hypothesis that HR mice have greater endurance than C in a graded-speed treadmill test (e.g., Lerman et al., 2002; Haubold et al., 2003). We also measured blood glucose and lactate concentrations at rest, during voluntary wheel running, and at the end of the endurance test. We hypothesized that wheel running would increase blood lactate concentrations relative to those at rest, and that concentrations after the endurance test would be even higher. We also hypothesized that blood glucose concentrations would be decreased following the endurance trial as compared with values at rest. In human beings, endurance-trained subjects exhibit lower lactate values after exhaustive exercise (Phillips, 2006). Even

without training (wheel access or forced exercise), HR mice exhibit several changes similar to those observed in trained athletes (or "athletic" species). Some of these changes include increased aerobic capacity (Swallow et al., 1998b; Rezende et al., 2006a; Rezende et al., 2006b) and increased insulin-stimulated glucose uptake in some hind limb muscles (Dumke et al., 2001). Therefore, we further hypothesized that HR mice would show a more moderate increase in lactate concentrations after the endurance test, as compared with C mice. Both sexes were measured because sex differences have been demonstrated for wheel-running distance and speed (Swallow et al., 1998a; Koteja et al., 1999a,b; Houle-Leroy et al., 2000; Swallow et al., 2001; Garland et al., 2002; Garland, 2003; Malisch et al., 2009; Rezende et al., 2006b; Rezende et al., 2009), such exercise-relevant subordinate traits as citrate synthase and hexokinase activities in mixed hind limb muscles (Houle-Leroy et al., 2000), the home-cage activity response to a high-fat diet (Vaanholt et al., 2008), and the wheel-running response to rimonabant, a selective endocannabinoid receptor antagonist (Keeney et al., 2008). Thus, motivation and/or ability for endurance exercise might also differ between the sexes.

A notable and unexpected feature of the selection experiment has been the increase in frequency in two of the four HR lines of a small-muscle phenotype, termed mini-muscle (Garland et al., 2002). This phenotype exhibits an approximately 50% reduction in hindlimb muscle mass. Pleiotropic effects of this Mendelian recessive allele include a doubling of mass-specific aerobic capacity and hexokinase activity (Houle-Leroy et al., 2003; Rezende et al., 2006c), alterations in muscle fiber type composition (fewer type IIb fibers) (Guderley et al., 2006; Bilodeau et al., 2009) and contractile

properties (Syme et al., 2005), increased myoglobin concentration and capillarity in medial gastrocnemius (Rezende et al., 2006c; Wong et al., 2009), increased glycogen concentration in gastrocnemius (Gomes et al., 2009), and increased heart ventricle mass (Garland et al., 2002; Swallow et al., 2005). Mini-muscle individuals exhibit elevated mass-adjusted $\dot{V}O_2max$ in hypoxia (Rezende et al., 2006a; but not in normoxia or hyperoxia), run faster on wheels, and run more total revolutions/day under certain conditions (Kelly et al., 2006; Gomes et al., 2009). Although the underlying gene has not yet been identified, it is known to act as a Mendelian recessive and to lie in a 2.6335-Mb interval on chromosome MMU11 (Hannon et al., 2008; Hartmann et al., 2008). Consequently, given the various unique characteristics of mice with the mini-muscle phenotype, we also compared endurance and other traits of mini-muscle individuals with HR counterparts lacking the mini-muscle phenotype.

Materials and methods

Experimental animals

Mice were sampled from the 49th generation of an artificial selection experiment for high voluntary wheel running (Swallow et al., 1998a; Garland, 2003; Swallow et al., 2009). The original progenitors of the selection experiment were outbred Hsd:ICR house mice (*Mus domesticus*). After two generations of random mating, 10 pairs of mice were used to create each of eight closed lines. Four are bred for high running on wheels (HR lines) and four are bred without regard to how much they run, thus serving as control (C

lines). For each generation, when the mice reach 6-8 weeks of age they are housed individually in standard cages (27 X 17 X 12.5 cm) attached to Wahman-type activity wheels (1.12 m circumference, 35.7 cm diameter, 10-cm-wide running surface: http://www.biology.ucr.edu/people/faculty/Garland/Mice_on_Running_Wheels_by_Ted_Garland.jpg). Wheels are interfaced to a computer and revolutions are recorded in 1-minute intervals, continuously for 6 days; the selection criterion is the number of revolutions run on days five plus six. Within each HR family, the highest-running male and female are chosen as breeders to produce the next generation. Within C families, a male and female are chosen at random. Sibling matings are disallowed in all lines.

Ninety six mice from generation 49 (half male, half female) were weaned at 21 days of age, then housed randomly in groups of 4/cage. As in the selection routine, all mice had water and food [Harlan Teklad Laboratory Rodent Diet (W)-8604] available ad libitum. Room temperature was ~73° Fahrenheit and photoperiod was 12:12, with lights on at 0700.

Measurement chronology, wheel testing, and endurance protocol

At an average of 72 days of age (SD = 8.5, min = 58, max = 86), mice were given wheel access for six days in accordance with the routine selection protocol. After downloading of wheel data at the end of day six, mice were allowed to remain with wheel access. On the seventh night of wheel access, blood samples were taken two hours after lights-off, which corresponds to peak wheel-running activity (Girard et al., 2001; Rhodes et al., 2003; Malisch et al., 2008). Over the following three days, while still allowed

wheel access, mice were trained to run on a two-lane, motorized treadmill for 15 min/day. Treadmill speed for each training day was 10, 14 and 18 m/min, respectively. The treadmill was set to an incline of 25^{o}, which has been shown to elicit maximum O_2 consumption in mice (Kemi et al., 2002; see also Rezende et al. 2006a). The chamber in which the mouse ran was made of clear Plexiglas with dimensions 6.5 cm wide, 12.5 cm high, and 44 cm long. After the 3-day training period, on each of the following two days, mice were tested using a graded exercise endurance test, following the protocol of Lerman et al. (2002) and Haubold et al. (2003). Starting belt speed was 20 m/min and speed was increased 1.5 m/min every 2 minutes. An air gun and an electrical grid with a mild current (adjustable-amperage, 0-12 mA) were placed at the back of the treadmill to provide motivation. Mice were judged to be exhausted when they showed an inability to maintain workload and remained on the electrical grid for four seconds (Lerman et al., 2002; Haubold et al. 2003). For both trial days, values were excluded from statistical analyses if mice were judged to be non-cooperative. Blood samples (see below) were taken after exhaustion on the second day (Djawdan, 1993). Five days after the second treadmill test, resting blood samples were obtained during the photophase.

Although mice are active primarily at night and circadian variation in performance is known to occur in some mammals (e.g., Colquhoun, 1981), for logistical reasons we tested endurance during the photophase. So far as we are aware, possible circadian variation in running endurance of laboratory mice has not been studied. However, in a study of maximal sprint speeds of several species of wild rodents, Djawdan and Garland (1988, p. 766) stated that "Preliminary tests indicated that animals

tested at night were not significantly faster than those tested during the day ..."

Blood sampling, glucose, lactate, and organ masses

Blood samples (one 70 µl microcapillary tube) were collected as rapidly as possible from the submandibular vein using Goldenrod animal lancets (Medipoint, Inc., Mineola, NY, USA). These were immediately subsampled with 20 ul tubes for determination of glucose and lactate concentrations from whole blood in duplicate. During the final (resting) blood sample, we took an additional 70 µl sample to determine hematocrit after centrifugation.

Glucose was measured with an Ascensia Contour portable glucose monitor (Bayer Mishawaka, IN, USA) and test strips (7098B). This instrument displays the [glucose] ~5 seconds after the blood is applied to the test strip. Lactate was measured using an Accutrend/Accusport Lactate Portable Analyzer and Bm-Lactate strips (Roche Diagnostics Gmbh, Mannheim, Germany; see Bishop, 2001). This instrument displays the [lactate] ~60 seconds after the blood is applied to the test strip.

Immediately after collection of the resting blood samples (see above), mice were euthanized by CO_2 inhalation and the heart ventricles, lungs, liver, spleen, and both triceps surae muscles (Garland et al., 2002) were dissected and weighed. All organs were then placed in a drying oven at 55^{o} Fahrenheit for four days to achieve constant weight, then re-weighed.

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. Sex, mini-muscle status, and line type (HR or C) were fixed factors; line was nested within line type as a random effect. The sex X line type interaction was tested relative to sex X line(line type). Covariates varied with the trait analyzed and included age, time of day, bleed delay time, and amount of wheel running during the 20 minutes prior to blood sampling (preliminary analyses indicated that wheel running over this time interval had higher predictive ability than over 10, 30 or 40 minutes prior to blood sampling). Whenever possible, we used directional hypotheses, e.g., HR mice will have greater endurance.

For measures of wheel running (total revolutions/day, number of intervals with at least one revolution, mean revolutions/min for the active intervals, highest single 1-min interval), we analyzed mean values from days 5 and 6 of wheel access because those are the days used in the routine selective breeding protocol (Swallow et al., 1998a). We analyzed the longer of the two endurance trials for each mouse because we were interested in the maximum performance value of each individual (e.g., Dohm et al., 1996; Swallow et al., 1998b; Rezende et al., 2006a). For glucose and lactate, which were measured on three occasions (resting, wheel running, and exhaustion), values were analyzed separately using age, time of day, (z-transformed time)^2 (this term allows for a curvilinear relation between the trait and time), and bleed delay time as covariates. Prior wheel running (over 20 minutes) was used as an additional covariate for blood samples

obtained during wheel running. To compare values at rest, during wheel running, and at treadmill exhaustion, we used a priori contrasts in SAS Procedure Mixed for a repeated-measures ANOCVA with covariates of age and bleed delay time.

For endurance, we also tested for differences among the four replicate lines within HR or C groups, using two different approaches. First, we considered the combined analysis of HR and C lines and compared the restricted maximum likelihood (REML) of models with and without line and the sex X line(line type) interaction term. Twice the difference in the ln REML of the two models is distributed asymptotically as a χ^2 with 2 degrees of freedom. If this value exceeds 5.991, then differences among the replicate lines are statistically significant for one or both sexes. Second, we performed separate two-way ANOVAs for the HR lines and for the C lines, including sex, line, and the sex X line interaction term (mini-muscle status was not included as an independent variable because it is highly confounded with HR line membership -- line 3 is fixed for the mini muscle allele: see Garland et al., 2002; Syme et al. 2005; and Introduction). Covariates were age, time of day, and (z-transformed time)^2.

Finally, we tested whether line means for wheel running could be predicted by endurance. To obtain the estimates of line means, we performed a 2-way ANCOVA of the square root of endurance running time, with factors of line, sex, and the sex X line interaction, and covariates of age, time of day, and (z-transformed time)^2. To obtain estimates of line means for average wheel running on days 5 and 6 of the 6-day test, we performed a 2-way ANCOVA with factors of line, sex, and the sex X line interaction, and covariates of age and wheel freeness, which is a measure of how many revolutions a

wheel spins following acceleration to a standard velocity. Separately for each sex, we then regressed wheel-running line means on endurance line means and also tested whether these regression models were improved (based on adjusted r^2 and partial *F* tests) by addition of dummy variables to code for line type or line 3 (which is fixed for the mini-muscle gene: see above and Syme et al., 2005).

Results

Voluntary wheel running

As expected from previous studies of these mice, in this sample the daily wheel-running distance of the HR lines was almost 3-fold higher than for the C lines (2-tailed $P = 0.0014$, based on analysis of revolutions raised to the 0.9 power to improve normality of residuals), and this increase was related primarily to higher average and maximum (single highest minute) voluntary running speeds in HR versus C mice ($P = 0.0020$ and $P = 0.0011$, respectively) (Table 1.1). Also as expected from previous studies, females ran significantly more revolutions/day than males ($P = 0.0473$; the sex X line type interaction was not significant: $P = 0.3039$). Females did not run significantly faster than males based on either average ($P = 0.2673$) or maximum ($P = 0.1839$) values, and the sex X linetype interaction was not significant ($P = 0.2396$ and $P = 0.2729$, respectively).

Endurance

As shown in Figure 1, endurance time was highly repeatable between days ($r = 0.785$, $P < 0.0001$), and on average mice ran 8.8% longer on the second test day (paired t

= 4.57, d.f. = 73, $P < 0.0001$). Figure 2 shows a "survivorship curve" based on the higher endurance time for each mouse. The HR lines had significantly higher endurance (Table 1.2; 1-tailed $P = 0.0088$), running an average of 32.6 and 35.0 minutes on the treadmill (males and females, respectively; backtransformed least squares means), whereas the C animals ran for an average of 26.3 and 28.1 minutes. Results were similar when body mass (which is reduced in the HR lines) or previous wheel running (which is increased in the HR lines) were included as covariates in the model (Table 1.2).

Total distance run during the endurance test averaged 1,060 ± 109, 1,183 ± 98, 794 ± 119, and 872 ± 119 meters for HR and C males and females, respectively (least squares means ± S.E.). The average ending speed obtained by the C animals in the endurance test was ~40 meters/minute, while the HR lines average ending speed was ~45 m/min (Table 1.1).

Line effects and correlation of line means

In the combined analysis of endurance for both HR and C lines, the significance level for line and the sex X line(line type) interaction term was $P = 0.0559$, based on a χ^2 of 5.77 with 2 d.f. In the separate analysis of C lines, the effects of sex ($P = 0.1456$), line ($P = 0.4518$), and the sex X line interaction ($P = 0.4454$) were not significant. In the HR lines, the sex effect was not significant ($P = 0.1457$), line was highly significant ($P = 0.0044$), and the sex X line interaction was marginally nonsignificant ($P = 0.0650$).

Figure 3 shows the generally positive relation between line means for wheel running and treadmill endurance, separately by sex. In females, the best-fitting model for

predicting wheel running included both endurance running time (P = 0.0217) and line type (P = 0.0185) (adjusted r^2 = 0.888). In males, the best model for predicting wheel running included both endurance (P = 0.0044) and the dummy variable for line 3 (P = 0.0133) (adjusted r^2 = 0.805).

Organ masses

As shown in numerous previous studies of these lines of mice, HR were smaller in body mass than C, and females were smaller than males (Table 1.3). Controlling for variation in body mass, HR and control lines did not significantly differ for any organ mass or hematocrit, nor was the sex effect or sex X line type interaction significant (Table 1.3). As expected, mini-muscle mice had smaller triceps surae (P = 0.0001). Mini-muscle mice also had significantly larger ventricles, lungs, liver, and marginally larger spleens (P = 0.0546: Table 1.3).

Blood glucose and lactate concentrations

Glucose for each blood sample in our study was measured in duplicate. During wheel running, at exhaustion, and at rest the Pearson product moment correlation for the duplicates was 0.960, 0.991, and 0.960, respectively. A paired t-test indicate no statistical difference in mean values between the first and second duplicates (P = 0.520, 0.268, and 0.904, respectively).

Resting blood glucose values of individual mice ranged from 72.0 to 168.5 mg/dl. As shown in Figure 4, resting glucose did not differ statistically between HR and C mice

(P = 0.3820), but males had higher values (backtransformed least squares mean 118.5 with asymptotic 95% C.I. 106, 132 mg/dl) than females (98 with 95% C.I. 88, 108 mg/dl)(P = 0.0014) (sex X line type interaction P = 0.6400). Mini-muscle mice did not differ from normal individuals (P = 0.5366).

Individual glucose concentrations during peak voluntary wheel running ranged from 52.0 to 141.5 mg/dl. Males again tended to have higher concentrations than females (P = 0.0881), but again no effect of line type was present (P = 0.3680) (sex X line type interaction P = 0.3970) and mini-muscle mice did not differ from normal individuals (P = 0.2300). Wheel running during the 20 minutes prior to blood sampling ranged from 0 to 797 revolutions, but was not a significant predictor of [glucose] in the full nested ANCOVA model (P = 0.9920).

Post-endurance glucose concentrations were much lower than at rest or during wheel running, ranging from 12.0 to 120.5 mg/dl and averaging 52 mg/dl (simple mean). They did not vary significantly in relation to either sex or line type (P = 0.3318, P = 0.5923 respectively; sex X line type interaction P = 0.4699), but mini-muscle individual tended to have higher values (P = 0.0842).

Repeated-measures ANCOVA indicated that glucose concentrations during voluntary wheel running were not significantly lower than at rest (P = 0.1738), but values at treadmill exhaustion were lower than at rest ($P < 0.0001$) or during wheel running (P = 0.0001). Mini-muscle mice had higher glucose concentrations in general (P = 0.0140) and showed a significant interaction with measurement condition (P = 0.0265)(Figure 1.5). Glucose in normal mice decreased much more between rest (backtransformed least

squares mean = 105.5 mg/dl) and exhaustion (49.1 mg/dl) as compared with mini-muscle individuals (112.1 to only 84.4 mg/dl, respectively: Figure 1.5).

Lactate for each blood sample in our study was also measured in duplicate. During wheel running, at exhaustion, and at rest the Pearson correlation for duplicates was 0.869, 0.953, and 0.779, respectively. A paired t-test indicated no statistical difference in mean values (P = 1.00, 0.627, and 0.051, respectively).

At rest (Figure 1.6), blood lactate concentrations ranged from 2.0 to 4.8 mM/l and did not vary significantly in relation to sex (P = 0.3085), line type (P = 0.1926; sex X line type interaction P = 0.4516) or mini-muscle status (P = 0.0535). During wheel running, lactate ranged from 2.8 to 11.5 mM/l, and was lower in mini-muscle individuals (P = 0.0448) but did not vary in relation to sex or line type (P = 0.7652 and P = 0.5914, respectively; sex X line type interaction P = 0.6458). Wheel running from the 20 minutes prior to blood sampling was not a significant predictor of [lactate] in the full nested ANCOVA model (P = 0.6791). At exhaustion following treadmill exercise, lactate concentrations ranged from 3.5 to 13.5 mM/l, and did not vary in relation to mini-muscle (P = 0.2331), sex (P = 0.3480) or line type (P = 0.3255) (sex X line type P = 0.7570). A priori contrasts in the repeated-measures ANCOVA indicated that lactate concentrations during voluntary wheel running were significantly higher than at rest (P = 0.0031) and that values at treadmill exhaustion were higher than at rest (P < 0.0001) or during wheel running (P = 0.0024).

Discussion

Selective breeding for high voluntary wheel-running behaviour in four replicate

lines has produced mice that run almost three times more revolutions/day as compared with four unselected control lines. Before the start of the selection experiment, it was hypothesized that the evolution of high voluntary running would entail both increases in physical capacities for exercise and changes in the brain that affect motivation, willingness to run or the reward perceived from running (e.g., see Friedman et al., 1992; Garland, 2003; Swallow et al., 2009). Changes in the brain have clearly occurred and seem to indicate motivational alterations in the HR lines (e.g., Rhodes et al., 2005, 2009; Keeney et al., 2008). Previously, we reported that HR mice have elevated maximal oxygen consumption ($\dot{V}O_2max$) during forced treadmill exercise (Swallow et al., 1998b; Rezende et al., 2006a,b). Here, we report for the first time that HR mice of both sexes have elevated treadmill endurance-running capacity as compared with C mice. Thus, results of this mouse selection experiment are consistent with findings from a selection experiment using rats in which bidirectional selection for treadmill endurance has led to corresponding divergence between the up and down selected lines in both $\dot{V}O_2max$ and voluntary wheel running (Waters et al., 2008).

The mechanistic basis of high endurance is multifactorial (Myburgh, 2003), but in the HR lines it appears to lie partly in their elevated $\dot{V}O_2max$ (Swallow et al., 1998b; Rezende et al., 2006a,b,c), increased insulin-stimulated glucose in extensor digitorum longus muscle (Dumke et al., 2001), and probably an elevated ability to oxidize lipids to fuel sustained exercise (Gomes et al., 2009). However, based on the present results (Table 1.3), it is not related to differences from the C lines with respect to hematocrit or

the relative masses of heart ventricles, lungs, liver, spleen, triceps surae muscle (see also Swallow et al., 2005; Rezende et al., 2006c).

Our results suggest that both C and HR lines have higher endurance capacities than some standard inbred strains of mice (see also Lightfoot et al., 2001). In our study, with a 25^{o} incline, the average ending speed was 40.3 and 44.8 m/min for C and HR mice, respectively. One female HR mouse reached a speed of 63.5 m/min after running for 58 minutes. Lerman et al. (2002) and Haubold et al. (2003) used a very similar protocol as in the present study, but with only a 7^{o} incline. The former study reported ending speeds of about 32-40 m/min for six inbred strains of mice, whereas the latter reported 28 m/min for wild-type 129 mice. Finally, using a somewhat different protocol, Massett and Berk (2005) tested endurance in three strains of inbred mice and F1 hybrids, all of which ran substantially fewer meters than our C animals.

It is also of considerable interest to compare our values for endurance with those from recent studies using direct genetic (Tsao et al., 2001; Wang et al., 2004; Hakimi et al., 2007) or pharmacological manipulations (Narkar et al., 2008) in an attempt to alter endurance abilities of mice. For the most part, their endurance protocols were much less intensive than the one employed here because of lower speeds and/or lack of an incline. In Narkar et al. (2008), the endurance test (before some animals received 4 weeks of exercise training) involved gradually increasing treadmill speed from zero to 15 m/min, where the speed was then maintained until exhaustion. The average endurance times for control C57BL/6J and experimental treatment groups ranged from 30-100 minutes (for untrained mice). The treadmill protocol employed by Wang et al. (2004) used a constant

speed of 10 m/min for 60 minutes, followed by speed increases of 1 m/min every 15 minutes. The final treadmill speed was 12 m/min after ~88 minutes of running for control mice and 17 m/min after ~145 minutes of running for peroxisome proliferators-activated receptor δ (PPARδ) transgenics (their Figure 1.6A). Average times for treadmill running from both studies are nearly as long as or longer than the mice in this study, but Narkar et al. (2008) and Wang et al. (2004) used no slope and final speeds that were lower than the starting treadmill speed used in the present study. Our interpretation is that the experimentally manipulated mice of Wang et al. (2004) and Narkar et al. (2008) did not have exceptionally high endurance as compared with either our control lines or the HR lines. Additionally, those studies did not report any post-test physiological measure, such as blood lactate or glucose, so it is difficult to know if their animals stopped running because of physiological exhaustion or possibly "boredom."

Hakimi et al. (2007) used three separate endurance tests to better understand the role of phosphoenolpyruvate carboxykinase (PEPCK-C) in energy metabolism. Their most comparable endurance test used a 25^{o} slope and started at 10 m/min, then increased 2 m/min every 2 minutes. Average ending speeds were 23.4 m/min for controls and 36.6 m/min for mice over-expressing PEPCK-C. Compared with our study, where a very similar endurance protocol was used, their PEPCK-C animals approach the speeds reached by our control animals (40 m/min). The reported blood lactate concentrations for control animals from Hakimi et al. (2007) show a significant increase from rest to post-endurance and reach concentrations indicative of physiological fatigue. Hakimi et al. (2007) also report mass-specific maximal oxygen consumption obtained during forced

treadmill exercise, and comparison with values in Rezende et al. (2006a,b) indicates that their transgenic mice have values similar to our control lines, whereas our HR lines have significantly higher values.

Additionally, Tsao et al. (2001) did not measure endurance but did report that mice overexpressing the glucose transporter GLUT4 ran fourfold farther on wheels than controls. However, as noted by Lightfoot et al. (2004), even that elevated distance would rank fourth lowest as compared with the 13 inbred strains they studied. Overall, the foregoing comparisons indicate the efficacy of selective breeding (experimental evolution) as a tool to alter the performance capacities of rodents (see also Koch et al., 1998; Koch and Britton, 2001; Waters et al., 2008; Swallow et al., 2009).

Although HR lines as a group have statistically higher endurance capacity than their control lines, the two sets of lines do not show complete separation (Figure 1.3). Moreover, we found that endurance capacity differs significantly among the four HR lines, but not among the four C lines. Considering the mean values for each of the eight lines (which have been genetically separated for 50 generations, including generation zero, prior to the start of selective breeding: see Swallow et al., 1998a), we found a positive relation between endurance capacity and amount of voluntary wheel running. This association reinforces the association demonstrated by the general differences between the HR and C lines (i.e., the former run more on wheels and have higher endurance: Table 1.1, Figure 1.2). We also found that the line-mean relation between endurance and wheel running differs between the sexes (Figure 1.3). In females, the best-fitting model for predicting wheel running included endurance and line type, and the

graphical representation shows that, relative to C females, HR females run more on the wheels than would be predicted from their treadmill endurance capacity (Figure 1.3). In males, the best model included endurance and a dummy variable coding for HR line 3 (lab designation), which is fixed for the mini-muscle allele. Thus, in males, HR line 3 runs more on wheels than would be predicted from their endurance capacity (Figure 1.3). This sex difference in the wheel-running-endurance relation also reinforces the sex difference in how high wheel running has evolved in general in the HR lines, i.e., females have evolved higher running distances almost exclusively by increased running speed, whereas male HR mice show a significant increase in amount of time spent running in addition to average running speed (Garland, 2003; Rezende et al., 2009; references therein). The exercise-physiological underpinnings of this sex difference in response to selective breeding are not yet known, but a recently discovered sex difference in the response to an endocannabinoid receptor antagonist points to a "motivational" difference between HR males and females (Keeney et al., 2008).

Resting blood glucose and lactate values of both HR and C mice (Figures 1.4-1.6) are generally consistent with other studies of mice (Tsuboyama-Kasaoka et al., 1999; Banerjee et al., 2004; Pederson et al., 2005a, b; Ferreira et al., 2007; Svenson et al., 2007; Wende et al., 2007), rats (Pimenta et al., 2006), hamsters (Mistlberger et al., 2006), and human beings (Goldfarb et al., 1986; Rizzo et al., 2005; Scheen et al., 1998), although the lactate values are slightly higher than some of the previous mouse studies, possibly because our study is apparently the first to measure lactate in samples obtained from the submandibular vein using lancets. The observed higher resting glucose concentrations in

males are also consistent with previous studies of mice (Pederson et al., 2005a, b; Svenson et al., 2007). Glucose concentrations following exhaustive treadmill exercise were significantly reduced (Figures 1.4, 1.5), but did not vary in relation to line type, sex or mini-muscle status. These low blood glucose concentrations (overall mean ~52 mg/dl) are consistent with other studies of exhaustive treadmill exercise in laboratory mice (e.g., Pederson et al., 2005a).

Voluntary wheel running increased blood lactate concentrations of all mice to values significantly greater than at rest (Figure 1.6). Exhaustive treadmill exercise further increased blood lactate concentrations of both HR and C lines, to values consistent with the literature for laboratory house mice (Pederson et al., 2005a; Pimenta et al., 2006; Hakimi et al., 2007), with these values being somewhat lower than reported for wild rodents of other species (Djawdan, 1993).

Exercise is considered a key component of body weight regulation and a cornerstone for the treatment of metabolic syndrome and other metabolic disorders (Thorburn and Proietto, 2000). As worldwide rates of obesity (and associated mortality, e.g., Allison et al., 1999) continue to rise, understanding the determinants of voluntary physical activity is becoming increasingly important from a biomedical perspective. Diminished aerobic capacity, low expression levels of genes coding for proteins involved in oxidative phosphorylation, and mitochondrial dysfunction have been correlated with increased circulating triglyceride and glucose concentrations, insulin resistance, and type II diabetes (Mootha et al., 2003; Patti et al., 2003; Bernal-Mizrachi and Semenkovich, 2006). Conversely, high aerobic capacity and associated lower-level traits may protect

from components of the metabolic syndrome, and selective breeding of rats for high treadmill endurance capacity has made them resistant to the adverse effects of a high-fat diet (Noland et al., 2007). Selection for high voluntary wheel running in mice has led to correlated increases in $\dot{V}O_2$max and endurance capacity, reduced body fat (Swallow et al., 2005; Nehrenberg et al., 2009), reduced circulating leptin concentrations (Girard et al., 2007; but see Vaanholt et al., 2008), and increased circulating adiponectin concentrations (Vaanholt et al., 2007; depending on sex and diet: Vaanholt et al., 2008). These characteristics, along with alterations in dopamine and endocannabinoid signaling in the brain of HR mice (Rhodes et al., 2005; Keeney et al., 2009; cf. Stice et al., 2008), suggest that they may also show decreased susceptibility to obesity and cardiovascular disease risk factors, especially when challenged with a high-fat diet (Vaanholt et al., 2008). This hypothesis will be the focus of future research.

Chapter 1 Acknowledgements

We would like to thank Rachael L. Marsik and Rashmi S. Wijeratne for helping collect data. All animal procedures are in compliance with the UCR Institutional Animal Care and Use Committee and U.S. laws. This study was supported by U.S. NSF grant IOB-0543429 to T.G.

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Table 1.1. Comparison of least squares means for voluntary wheel-running traits and for ending treadmill speed during endurance test, including ratios

	Wheel N	Wheel Distance Run (m/day)	Wheel Mean Speed (m/min)	Wheel Maximum Speed in 1 min (m/min)	Endurance N	Treadmill Ending Endurance Speed (m/min)	Wheel Mean / Ending Endurance Speed	Wheel Maximum / Ending Endurance Speed
High Runner Male	23	8,668 ± 1,125	20.3 ± 2.04	35.3 ± 2.46	15	44.0 (26.0, 48.5)	0.461	0.802
High Runner Female	21	11,890 ± 1,115	24.3 ± 2.01	40.5 ± 2.41	22	45.5 (26.0, 50.0)	0.534	0.890
Control Male	26	2,909 ± 1,320	10.4 ± 2.37	21.4 ± 2.88	26	39.5 (24.5, 42.5)	0.263	0.552
Control Female	26	4,024 ± 1,324	10.3 ± 2.4	22.0 ± 2.88	25	41.0 (24.5, 44.0)	0.251	0.537

Values for wheel running are least squares means ± SE from SAS Procedure Mixed for averages of days 5 and 6 of a 6-day test. Values for endurance speeds are also SAS least squares means and 95% confidence intervals, based on back-transformation from the square root scale, for the higher of two endurance tests. See text for significant levels.

Table 1.2. F and P values from ANCOVA of endurance time with and without previous wheel running or body mass as a covariate

	F	d.f.	*P*	F	d.f.	*P*	F	d.f.	*P*
HR vs. C	10.54	1,6	**0.0088** [+]	5.94	1,6	**0.0254** [+]	5.77	1,6	**0.02655** [+]
Sex	2.01	1,6	0.2062 [+]	0.95	1,6	0.3682 [+]	0.23	1,6	0.6512 [-]
Interaction	0.01	1,6	0.9107	0.01	1,6	0.9179	0.14	1,6	0.7201
Mini-muscle	0.88	1,68	0.3517 [-]	1.53	1,67	0.2211 [-]	1.3	1,67	0.2530 [-]
Revolutions on day 5+6				2.42	1,67	0.1246			
Body mass							2.30	1,67	0.1338

Results from 2-way nested ANCOVA in SAS Procedure Mixed. Replicate line was included as random effects in analyses. Age, time of day, and time of day^2 were additional covariates (results not shown). + indicates direction HR>C, females>males, or mini>normal. *P* values are 2 tailed except for HR vs. C, where values are 1-tailed. Significant values ($P < 0.05$) are in **bold**.

Table 1.3. Least squares means (back transformed from logs), 95% confidence intervals, and P values for body mass, dry organ masses, and hematocrit

Trait	Line type	Male	Female	$P_{\text{line type}}$	P_{sex}	$P_{\text{sex X line type}}$	$P_{\text{mini-muscle}}$	$P_{\text{body mass}}$
Body Mass (g)	HR	30.1 (27.89, 32.27)	22.0 (19.88, 24.17)	**0.0145**	**< 0.0001**	0.6232	0.6321	--
	Control	33.8 (31.26, 36.35)	25.0 (22.44, 27.44)					
Ventricles (g)	HR	0.0337 (0.0309, 0.0367)	0.0334 (0.0301 0.0370)	0.1712	0.9065	0.3683	**0.0218**	**< 0.0001**
	Control	0.0309 (0.0277, 0.0345)	0.0315 (0.0284, 0.0350)					
Lungs (g)	HR	0.0412 (0.0384, 0.0441)	0.0453 (0.0416, 0.0493)	0.1213	0.0676	0.2916	**0.0044**	**< 0.0001**
	Control	0.0393 (0.0359, 0.0431)	0.0419 (0.0385, 0.0456)					
Liver (g)	HR	0.5444 (0.5093, 0.5818)	0.5246 (0.4843, 0.5682)	0.6252	0.2897	0.9780	**0.0011**	**< 0.0001**
	Control	0.5359 (0.4926, 0.5831)	0.5158 (0.4765, 0.5584)					
Spleen (g)	HR	0.0234 (0.0189, 0.0291)	0.0245 (0.0186, 0.0322)	0.1484	0.3606	0.2478	0.0546	**0.0303**
	Control	0.0267 (0.0201, 0.0355)	0.0313 (0.0239, 0.0409)					
Mean Triceps	HR	0.0269 (0.0256 0.0282)	0.0262 (0.0245, 0.0279)	0.6221	0.4774	0.6172	**0.0001**	**< 0.0001**
Surae (g)	Control	0.0271 (0.0253, 0.0289)	0.0267 (0.0251, 0.0283)					

Table 1.3. continued. Least squares means (back transformed from logs), 95% confidence intervals, and P values for body mass, dry organ masses, and hematocrit (Cont.)

Trait	Line type	Male	Female	$P_{\text{line type}}$	P_{sex}	$P_{\text{sex X line type}}$	$P_{\text{mini-muscle}}$	$P_{\text{body mass}}$
Hematocrit (%)	HR	42.12 (39.48, 44.94)	43.18 (39.58, 47.12)	0.1694	0.3917	0.5400	0.8990	0.4563
	Control	39.64 (36.22, 43.37)	41.70 (38.56, 45.09)					
Hematocrit (%)	HR	42.37 (39.79, 45.12)	42.33 (39.95, 44.84)	0.2131	0.6211	0.5870	0.9690	--
	Control	40.34 (37.60, 43.27)	41.23 (38.47, 44.21)					

Values are back transformed (from $\log_{10}$) least squares means from SAS Procedure Mixed, along with upper and lower 95% confidence limits in parentheses. All *P* values are 2-tailed. $\log_{10}$ body mass was included as a covariate in analyses of dry organ masses and hematocrit, no transform was used on body mass. Hematocrit was analyzed both with and without body mass as a covariate because it does not necessarily covary with body size (e.g., see Swallow et al., 2005). Significant values ($P < 0.05$) are in **bold**. Least squares means and 95% confidence intervals for mini-muscle verus normal individuals are as follows: body mass, 28.04 (27.05, 29.01), 27.42 (25.01, 29.82); ventricles, 0.0344 (0.0311, 0.0380), 0.0304 (0.0291, 0.0318); lungs, 0.0444 (0.0411, 0.0480), 0.0395 (0.0382, 0.0408); spleen, 0.0302 (0.0232, 0.0392), 0.0229 (0.0206, 0.0265); mean triceps surae, 0.0204 (0.0193, 0.0216), 0.0349 (0.0341, 0.0357); hematocrit with body mass as covariate, 41.74 (38.90, 44.79), 41.53 (40.42, 42.68); hematocrit without body mass as covariate, 41.52 (38.78, 44.47), 41.59 (40.49, 42.73).

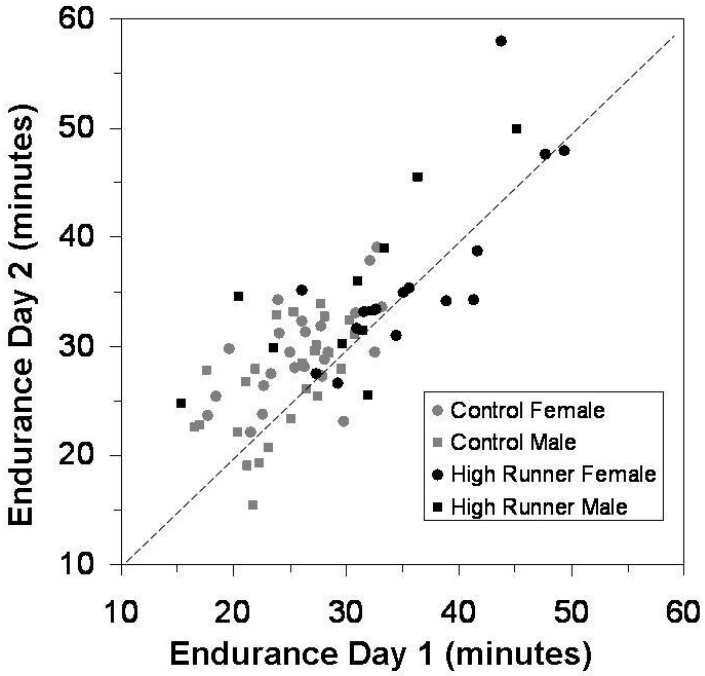


Figure 1.1 Endurance time in minutes, from two separate tests. Grey circles represent control females, grey squares represent control males. Black circles represent high runner (HR) females and black squares are the HR males. N= 74, r = 0.79, p <0.001.

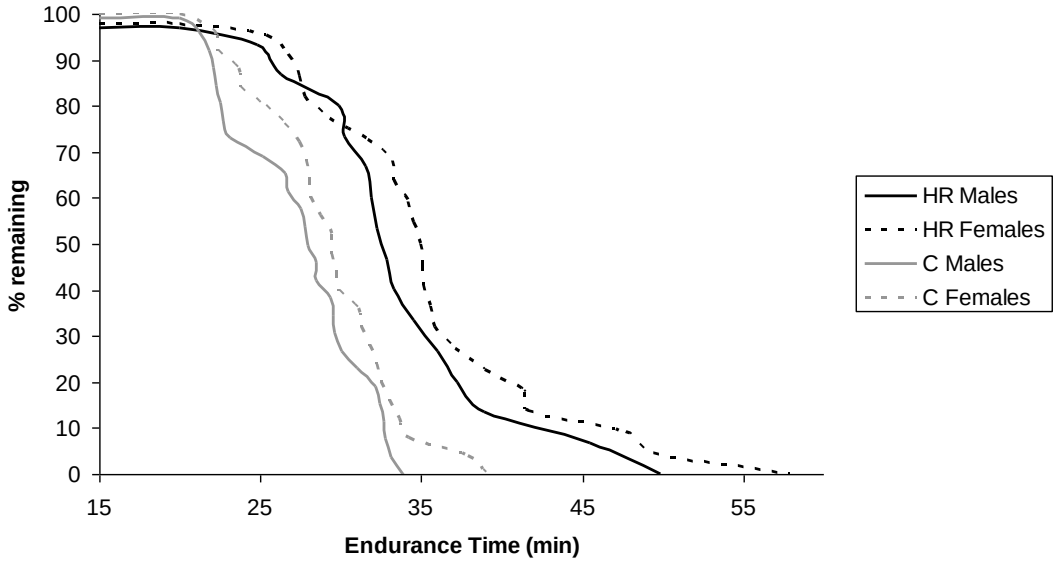
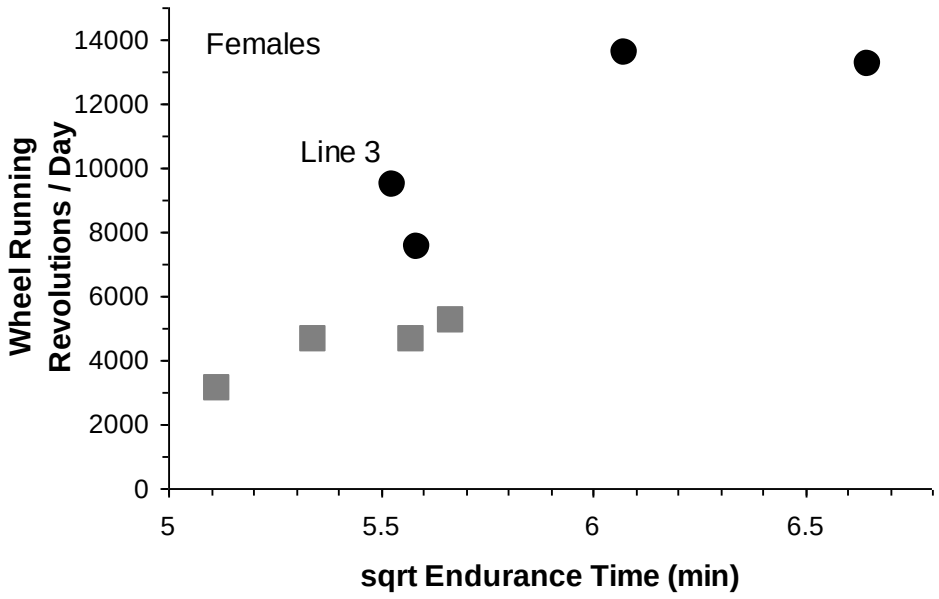


Figure 1.2 The percent of mice reaching any given time split by sex and line type. The solid black line indicates HR males (n= 15), dashed black line represents HR females (n= 22). The solid gray line represents the control males (n= 26) with the dashed grey line for female controls (n=25). The starting belt speed was 20 m/min and increased every 2 minute by 1.5 m/min. All mice lasted at least 20 minutes. Initial percents are 100 for each group but lines are slightly staggered for visual clarity.


Females
Line 3
Wheel Running
Revolutions / Day
14000
12000
10000
8000
6000
4000
2000
0
5
5.5
6
6.5
sqrt Endurance Time (min)

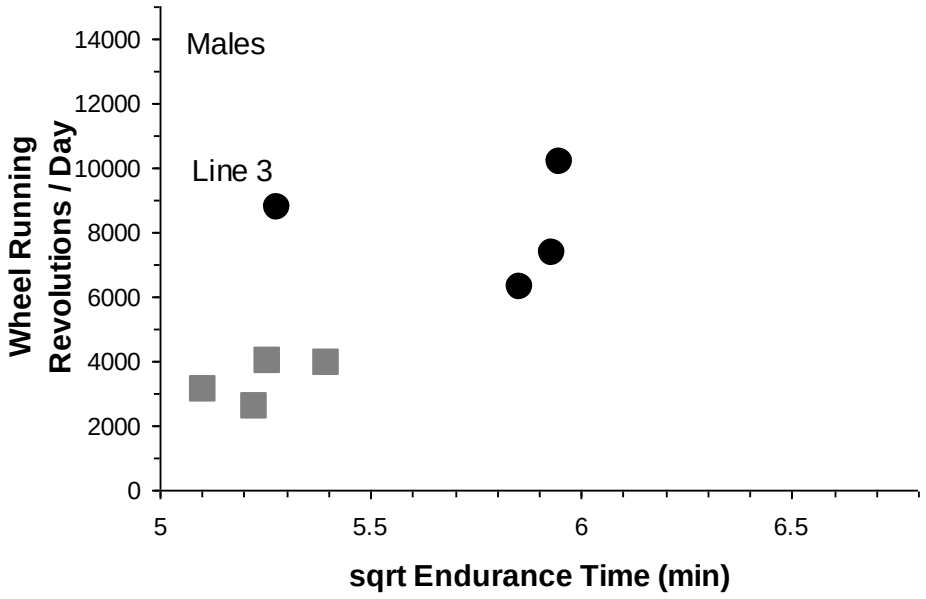

Males
Line 3
Wheel Running
Revolutions / Day
14000
12000
10000
8000
6000
4000
2000
0
5
5.5
6
6.5
sqrt Endurance Time (min)

Figure 1.3 Adjusted line means for endurance time (square-root transformed) and wheel running on nights 5+6 for males and females from all eight lines. In females, the best model for predicting wheel running included both endurance running time and line type, whereas in males it included endurance and a dummy variable coding for line 3 (i.e., line 3 males have unusually high wheel running for their endurance).

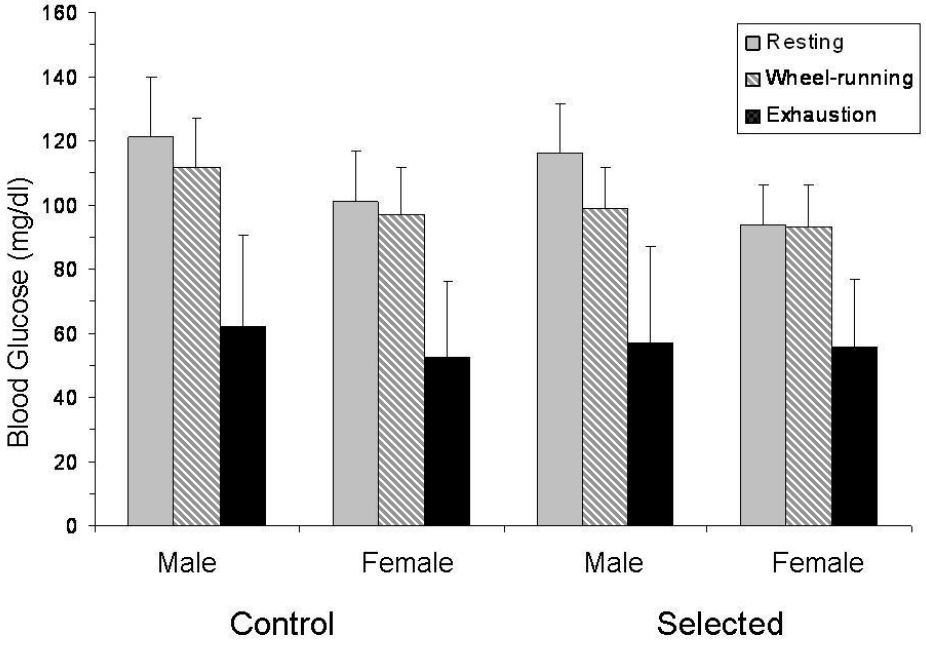


Figure 1.4 Plasma glucose levels (mg/dl) at rest during the day, during nightly wheel running, and at exhaustion following a treadmill endurance test. Values are back-transformed least squares means and upper 95% confidence limits. In addition, males have significantly higher resting glucose levels than females. Glucose levels do not differ between resting and wheel running, but post-endurance test (exhaustion) values are significantly lower than both wheel running and resting values. Age, time of day, time of day^2, handling time, and prior 20 minutes of wheel running were all used as covariates when applicable.

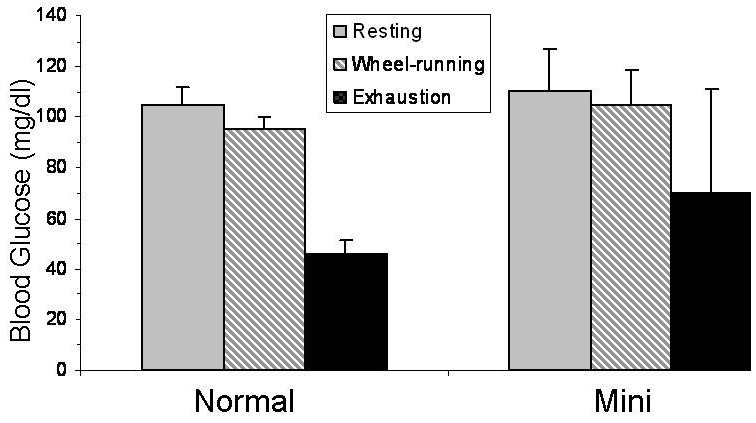


Figure 1.5 Plasma glucose levels (mg/dl) at rest, during nightly wheel running, and post-endurance test for mini-muscle and normal mice. Mini-muscle animals are found only in two selected lines. Mini-muscle mice have significantly higher glucose levels and show a significant interaction with measurement period. Values are back-transformed least squares means and upper 95% confidence limits. Age, time of day, time of day^2, handling time, and prior 20 minutes of wheel running were all used as covariates when applicable.

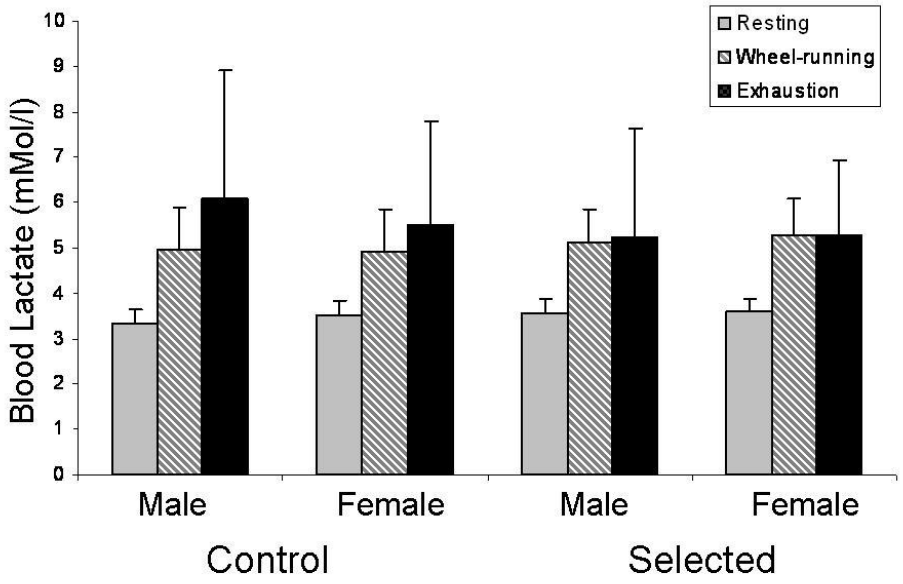


Figure 1.6 Plasma lactate levels (mMol/l) at rest during the day, during nightly wheel running, and at exhaustion following a treadmill endurance test. Values are back-transformed least squares means and upper 95% confidence limits. No significant differences exist between HR and C mice, between the sexes or between mini-muscle and normal mice for any measure. However, a priori contrasts in a repeated-measures ANCOVA indicated that lactate levels during voluntary wheel running were significantly higher than at rest, and that values at treadmill exhaustion were significantly higher than at rest or during wheel running (see Results). Age, time of day, time of day^2, handling time, and prior 20 minutes of wheel running were all used as covariates when applicable.

Chapter 2

Effects of Western diet and wheel access on Metabolic Syndrome profiles in mice bred for high voluntary exercise

Thomas H. Meek[1], Joey C. Eisenmann[2], Brooke K. Keeney[1], Robert M. Hannon[1], Elizabeth M. Dlugosz[1], Theodore Garland, Jr.[1*]

[1]Department of Biology, University of California, Riverside, Riverside, CA 92521, USA

[2]Departments of Kinesiology and Pediatrics and Human Development, Helen DeVos Children's Hospital, Grand Rapids, MI 49503, USA

Abstract

Increased consumption of high-fat/high sugar foods (i.e., Western diet) and a sedentary lifestyle are two key factors associated with the increased rates of human obesity and cardiovascular disease. However, experimental studies of diet and exercise have shown varying effects on metabolic syndrome risk factors in both humans and rodents, sometimes depending on genetic background. To examine the potential interactive effects of diet, exercise, and genetic background on components of the metabolic syndrome, we studied mice from four replicate lines bred for high voluntary wheel running and from four unselected control lines. Starting 3 days post-weaning (24 days of age), males of both genetic linetypes, high runner (HR) or control, were housed for 60 days with or without wheels and fed either a standard chow (14% kcal from fat) or Western diet (WD, 42% kcal from fat). Four blood samples were taken over the course of the experiment to measure fasting plasma total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), and glucose. Our findings show that WD was obesogenic for all mice (both HR and control, irrespective of wheel access), leading to increased retroperitoneal fat and liver mass, even after accounting for amount of wheel running and calories consumed as covariates in statistical analyses. Neither genetic selection history nor wheel access had an appreciable impact on the juvenile or adult blood lipid profiles. As expected from results of previous studies with diets high in saturated fats, WD decreased TG. WD also significantly increased fasting glucose levels, and raised TC and HDL-C concentrations, leading to an improved total cholesterol/HDL ratio, which may offset some of the negative aspects of the diet. The linetypes showed

divergent ontogenies for body mass and food consumption (adjusted for body mass). These differences were only perceptible through interactive effects with diet or wheel access. Environmental factors of diet and wheel access also had pronounced effects on the ontogenetic trajectories of body mass, food consumption, and fasting glucose concentrations. These data underscore the importance (and often unpredictable nature) of genotype-by-environment and environment-by-environment interactions when studying body weight regulation.

Introduction

Habitual physical activity is associated with many health-related benefits, and many physiological functions have been shown to improve in response to exercise training (e.g., Allen et al., 2001; Baar et al., 2002; Joyner and Green, 2009; Mestek et al., 2010). The metabolic alterations associated with exercise have been linked to decreases in blood pressure, insulin resistance, blood lipids, and adiposity, all of which are major risk factors for cardiovascular disease and death (Must et al., 1999; U.S. Department of Health and Human Services 2008). In addition, exercise-induced metabolic modifications have been associated with the prevention of Type II diabetes mellitus (NHLBI, 1998), stroke (NHLBI, 1998), some forms of cancer (Farrell et al., 2007), and can stimulate some types of learning or help maintain cognitive function throughout life (Hillman et al., 2008; Lautenschlager et al., 2008).

Although exercise per se has many benefits, high aerobic capacity (maximal oxygen consumption) may independently provide resistance to diet-induced obesity and associated co-morbidities (Stevens et al., 2002). Evidence in humans demonstrates higher rates of metabolic syndrome (i.e., co-occurrence of CVD risk factors) and cardiovascular disease mortality in unfit individuals (as measured by a standard treadmill test) than in physically fit individuals (Blair et al., 1995; LaMonte et al., 2005; Church, 2010). Regular exercise training can enhance aerobic capacity, but this response itself is under partial genetic control (Bouchard and Rankinen, 2001) making it difficult to distinguish between health benefits derived from exercise per se or 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 were sedentary showed nearly a 3-fold decrease in all-cause mortality when compared to 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).

Data from rodent studies provide similar lines of evidence. An artificial selection experiment using laboratory rats bred for high or low treadmill endurance during forced exercise showed a 171% divergence in treadmill running distance after six generations (Koch and Britton, 2001). Without exercise training, the low-endurance rats have a decreased maximal oxygen consumption (VO_2max) that is associated with higher levels of blood glucose and insulin, elevated levels of circulating triglycerides and free fatty acids, elevated blood pressure, and increased percent body fat (Wisloff et al., 2005), all of which are recognized as contributing to disease states. Thus, understanding variation in physical activity, innate physical capacities, and environmental and genetic influences on voluntary activity has important implications for the prevention of metabolic disorders (Garland et al., 2011b; Kelly et al., 2011).

In a selection experiment using laboratory house mice, breeding for high voluntary wheel running has led to several phenotypes 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 lifestyle. Some of these correlated responses to selection include innately low body fat (Swallow et al., 2005; Nehrenberg et al., 2009), increases in aerobic performance (VO_2max: Rezende et al., 2006a; endurance: Meek et al., 2009), elevated muscle oxidative capacity (especially when housed with wheels)(Houle-Leroy et al.,

2000; Bilodeau et al., 2009; Meek et al., chapter 4), increased insulin-stimulated glucose uptake in isolated extensor digitorum longus muscle (Dumke et al., 2001), increased upregulation of GLUT-4 transporter in gastrocnemius muscle in response to wheel running (Gomes et al., 2009), and increased circulating adiponectin levels (Vaanholt et al., 2007). Using these unique lines mice, we addresses two main questions. First, does a genetic propensity for high voluntary exercise (the high runner [HR] lines) prevent or at least ameliorate risk factors associated with cardiovascular disease, even in the absence of exercise (i.e., if HR mice housed without wheel access are resistant to diet-induced obesity and health-related consequences). Second, does diet and/or exercise interact with genetic background to produce differential responses in metabolic risk factors (glucose, cholesterol, and triglycerides) and body fat. HR mice have been shown to respond to Western diet (high in saturated fat plus added sucrose) by increasing total running by as much as 75%, totaling 13,000 meters per night, whereas mice from the non-selected control (C) lines show no statistical change in running when given this diet (Meek et al., 2010). This gene-by-environment interaction may directly or indirectly contribute to large differences in susceptibility to weight gain or diet-induced metabolic abnormalities.

Materials and methods

Subjects

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 (Rhodes et al., 2005; Swallow et al.,

2009; Garland et al., 2011a,b). The original progenitors were outbred, genetically variable Hsd:ICR house mice (*Mus domesticus*). Four closed lines were bred for high 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 six days when they reach 6-9 weeks of age. Within each 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 the present 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 ~73° Fahrenheit and photoperiod was 12:12, with lights on at 0700 Pacific Time.

Experimental Groups

As described in a previous report (Meek et al., 2010; see Appendix 2.16), at an average of 24 ± 0.05 (S.E.) days of age, mice were placed into experimental groups (experimental day 1). 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 X 17 X 12.5 cm), as used during routine wheel testing for the selection experiment, while 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% kJ from fat) or Western diet (WD) (Harlan Teklad TD.88137 Western Diet, 42% kJ from fat with added sucrose).

Mice and food hoppers were weighed on experimental days 1, 8, 16, 30, 43, and 58. 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, we converted food consumption from grams to energetic intake, using total kJ of metabolizable energy of 12.98 and 19.01 for SD and WD, respectively, based on Harlan Teklad's reported caloric content for each diet.

For wheel-access animals, wheel revolutions were recorded in one-minute bins for approximately 23 hours 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

Four blood samples were taken during the eight-week time course of the experiment, all during the diurnal phase (see Appendix 2.16). All mice were fasted and restricted from wheel access for 4-6 hours before blood sampling. The first was obtained before the start of the experiment when mice were 24 days old. The second and third samples were taken during the second and third week of the experiment to follow the ontogeny of biomarkers related to the metabolic syndrome. The first three blood samples

were 60 µl each, obtained from the submandibular vein using Goldenrod animal lancets (Medipoint, Inc., Mineola, NY, USA). The final sample was taken as trunk blood after sacrifice by decapitation during the eighth week of the experiment.

35 µl from all blood samples were immediately divided into aliquots and used to determine total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), triglycerides (TG), and glucose in whole blood with an automated Cholestech LDX analyzer (Hayward, CA, USA) and cassettes (10-991). Values outside the normal operating range of the Cholestech machine were determined in duplicate with the remaining plasma sample using Pointe Scientific (Canton, Michigan, USA)(HDL-C) or Wako Diagnostics (Richmond, VA, USA)(TG) commercially available kits calibrated to the same standards as the Cholestech machine.

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 X diet, linetype X wheel access, wheel access X diet, linetype X wheel access X diet interaction were tested relative to the variance among replicate lines, and degrees of freedom were always 1 and 6. 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). Due to the known differences in physiology, mini-muscle status was included as an independent variable in all analyses that involved the HR lines.

Results

Body Mass

Body mass did not differ statistically between the linetypes (HR or C lines) or between mini-muscle and normal mice when measured before the start of the experiment (Table 2.1). After only one week, Western diet significantly increased body mass, and this effect remained significant throughout the experiment. Even using body length as a covariate, WD significantly increased body mass for week 2 and week 8 measurements (length was not recorded for weeks 4 and 6). Wheel access did not significantly reduce body mass until week 4 (Table 2.1). Wheel access caused a much more appreciable reduction in body mass for mice fed Western diet, as evident by the significant wheel access by diet interaction (Table 2.1). Western diet mice in the sedentary groups greatly

exceeded the body mass of standard chow mice (30% by the eighth week; Figure 2.1), but the difference between diet groups for mice with wheel access was much less (13%).

Although the effect of Western diet on body mass was statistically significant for all weeks, the gain in body mass was progressive throughout the study (Figure 2.1). This is further supported by a repeated-measures ANCOVA of body mass (Appendix 2.6A): the time x diet interaction was highly significant ($P < 0.0001$).

HR and C lines did not significantly differ in body mass for any weekly analysis alone. However, in the same repeated-measures ANCOVA as above, there was also a significant interaction between time and linetype ($P = 0.0153$). This interaction remained significant ($P = 0.0238$) even when body length and total calories consumed were added as covariates (Appendix 2.8). The interaction implies that the trajectories of body mass (and for body mass adjusting for body length and caloric intake) throughout the experiment were indeed different between the linetypes.

The prevention of body mass gain due to wheel running was accompanied by significantly lower retroperitoneal fat pad and liver mass upon dissection at the end of the study (Table 2.4 and Figure 2.4). Western diet had a significant, positive effect on all organ masses (adjusted for body length), which remained significant even after adding calories consumed as an additional covariate. When controlling for caloric consumption, HR mice did not have significantly lower retroperitoneal fat pad mass except when allowed access to wheels (significant linetype x wheel access interaction).

Food Consumption

Mice ate significantly more Western diet (kJoule/day) for nearly all weeks (Table 2.2). Interestingly, sedentary mice consumed substantially more calories when on Western diet compared to standard chow (Figure 2.2). This differential effect, however, was not observed in wheel-access mice, where (on average) no overall difference in food consumption occurs on the Western diet beyond the second week. This finding is supported by the significant wheel access by diet interaction (Table 2.2).

Overall, wheel-access mice consumed more calories regardless of diet. Food consumption also differed between linetypes (linetype x wheel access), probably driven by the behavioral response of increased running in HR mice eating Western diet. Mini muscle individuals ate significantly more during week 2 and weeks 3 & 4 (Table 2.2).

Lipid and Glucose Profile

We found no statistically significant linetype or wheel access differences for any plasma metabolite, but did find effects of diet. Western diet significantly increased fasting glucose concentrations by the end of the experiment (Table 2.3). This rise in glucose likely represents a progression towards insulin resistance (not measured), with final glucose concentration of Western diet mice being the highest (Supplemental figure 2.1). Repeated-measures ANCOVA (with age, fasting time, and handling time as covariates) for glucose showed a significant time x diet interaction ($P = 0.0049$; Appendix 2.10), an effect particularly evident in sedentary mice at week 8 of the study (see Table 2.3). Fasting plasma TG concentrations were decreased in response to

Western diet at week 1, week 8, and nearly in week 2 (Table 2.3).

Western diet also increased TC by the final blood sample. Western diet had a large, positive influence on HDL-C at all time points, which showed a factorial increase greater than the observed increase in TC (Supplemental figure 2.1). Concomitantly, WD resulted in decreased non-HDL-C, including both low density lipoprotein (LDL) and very low density lipoprotein (VLDL).

Discussion

Voluntary physical activity is a key component of energy balance and can positively impact many aspects of both physical and mental health (Hillman et al., 2008; Lautenschlager et al., 2008; Garland et al., 2011b). Many chronic diseases are associated with low physical activity (sedentary), particularly in concert with an obesogenic diet. Consequently, we expected that HR mice, which are genetically predispositioned for high activity levels (both on wheels and in cages when wheels are not provided), to better resist the detrimental effects of the high-fat Western diet. However, while we found several effects of selection for high voluntary running, the only metabolic risk factor to show an interaction with selection history was retroperitoneal fat pad mass.

Eight weeks of high fat feeding is likely insufficient for the development of cardiovascular abnormalities and severe metabolic disorders in rodents that are neither genetically predisposed nor pharmacologically manipulated. Still, this time frame can illustrate the detrimental influence of WD or a sedentary environment on body mass, food consumption, organ mass, and fasting glucose concentration, all of which show a

significant adverse response by the end of 8 weeks (Figure 2.1, Figure 2.2, Figure 2.4, Supplemental figure S2.1). Furthermore, some physiological traits, such as body mass and food consumption, show significant differences in ontogenetic trajectories (Figure 2.1, Figure 2.2), as does the behavioral trait of wheel running (Figure 2.3).

In humans, it has been suggested that daily activity levels are regulated in such a way that beyond a certain "set point" for activity, compensatory behaviors such as increased food consumption and/or decreases in activity counteract weight loss and sometimes result in net energy gain (Wilkin et al., 2006; Sonneville and Gortmaker, 2008). In our study, wheel access always had a positive effect on food consumption (kJ/day) after the first experimental week (Table 2.2). Despite the increase in food consumption caused by wheel access, voluntary exercise resulted in less gain in body mass over the experiment (Table 2.1, Table 2.4). Exercise prevented body mass gain the most in mice fed a Western diet. One important distinction to note, however, is that any activity measured in this study was purely voluntary. In contrast, exercise intervention studies where subjects are required to exercise more may not truly represent voluntary exercise. As a consequence, different levels of psychological "stress" may come into play, much as they do for rodent studies that employ voluntary wheel running versus forced exercise on a motorized treadmill (e.g., see Girard and Garland, 2002; Brown et al., 2007; Holmes et al., 2009; Fuss and Gass, 2010). To provide efficient treatment of obesity, it is essential to understand the role of voluntary (habitual) verses forced exercise in weight loss, and what mechanisms underlie the compensatory responses in each paradigm (see also Garland et al., 2011b).

Mice in our study gained body mass when a typical voluntary behavior -- wheel running -- was prevented (Figure 2.1). Mass gain was further increased by Western diet. When animals were allowed to exercise, body mass (and fat) was maintained at much lower levels, with a smaller mass gain when exposed to the Western diet. This response is likely attributable to both physical activity (using more energy) and modulated food consumption (not ingesting enough calories to compensate for increased activity).

The interaction between wheel access and diet in the analysis of food consumption is pertinent (Table 2.2, Figure 2.2). In sedentary conditions, mice fed Western diet ate significantly more calories per day compared to mice fed standard chow. However, averaged across linetypes, wheel-access mice significantly decreased consumption of Western diet compared to standard chow from weeks 5-8 after accounting for total revolutions run (Supplemental Table 2.8). A decrease in calorie consumption on a high fat diet is not necessarily uncharacteristic when comparing different studies or strains of animals (West and York, 1998), but this result is in stark contrast with a distinctive increased intake of calories from Western diet in sedentary conditions within the present study. Relative to mice fed standard diet, sedentary mice became hyperphagic on Western diet while wheel-access mice did not. Thus, hyperphagia appears environmentally dependent.

The amelioration of body mass and fat mass gain in wheel-access groups is likely attributable to both greater energy expenditure during locomotion and only a marginal increase in caloric intake. We do not know why the homeostatic mechanisms for weight control appear to have different "baselines" or "setpoints" in sedentary verses active

conditions. Perhaps the neurological reward for exercise is substituting for reward generated from eating a highly palatable diet, as has been found with nicotine activation of pro-opiomelanocortin neurons, which leads to reduced food intake and body mass in mice (Mineur et al., 2011). Either way, hyperphagic behaviors will need to be interpreted through this environmentally dependent response.

The phenotypic response to a high fat diet depends on the composition of the diet (Dorfman et al., 2004), individual genetic background, and additional environmental factors, such as room temperature, housing conditions or access to a wheels. Unlike fasting glucose concentrations, the diet effect for TC cannot be attributed to any adverse effects of the diet, but rather the large increase in HDL-C. The decrease of TG and increase of HDL-C are common responses to eating highly fatty diets (Albers et al., 1999; Mensink et al., 2003; Svenson et al., 2007). However, during caloric abundance or excess saturated lipid consumption, many cellular factors are initially up- or down-regulated to compensate for the dietary shift in lipid availability.

Recent evidence shows many of these changes revert back to baseline levels or beyond after extended exposure to high fat diet. For instance, Chan et al. (2008) showed an initial up-regulation of fatty acid oxidation genes and down-regulation of lipogenic genes, and the abundance of their protein products in C57BL/6J mice on a high fat diet. After four weeks there was a reversal of this gene expression profile, which corresponded to the onset of weight gain. Sparks et al. (2005) found genes involved in oxidative phosphorylation and peroxisome proliferator–activated receptor γ coactivator (PGC)-1α (and PGC-1β) from muscle samples were down-regulated after only 3 days of high fat

feeding in human males. In the same study, cross-sectional measurements using C57BL/6J mice showed a similar decrease in gene expression and protein content in the aforementioned traits after 3 weeks on a high fat diet. This reversion has been hypothesized to underlie the progression of disease states as a result of cellular lipid accumulation. The findings of Sparks et al. (2005) have not always been repeatable. Turner et al. (2007) fed the same strain of mice a very similar high fat diet (with respect to % fat, protein, and carbohydrate). After 5 or 20 weeks, fat fed mice had higher palmitoyl-CoA oxidation in isolated mitochondria, as well as increased oxidative enzyme activity and PGC-1α when compared to controls fed standard chow. Interestingly, high fat fed mice still had higher circulating insulin, impaired glucose tolerance, and reduced insulin-stimulated glucose clearance. Furthermore, differences in the metabolic syndrome phenotype often do not emerge in 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 still showed an appreciable increased HDL-C after 17 weeks on an atherogenic diet (15% dairy fat, 50% sucrose, 20% casein, 0.5% cholic acid, 1.0% cholesterol). In the same study, males from four strains showed a decrease in plasma TG after 17 weeks. For both sexes, this wide array of among-strain responses is mirrored in other traits composing the metabolic syndrome (Albers et al., 1999; Svenson et al., 2007).

Removal of an adverse stimulus, such as WD, helps reduce body mass; however, this is not sufficient to restore normal body weight in mice (Guo et al. 2009). The trajectories of body mass for the different diet groups in our study are significantly different (Appendix 2.8, time x diet interaction $P < 0.0001$). The progression of body

mass in Figure 1 shows that mice fed standard diet did not simply have slower mass gain compared to mice eating Western diet. Rather, body mass appears to plateau with standard diet but continually trend upwards in mice fed WD, suggesting a fundamental difference in body mass regulation between these two diets. Overweight and obese adolescent humans typically remain in their respective weight categories well into adulthood, thus indicting 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 is essential.

The effects of wheel access and Western diet were consistent throughout our study and the trajectories of each group, while only modestly different during the juvenile period, resulted in substantial differences after only eight weeks. By the end of the study, Western diet increased body mass by 22% and wheel running prevented weight gain by 12% when the groups are pooled (Figure 2.1). A comparison of the most extreme groups, in terms of genetic and environmental factors that might affect body fat (control linetype, sedentary environment, WD *versus* HR linetype, wheel access, and SD), shows a 49% difference in body mass (42g vs. 28g; Figure 2.1) and a 454% difference in retroperitoneal fat mass (2.0g vs. 0.36g; Figure 2.4). These dramatic differences emphasize the importance of understanding longitudinal development and how genetic and environmental influences can significantly change the shape of physiological and behavioral ontogenies (Morgan et al., 2003).

Several of our findings are relevant to understanding the complicated interrelationships of energy intake, diet, voluntary exercise, and energy balance (review in Garland et al., 2011b). The effects of WD were nearly omnipresent, as almost every trait studied, at some time point, showed a significant response. Western diet is obesogenic, as it increased the mass of the retroperitoneal fat pad (see also Meek et al., 2010) and liver, even when adjusting for the increased caloric intake (Figure 2.4). This implies either that WD is absorbed or metabolized differently, and/or that energy expenditure is affected by Western diet. 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 had a large effect on body mass and its ontogeny. Exercise prevented fat mass gain and helped to prevent hyperphagia on Western diet. 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. 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 weight.

Chapter 2 Acknowledgements

Supported by MSU Seed Grant to JCE and NSF IOB-0543429 and IOS-1121273 to TG.

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Table 2.1 *P* values from analysis of variance or analysis of covariance (ANCOVA) for body mass or body length.

	Week 0 (n = 194)		Week 1 (n = 195)		Week 2 (n = 198)		Week 4 (n = 198)	Week 6 (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+**		**<0.0001+**		
Age	0.0126+	0.0088+	0.0299+	0.2942+	0.4462+	0.5276+		

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 to rump (not anesthetized). 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 (results not shown). + 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.

Table 2.1 Continued

	Week 8 (n = 197)		Week 8 (n = 197) (*NR covariate)	Body Length Measures: Week 8 (n = 198) (#nose-rump)	Body Length Measures: Week 8 (n = 197) (#crown-rump)
	P	*P*	*P*	*P*	*P*
Linetype (HR vs. C)	0.1715-	0.2371-	0.2211-	0.1128-	0.0613-
Wheel access (WhlAcc)	**0.0096-**	**0.0243-**	**0.0158-**	**0.0136-**	**0.0068-**
Diet	**0.0023+**	**0.0027+**	**0.0021+**	0.0965+	0.0586+
Mini-muscle	0.1625-	0.2183-	0.2051-	0.8334-	0.8736-
Linetype X WhlAcc	0.6990	0.3614	0.3853	0.0621	0.0558
Linetype X Diet	0.6216	0.5915	0.5789	0.9939	0.9428
Wheel access X Diet	**0.0183**	**0.0365**	**0.0334**	0.1486	0.1132
Linetype X WhlAcc X Diet	0.6227	0.5152	0.6668	0.8456	0.4424
Body length		**<0.0001+**	**<0.0001+**		

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 to rump (not anesthetized). 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 (results not shown). + 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. These measures were taken on anesthetized mice. Note that wheel access significantly reduced body length by the end of the study.

Table 2.2 F and *P* values from ANCOVAs for caloric intake (kJoule/day)

	Week 1 (n = 188)		Week 2 (n = 196)		Weeks 3 & 4 (n = 192)		Weeks 5 & 6 (n = 193)	
	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+**
Wheel access (WhlAcc)	0.86	0.3893-	7.69	**0.0323+**	23.41	**0.0029+**	39.89	**0.0007+**
Diet	180.40	**<0.0001+**	37.00	**0.0009+**	5.45	0.0583+	5.40	0.0592+
Mini-muscle	3.11	0.0798+	6.34	**0.0128+**	6.89	**0.0095+**	1.17	0.2817+
Linetype X WhlAcc	0.03	0.8618	6.80	**0.0403**	2.31	0.1797	8.04	**0.0298**
Linetype X Diet	0.01	0.9195	2.69	0.1520	2.88	0.1407	1.59	0.2540
Wheel access X Diet	11.84	**0.0138**	3.52	0.1096	6.04	**0.0492**	9.96	**0.0197**
Linetype X WhlAcc X Diet	0.07	0.7935	1.88	0.2192	1.48	0.2696	3.84	0.0977
log Body mass	239.28	<0.0001+	105.78	<0.0001+	89.13	<0.0001+	56.93	<0.0001+
Age	0.01	0.9128-	1.09	0.2990-	2.55	0.1124-		

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). Food consumption analyzed as log (kilojoules per day). Age was an additional covariate in weeks 1, 2, and 3&4. + indicates direction HR > C, wheel access > sedentary, Western diet > standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (S 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.

Table 2.2 Continued

	Weeks 7 & 8 (n = 193)	
	F	*P*
Linetype (HR vs. C)	2.61	0.1573+
Wheel access (WhlAcc)	16.12	**0.0070+**
Diet	6.43	**0.0443+**
Mini-muscle	0.02	0.8953+
Linetype X WhlAcc	6.86	**0.0397**
Linetype X Diet	0.14	0.7253
Wheel access X Diet	10.07	**0.0192**
Linetype X WhlAcc X Diet	2.81	0.1444
log Body mass	34.38	<0.0001+

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). Food consumption analyzed as log (kilojoules per day). Age was a covariate in weeks 1, 2, and 3 (results not shown). + indicates direction HR > C, wheel access > sedentary, Western diet > standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (S 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 values ($P < 0.05$) are in bold.

Table 2.3 *P* values from ANCOVA for fasting plasma metabolites

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *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
Lintype 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
Lintype x Whlacc x Diet		0.2189	0.7691	0.4851

Table 2.3 continued. *P* values from ANCOVA for fasting plasma metabolites

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
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
Lintype 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
Lintype x Whlacc x Diet		0.9522	0.2346	0.5224

Table 2.3 continued. *P* values from ANCOVA for fasting plasma metabolites

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
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
Lintype x Whlacc x Diet		0.9753	0.6290	0.8200

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. It was 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.

Table 2.4 *P* values from ANCOVAs for organ masses with body length (crown-rump) and caloric intake.

	log Retroperitoneal fat N = 196		Triceps surae N = 194		log Ventricles N = 194		log Liver N = 197	
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Linetype (HR vs. C)	0.2066-	0.1953-	0.9545-	0.9220-	0.9713+	0.9346-	0.2949-	0.2690-
Wheel access (WhlAcc)	**0.0020-**	**0.0014-**	0.6347+	0.8053-	**0.0147+**	**0.0416+**	**0.0302-**	**0.0108-**
Diet	**<0.0001+**	**<0.0001+**	**0.0011+**	**0.0033+**	**0.0028+**	**0.0133+**	**0.0023+**	**0.0046+**
Mini-muscle	0.1012+	0.0945	**<.0001-**	**<.0001-**	0.6247+	0.7134+	0.9099+	0.8767+
Linetype X WhlAcc	0.0743	**0.0487**	0.6111	0.2417	0.1929	0.4574	0.8480	0.4352
Linetype X Diet	0.6000	0.6280	**0.0406**	**0.0288**	0.1447	0.1093	0.9215	0.9216
Wheel access X Diet	0.2435	0.4109	0.5293	0.6060	0.2023	0.7081	**0.0058**	**0.0148**
Linetype X WhlAcc X Diet	0.4573	0.3613	0.5711	0.2761	0.3210	0.5568	0.7599	0.3546
Log body length (CR)	**0.0005+**	**0.0031+**	**<.0001+**	**0.0003+**	**<.0001+**	**0.0213+**	**<.0001+**	**0.0022+**
Log Caloric Intake		**0.1688+**		**0.0022+**		**<.0001+**		**0.0020+**

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 (crown to rump length), and caloric intake. Log values were analyzed except triceps surae which had no transform. + 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), diet, and their interaction. 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.

Table 2.4B *P* values from ANCOVAs for organ masses with body length (nose-rump) and caloric intake.

	log Retroperitoneal fat N = 196		Triceps surae N = 198		log Ventricles N = 198		log Liver N = 198	
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Linetype (HR vs. C)	0.2165-	0.2024-	0.9276-	0.8942-	0.9152-	0.8564-	0.2892-	0.2542-
Wheel access (WhlAcc)	**0.0016-**	**0.0011-**	0.7863+	0.5987-	**0.0146+**	**0.0387+**	**0.0192-**	**0.0070-**
Diet	**<.0001+**	**<.0001+**	**0.0008+**	**0.0029+**	**0.0021+**	**0.0107+**	**0.0025+**	**0.0049+**
Mini-muscle	0.1100+	0.1009+	**<.0001-**	**<.0001-**	0.6709+	0.7572+	0.9812+	0.9400+
Linetype X WhlAcc	0.0579	**0.0390**	0.6294	0.2465	0.1903	0.5026	0.9014	0.4546
Linetype X Diet	0.6109	0.6547	0.0367	0.0299	0.1507	0.1266	0.9185	0.9407
Wheel access X Diet	0.2386	0.4089	0.4829	0.6009	0.1984	0.6493	**0.0051**	**0.0151**
Linetype X WhlAcc X Diet	0.5273	0.4222	0.7357	0.3766	0.2600	0.5270	0.9351	0.4476
Log body length (NR)	**<.0001+**	**0.0003+**	**<.0001+**	**0.0007+**	**0.0003+**	**0.0268+**	**0.0006**	**0.0128+**
Log Caloric Intake		0.1701+		**0.0012+**		**<.0001+**		**0.0009+**

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), and caloric intake. Log values were analyzed except triceps surae which had no transform. + 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), diet, and their interaction. 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.

Figure 2.1

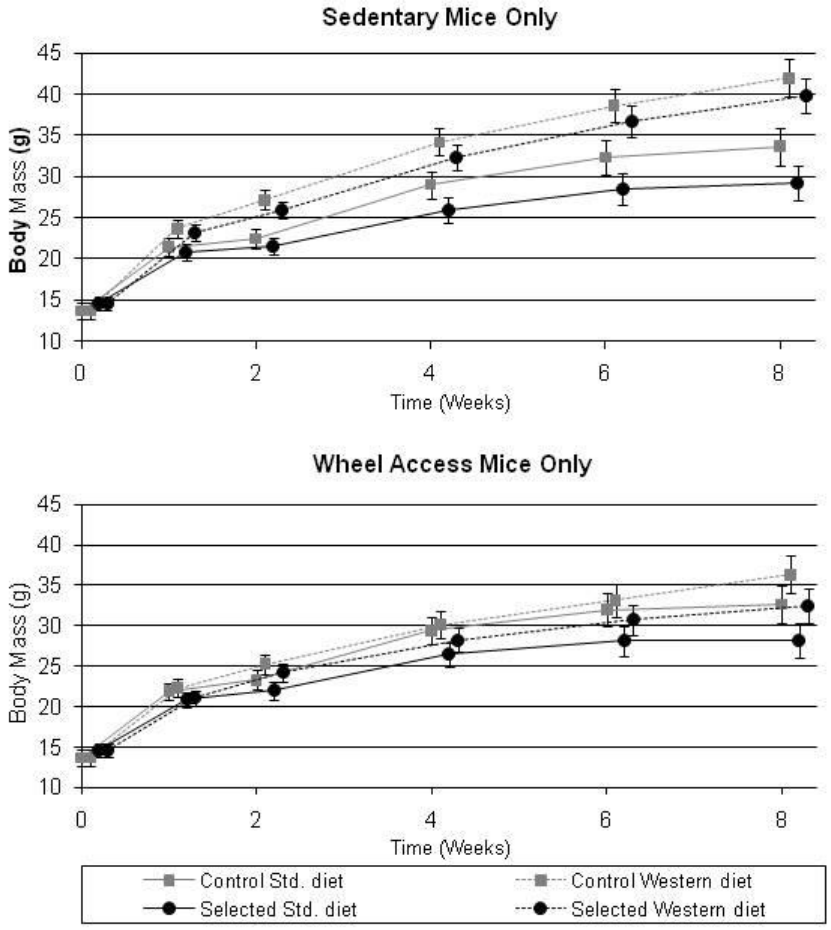


Figure 2.1 Least squares means and standard errors for body mass of selected (high runner- HR) and control (C) mice housed with and without wheels. At week 0 of experiment mice were 24 days old, and separated into diet groups. Points are slightly staggered along the X-axis for visual clarity of standard errors. See Table 2.1 for statistical results.

Figure 2.2

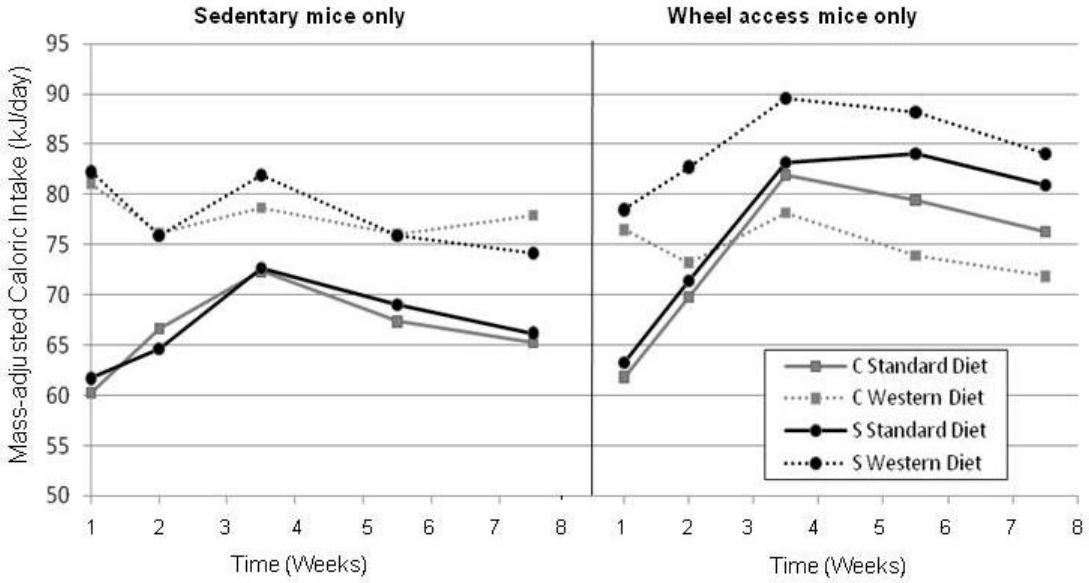


Figure 2.2 Mass-adjusted caloric intake (ANCOVA) as measured in kJ per day. Values are least squares means, back transformed from log scale. The left panel shows caloric intake for sedentary mice and the right panel for wheel-access mice only. Standard errors are not presented, because back-transformed values require separate upper and lower confidence limits, rather than standard errors, which encumbers the visual presentation. See Table 2.2 for statistical results.

Figure 2.3

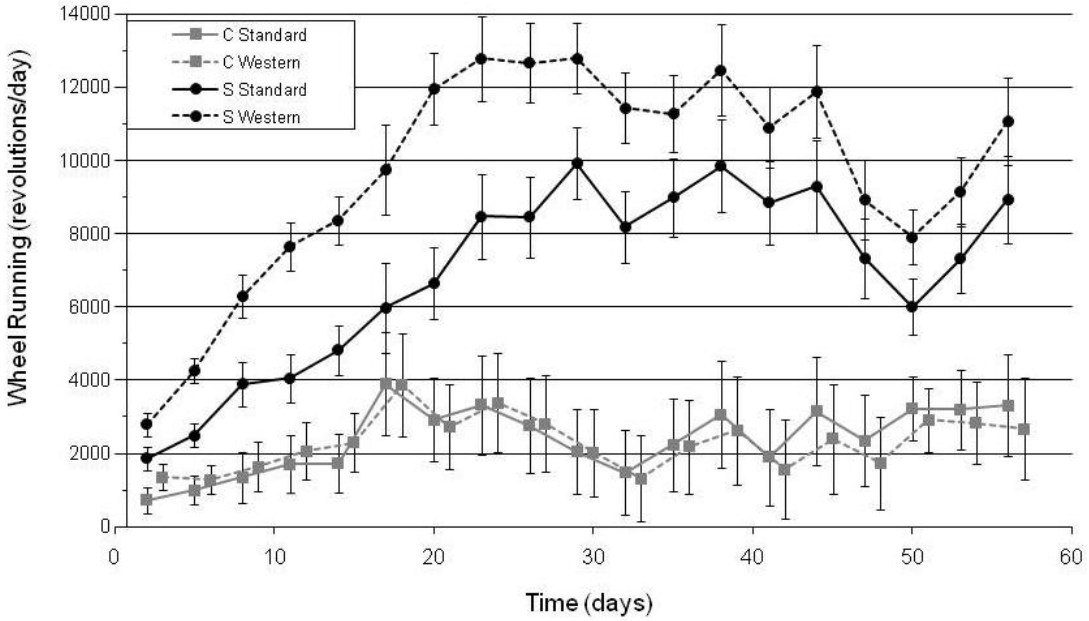


Figure 2.3 Least squares means and standard errors for wheel running for both C (points staggered along the X-axis for clarity) and HR mice on both diets condensed into 3 day averages. HR (selected) mice ran substantially more than C mice when on standard diet, but this differential was greatly increased on Western diet (Significant Linetype by diet interaction at nearly all points between day 10 and 50).

Figure 2.4

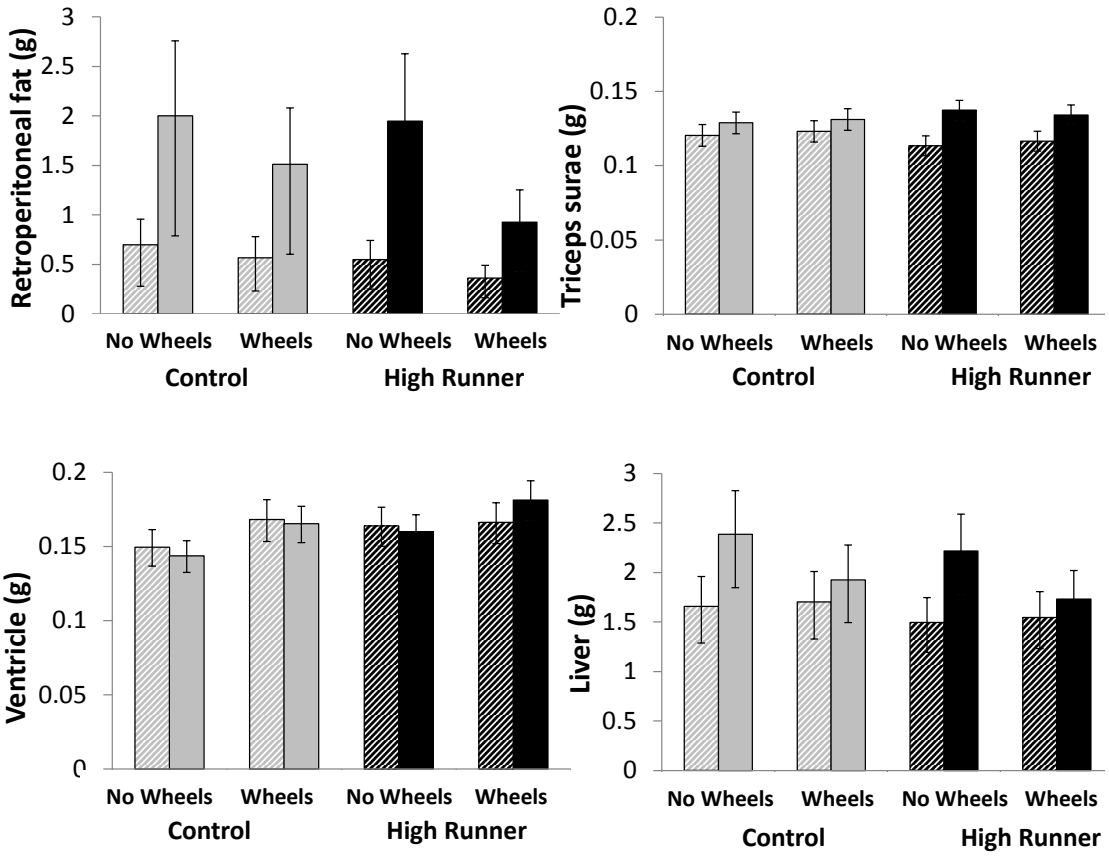


Figure 2.4 Least square means for organ masses for all eight groups. Values for fat pad mass, ventricle mass, and liver mass are back-transformed from values obtained from analysis of log-transformed data, with asymmetrical 95% confidence intervals. Values for triceps surae mass were not log-transformed, so bars represent one standard errors. Hashed bars represent mice fed standard diet and solid bars represent those fed Western diet. See Table 2.4 for results of statistical analyses.

Supplemental Table 2.1 *P* values from ANCOVA for Body Mass of Wheel-Access Mice Only

	Week 1 (n = 94)		Week 2 (n = 98)		Week 3 (n = 99)		Week 4&5 (n = 100)	Week 6&7 (n = 99)	Week 8 (n = 99)	
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Linetype (HR vs. C)	0.5521+	0.6310+	0.3003-	0.3463-	0.3003-	0.3025-	0.1606-	0.1984-	0.1540-	0.1673-
Diet			0.7372+	0.9796+	**0.0388+**	**0.0386+**	0.1085+	0.0692+	**0.0198+**	**0.0368+**
Mini-muscle	0.3977-	0.2886-	0.4302-	0.3902-	0.1390-	0.1853-	0.4014-	0.1707-	0.3962-	0.4373-
Linetype X Diet			0.9001	0.5688	0.7948	0.8835	0.7438	0.7316	0.8496	0.9488
Body length		**0.0014+**		**<0.0001+**		**<0.0001+**				**0.0004+**

Results from nested ANCOVA with categorical factors of linetype (HR vs. C), standard or Western diet, and mini-muscle status, and covariates of body length measured as crown (between the eyes) to rump length . 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 (results not shown). + indicates direction HR > C, 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 79. All *P* values are 2 tailed. Significant main effect values ($P < 0.05$) are in bold.

Supplemental Table 2.2 *P* values from ANCOVA for Body Mass of Sedentary Mice Only

	Week 1 (n = 100)		Week 2 (n = 98)		Week 3 (n = 99)		Week 4&5 (n = 99)	Week 6&7 (n = 99)	Week 8 (n = 99)	
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Linetype (HR vs. C)	0.6024+	0.5518+	0.9347-	0.8679-	0.4260-	0.4784-	0.2460-	0.1397-	0.1149-	0.1807-
Diet			**0.0249+**	**0.0322+**	**0.0019+**	**0.0012+**	**0.0047+**	**0.0048+**	**0.0021+**	**0.0021+**
Mini-muscle	0.2662-	0.2715-	0.3951-	**0.5217-**	0.4113-	**0.3508-**	0.3325-	0.8301-	0.9267-	0.6026+
Linetype X Diet			0.7822	0.3146	0.7678	0.8121	0.6831	0.6048	0.5882	0.4683
Body length		0.2296+		**<0.0001+**		**0.0103+**				**<0.0001+**

Results from nested ANCOVA with categorical factors of linetype (HR vs. C), standard or Western diet, and mini-muscle status, and covariates of body length measured as crown to rump length . 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 (results not shown). + indicates direction HR > C, 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 79. All *P* values are 2 tailed. Significant main effect values ($P < 0.05$) are in bold.

Supplemental Table 2.3 *P* values from ANCOVA for Body Mass of Standard Diet Mice Only

	Week 1 (n = 99)		Week 2 (n = 100)		Week 3 (n = 101)		Week 4&5 (n = 102)	Week 6&7 (n = 101)	Week 8 (n = 101)	
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Linetype (HR vs. C)	0.5931+	0.5668+	0.5296-	0.9470-	0.1720-	0.1947-	0.0593-	**0.0397-**	**0.0377-**	**0.0457-**
Wheel Access			0.5156+	0.4635+	0.4266+	0.3269+	0.5740+	0.5503-	0.1838-	0.3104-
Mini-muscle	0.3658-	0.3655-	0.9608-	0.7902-	0.3987-	0.4339-	0.5661-	0.4365-	0.3754-	0.4530-
Linetype X Wheel Access			0.5553	0.4355	0.7532	0.9886	0.9558	0.9975	0.9527	0.6637
Body length		0.4552		**<0.0001+**		**<0.0001**				**<0.0001+**

Results from nested ANCOVA with categorical factors of linetype (HR vs. C), wheel access or sedentary environment, and mini-muscle status, and covariates of body length measured as crown to rump length . 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 (results not shown). + indicates direction HR > C, wheel access > sedentary, 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 79. All *P* values are 2 tailed. Significant main effect values ($P < 0.05$) are in bold.

Supplemental Table 2.4 *P* values from ANCOVA for Body Mass of High Fat Diet Mice Only

	Week 1 (n = 95)		Week 2 (n = 96)		Week 3 (n = 97)		Week 4&5 (n = 97)	Week 6&7 (n = 97)	Week 8 (n = 96)	
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Linetype (HR vs. C)	0.5196+	0.5434+	0.6847-	0.5624-	0.6631-	0.7071-	0.4680-	0.4945-	0.3936-	0.5581-
Wheel Access			**0.0270-**	**0.0351-**	**0.0104-**	**0.0125-**	**0.0128-**	**0.0130-**	**0.0096-**	**0.0369-**
Mini-muscle	0.2478-	0.2674-	0.1167-	0.1819-	**0.0387-**	**0.0282-**	0.1659-	0.2094-	0.4548-	0.5171-
Linetype X Wheel Access			0.4992	0.6528	0.8807	0.9869	0.8020	0.6931	0.6455	0.3686
Body length		**0.0078+**		**<0.0001+**		**0.0004**				**0.0003+**

Results from nested ANCOVA with categorical factors of linetype (HR vs. C), wheel access or sedentary environment, and mini-muscle status, and covariates of body length measured as crown to rump length . 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 (results not shown). + indicates direction HR > C, wheel access > sedentary, 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 79. All *P* values are 2 tailed. Significant main effect values ($P < 0.05$) are in bold.

Supplemental Table 2.5 *P* values from ANCOVA for Caloric Intake for Sedentary Mice Only

	Week 1 (n = 99) *P*	Week 2 (n = 99) *P*	Week 3&4 (n = 94) *P*	Week 5&6 (n = 94) *P*	Week 7&8 (n = 96) *P*
Linetype (HR vs. C)	0.1607+	0.7795+	0.4560+	0.6352+	0.6436-
Wheel Access	**<0.0001+**	**0.0013+**	**0.0224+**	**0.0105+**	**0.0146+**
Mini-muscle	0.7699-	0.4878+	0.3015+	0.2674+	0.9769-
Linetype X Wheel Access	0.9975	0.8743	0.6163	0.6049	0.4761
Log Body Mass	**<.0001+**	**<.0001+**	**<.0001+**	**<.0001+**	**0.0008+**

Results from nested ANCOVA with categorical factors of linetype (HR vs. C), standard or Western diet, and mini-muscle status, and covariates of body length measured as crown to rump length . 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 (results not shown). + indicates direction HR > C, 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 79. All *P* values are 2 tailed. Significant main effect values ($P < 0.05$) are in bold.

Supplemental Table 2.6 *P* values from ANCOVA for Caloric Intake for Standard Diet Mice Only

	Week 1 (n = 95) *P*	Week 2 (n = 100) *P*	Week 3&4 (n = 99) *P*	Week 5&6 (n = 101) *P*	Week 7&8 (n = 95) *P*
Linetype (HR vs. C)	0.2925+	0.7386+	0.2319+	**0.0188+**	0.0627+
Wheel Access	0.2796+	**0.0189+**	**0.0001+**	**<0.0001+**	**0.0002+**
Mini-muscle	0.3221+	0.0618+	0.2858+	0.8842-	0.5396-
Linetype X Wheel Access	0.7755	0.4046	0.7295	0.3336	0.3310
Log Body Mass	**<.0001+**	**<.0001+**	**<.0001+**	**<.0001+**	**0.0018+**

Results from nested ANCOVA with categorical factors of linetype (HR vs. C), standard or Western diet, and mini-muscle status, and covariates of body length measured as crown to rump length . 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 (results not shown). + indicates direction HR > C, 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 79. All *P* values are 2 tailed. Significant main effect values ($P < 0.05$) are in bold.

Supplemental Table 2.7 *P* values from ANCOVA for Caloric Intake for High Fat Diet Mice Only

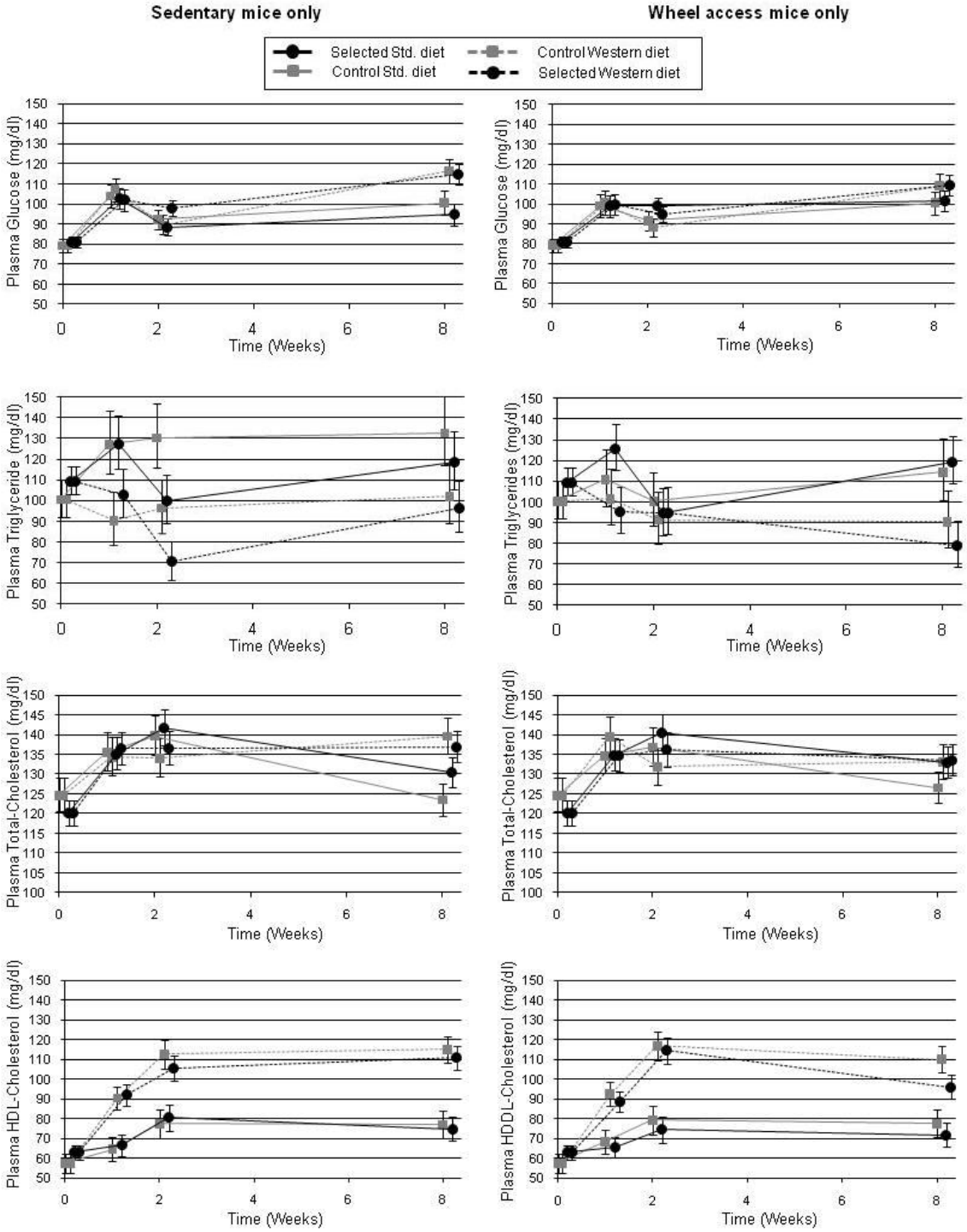
	Week 1 (n = 93) *P*	Week 2 (n = 97) *P*	Week 3&4 (n = 92) *P*	Week 5&6 (n = 90) *P*	Week 7&8 (n = 93) *P*
Linetype (HR vs. C)	0.1867+	0.4515+	0.1858+	0.0524+	0.3769-
Wheel Access	**0.0041-**	0.4610+	0.5895+	0.3286+	0.9997=
Mini-muscle	0.9876+	0.0797+	**0.0323+**	0.3791+	0.5555+
Linetype X Wheel Access	0.7797	**0.0388**	0.1401	**0.0249**	**0.0426**
Log Body Mass	**<.0001+**	**<.0001+**	**<.0001+**	**<.0001+**	**0.0018+**

Results from nested ANCOVA with categorical factors of linetype (HR vs. C), standard or Western diet, and mini-muscle status, and covariates of body length measured as crown to rump length . 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 (results not shown). + indicates direction HR > C, 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 79. All *P* values are 2 tailed. Significant main effect values ($P < 0.05$) are in bold.

Supplemental Table 2.8 *P* values from ANCOVA for caloric intake (kJoule/day) wheel access mice only

	Week 1 (n = 92) *P*	Week 2 (n = 99) *P*	Weeks 3 & 4 (n = 98) *P*	Weeks 5 & 6 (n = 98) *P*	Weeks 7 & 8 (n = 98) *P*
HR vs. C	0.8699 [+]	0.4531 [+]	0.8238 [+]	0.7614 [-]	0.8990 [-]
Diet	**<0.0001 [+]**	**0.0228 [+]**	0.2834 [-]	**0.0088 [-]**	**0.0216 [-]**
Linetype X Diet	0.7966	0.0907	0.0734	0.0517	0.2219
Mini-muscle	0.1583 [+]	**0.0028 [+]**	**0.0083 [+]**	**0.0111 [+]**	0.1225 [+]
log Body mass	**<0.0001 [+]**	**<0.0001 [+]**	**<0.0001 [+]**	**<0.0001 [+]**	**<0.0001 [+]**
Wheel running	0.2124 [+]	0.1980 [+]	**<0.0001 [+]**	**<0.0001[+]**	**<0.0001 [+]**

Results from nested ANCOVA in SAS Procedure Mixed with categorical factors of linetype (HR vs. C), diet, and mini-muscle status, and covariates of body mass and wheel running. Replicate line was included as a random effect in all analyses. Food consumption analyzed as log (kilojoules per day). Log body mass was a covariate in all analyses, and age was a covariate in weeks 1, 2, 3, and 4 (results not shown). + indicates direction HR > C, Western diet > Standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (HR vs. C), diet, and their interaction. For tests of mini muscle, log-transformed body mass, and wheel running, d.f. are 1 and approximately 78. All *P* values are 2 tailed. Two outliers were removed from week 1 analysis and a single outlier was removed from all other weeks.



Supplemental Figure 2.1 Least squares means and standard errors at four time points for fasting plasma metabolites. Data are split into sedentary mice (left graph) and wheel-access mice (right graph) for fasting plasma glucose, triglycerides, total cholesterol, and HDL cholesterol. See Table 3 for corresponding statistical tests.

Appendix 2.1 Least-squares (adjusted) means and 95% CI corresponding to tests in Supplemental table 2.2. See also Figure 2.4.

		Sedentary		Active	
	Linetype	Std. Diet	Western diet	Std. Diet	Western diet
Retroperitoneal fat (g)	HR	0.5483 (0.1933, 0.2986)	1.9454 (0.6825, 1.0513)	0.3616 (0.1284, 0.1991)	0.9270 (0.3256, 0.5019)
	C	0.6958 (0.2609, 0.4174)	2.0026 (0.7561, 1.2148)	0.5681 (0.2116, 0.3371)	1.5122 (0.5684, 0.9106)
Triceps surae (g)	HR	0.1134 (0.0067)	0.1373 (0.0067)	0.1165 (0.0067)	0.1342 (0.0067)
	C	0.1204 (0.0073)	0.1288 (0.0073)	0.1231 (0.0072)	0.1311 (0.0073)
Ventricles (g)	HR	0.1638 (0.0126, 0.0137)	0.1597 (0.0116, 0.0125)	0.1662 (0.0132, 0.0143)	0.1813 (0.0130, 0.0140)
	C	0.1495 (0.0118, 0.0128)	0.1435 (0.0103, 0.0111)	0.1680 (0.0135, 0.0147)	0.1652 (0.0118, 0.0127)
Liver (g)	HR	1.4952 (0.2511, 0.3018)	2.2192 (0.3703, 0.4445)	1.5453 (0.2612, 0.3143)	1.7306 (0.2893, 0.3474)
	C	1.6569 (0.3024, 0.3699)	2.3862 (0.4403, 0.5399)	1.7026 (0.3075, 0.3752)	1.9253 (0.3525, 0.4315)

Results are from corresponding table S2 with only body length (crown to rump length) as a covariate. Values presented are adjusted means with asymmetric 95% confidence intervals, except for triceps surae where standard errors are shown.

Appendix 2.2 P values from ANCOVA for fasting plasma metabolites for Wheel-access mice only

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
Glucose	(n = 175)	(n = 86)	(n = 85)	(n = 96)
Linetype (HR vs. C)	0.6780 [+]	0.9363 [-]	0.2845[+]	0.8297[+]
Diet		0.6347 [+]	0.2105[-]	0.0497[+]
Mini-muscle	0.7626 [-]	0.2923 [+]	0.9926[-]	0.2648[-]
Linetype x Diet		0.9352 [+]	0.9036[-]	0.8571[+]
S2AGE		0.8874 [+]	0.0851[-]	
FTIME2		0.9849 [+]	0.8182[-]	0.4628 [-]
LBLEED2		0.7950 [-]	0.7513[-]	0.0267 [+]
Triglycerides (Square root)	(n = 176)	(n = 93)	(n = 85)	(n = 94)
Linetype (HR vs. C)	0.6780 [+]	0.8573 [+]	0.9064 [-]	0.8029[-]
Diet		0.1328[-]	0.7072 [-]	0.0625[-]
Mini-muscle	0.7626 [-]	0.6412[+]	0.4950 [+]	0.3593[+]
Linetype x diet		0.3880[-]	0.6730 [-]	0.5702[-]
S2AGE		0.7922[-]	0.8370 [-]	
FTIME2		0.7854[-]	0.6178 [+]	0.5685[+]
Cholesterol (Log)	(n = 177)	(n = 88)	(n = 84)	(n = 97)
Linetype (HR vs. C)	0.6780 [+]	0.6381[-]	0.6201 [+]	0.5631[+]
Diet		0.4284[+]	0.2469 [-]	0.4654[+]
Mini-muscle	0.7626 [-]	0.4598[-]	0.4047 [-]	0.5469[-]
Linetype x Diet		0.5508[+]	0.9818 [-]	0.4699[+]
S2AGE		0.1587[-]	0.7969 [-]	
FTIME2		0.3981[+]	0.0913[+]	0.3235 [+]
HDL-Cholesterol	(n = 183)	(n = 95)	(n = 97)	(n = 97)
HR vs. C	0.3438 [+]	0.8475 [-]	0.7935[-]	0.2493[-]
Mini-muscle	0.3155 [-]	0.2927 [+]	0.9302[+]	0.0003[+]
Diet		0.0001[-]	<.0001[-]	0.4418[+]
Linetype x Diet		0.8126[+]	0.6923[+]	0.2596[+]
S2AGE		0.2946[+]	0.3213[+]	
FTIME2		0.7294[+]	0.6872[+]	0.1363 [+]

Appendix 2.2 continued. P values from ANCOVA for fasting plasma metabolites for Wheel-access mice only

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
Non HDL-Chol (LDL+VLVL)	(n = 175)	(n = 87)	(n = 85)	(n = 96)
Linetype (HR vs. C)	0.6780 [+]	0.6953[-]	0.8351[+]	0.1861[+]
Diet		0.0041[-]	0.0001[-]	0.0032[-]
Mini-muscle	0.7626 [-]	0.3201[+]	0.8081[-]	0.3917[-]
Linetype x Diet		0.8112[-]	0.9517[-]	0.8967[-]
FTIME2		0.6887[+]	0.5685[+]	
S2AGE		0.0154[-]	0.1595[-]	0.9308 [-]

Appendix 2.3 P values from ANCOVA for fasting plasma metabolites for Sedentary mice only

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
Glucose	(n = 175)	(n = 86)	(n = 81)	(n = 94)
Linetype (HR vs. C)	0.6780 [+]	0.9363 [-]	0.3935[+]	0.4760[-]
Diet		0.6347 [+]	0.1897[+]	0.0197[+]
Mini-muscle	0.7626 [-]	0.2923 [+]	0.0812[-]	0.4697[-]
Linetype x Diet		0.9352 [+]	0.0667[-]	0.8593[+]
S2AGE		0.8874 [+]	0.1266[-]	
FTIME2		0.9849 [+]	0.0096[-]	0.2455 [-]
LBLEED2		0.7950 [-]	0.4529[-]	0.1150 [+]
Triglycerides (Square root)	(n = 176)	(n = 92)	(n = 86)	(n = 94)
Linetype (HR vs. C)	0.6780 [+]	0.4649 [+]	0.0821 [-]	0.5954[-]
Diet		0.0175[-]	0.0215 [-]	0.0194[-]
Mini-muscle	0.7626 [-]	0.7337[+]	0.5330 [+]	0.5824[+]
Linetype x diet		0.5169[-]	0.9966 [-]	0.6909[-]
S2AGE		0.0713[-]	0.2091 [-]	
FTIME2		0.0503[-]	0.1964 [+]	0.0039 [-]
Cholesterol (Log)	(n = 177)	(n = 81)	(n = 82)	(n = 95)
Linetype (HR vs. C)	0.6780 [+]	0.6729[-]	0.4183 [+]	0.3959[+]
Diet		0.7289[+]	0.2653 [-]	0.0158[+]
Mini-muscle	0.7626 [-]	0.8353[-]	0.0283 [-]	0.0774[-]
Linetype x Diet		0.8088[+]	0.9379 [-]	0.2030[+]
S2AGE		0.0045[+]	0.9114 [+]	
FTIME2		0.1318[+]	0.5689[-]	0.6979 [+]
HDL-Cholesterol	(n = 183)	(n = 91)	(n = 98)	(n = 97)
HR vs. C	0.3438 [+]	0.7268 [+]	0.9260[-]	0.2493[+]
Mini-muscle	0.3155 [-]	0.6957 [+]	0.9161[+]	0.0003[+]
Diet		0.0006[-]	0.0002[-]	0.4418[+]
Linetype x Diet		0.9793[+]	0.1969[+]	0.2596[+]
S2AGE		0.4694[-]	0.7388[-]	
FTIME2		0.0950[+]	0.0846[+]	0.1363 [+]

Appendix 2.3 continued P values from ANCOVA for fasting plasma metabolites for Sedentary mice only

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
Non HDL-Chol (LDL+VLVL)	(n = 175)	(n = 80)	(n = 82)	(n = 92)
Linetype (HR vs. C)	0.6780 [+]	0.5435[-]	0.6878[+]	0.4366[+]
Diet		0.0043[-]	0.0005[-]	0.0004[-]
Mini-muscle	0.7626 [-]	0.6193[+]	0.3174[-]	0.5601[-]
Linetype x Diet		0.9573[-]	0.3872[-]	0.5023[-]
FTIME2		0.8827[-]	0.0657[-]	
S2AGE		0.0141[+]	0.8482[+]	0.0369 [+]

Appendix 2.4 P values from ANCOVA for fasting plasma metabolites for Standard diet only

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
Glucose	(n = 175)	(n = 87)	(n = 84)	(n = 97)
Linetype (HR vs. C)	0.6780 [+]	0.7732 [+]	0.8511[+]	0.3408[-]
Wheel access		0.2442[-]	0.0796[+]	0.1972[+]
Mini-muscle	0.7626 [-]	0.7124 [-]	0.7029[-]	0.2122[+]
Linetype x Whlacc		0.7009[-]	0.0503[-]	0.2622[+]
S2AGE		0.3133[+]	0.1150[-]	
FTIME2		0.4644 [-]	0.3299[-]	0.1233 [-]
LBLEED2		0.4794 [-]	0.9287[-]	0.6767 [+]
Triglycerides (Square root)	(n = 176)	(n = 94)	(n = 84)	(n = 95)
Linetype (HR vs. C)	0.6780 [+]	0.6018 [+]	0.1973 [-]	0.5679[-]
Wheel access		0.3169[-]	0.1931 [-]	0.2396[-]
Mini-muscle	0.7626 [-]	0.9344[-]	0.4309 [+]	0.3783[+]
Linetype x Whlacc		0.4782[-]	0.3200 [-]	0.7388[-]
S2AGE		0.3246[-]	0.9952 [+]	
FTIME2		0.9857[+]	0.0970 [+]	0.0652 [-]
Cholesterol (Log)	(n = 177)	(n = 89)	(n = 84)	(n = 98)
Linetype (HR vs. C)	0.6780 [+]	0.6692 [+]	0.7339 [+]	0.0960 [+]
Wheel access		0.8071 [-]	0.5842 [-]	0.3607 [+]
Mini-muscle	0.7626 [-]	0.1165 [-]	0.4529 [-]	0.2748 [-]
Linetype x Whlacc		0.9709[-]	0.8466 [-]	0.9233 [+]
S2AGE		0.4775 [-]	0.5731 [-]	
FTIME2		0.5888 [+]	0.2054 [+]	0.0085 [+]
HDL-Cholesterol	(n = 183)	(n = 95)	(n = 100)	(n = 99)
HR vs. C	0.3438 [+]	0.7788 [+]	0.9055[-]	0.5722[-]
Mini-muscle	0.3155 [-]	0.2352 [+]	0.4419[-]	0.5936[-]
Wheel access		0.5991[-]	0.7441[+]	0.6900[+]
Linetype x Whlacc		0.5338[+]	0.2282[+]	0.5223[+]
S2AGE		0.8846[-]	0.9119[-]	
FTIME2		0.5283[+]	0.1976[+]	0.7436 [-]

Appendix 2.4 continued P values from ANCOVA for fasting plasma metabolites for Standard diet only

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
Non HDL-Chol (LDL+VLVL)	(n = 175)	(n = 87)	(n = 84)	(n = 96)
Linetype (HR vs. C)	0.6780 [+]	0.8459 [-]	0.9661[+]	0.2861 [+]
Wheel access		0.6617 [-]	0.8777[+]	0.3624 [+]
Mini-muscle	0.7626 [-]	0.8100 [+]	0.8225[-]	0.6347 [-]
Linetype x Whlacc		0.9237 [-]	0.6882[-]	0.5312 [+]
FTIME2		0.9582 [+]	0.6866[-]	
S2AGE		0.4396 [-]	0.2839[-]	0.0384 [+]

Appendix 2.5 P values from ANCOVA for fasting plasma metabolites for Western diet only

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
Glucose	(n = 175)	(n = 79)	(n = 82)	(n = 93)
Linetype (HR vs. C)	0.6780 [+]	0.6866 [-]	0.2291 [+]	0.8005[+]
Wheel access		0.2144[+]	0.4274[+]	0.3028[-]
Mini-muscle	0.7626 [-]	0.3557 [-]	0.5972 [-]	0.0325[-]
Linetype x Whlacc		0.6370[-]	0.8119 [-]	0.7230[-]
S2AGE		0.6295[-]	0.3006 [-]	
FTIME2		0.1247 [+]	0.2017[-]	0.6783[-]
LBLEED2		0.7561 [-]	0.6941 [-]	0.0105[+]
Triglycerides (Square root)	(n = 176)	(n = 91)	(n = 87)	(n = 95)
Linetype (HR vs. C)	0.6780 [+]	0.9822 [+]	0.3927 [-]	0.5679[-]
Wheel access		0.8884[+]	0.3111 [+]	0.2396[-]
Mini-muscle	0.7626 [-]	0.2532[+]	0.5012 [+]	0.3783[+]
Linetype x Whlacc		0.3988[+]	0.1793 [-]	0.7388[-]
S2AGE		0.7376[-]	0.1813 [-]	
FTIME2		0.0332[-]	0.7777 [+]	0.0652 [-]
Cholesterol (Log)	(n = 177)	(n = 80)	(n = 83)	(n = 94)
Linetype (HR vs. C)	0.6780 [+]	0.4651 [-]	0.4707 [+]	0.8237 [+]
Wheel access		0.7911 [+]	0.5674 [-]	0.3897 [-]
Mini-muscle	0.7626 [-]	0.9569 [-]	0.0748 [-]	0.4662 [-]
Linetype x Whlacc		0.3776[+]	0.7432 [-]	0.7169 [-]
S2AGE		0.2155 [+]	0.2312 [+]	
FTIME2		0.2508 [+]	0.7747 [-]	0.5649 [-]
HDL-Cholesterol	(n = 183)	(n = 91)	(n = 98)	(n = 94)
HR vs. C	0.3438 [+]	0.7380 [-]	0.9260 [-]	0.4717 [-]
Mini-muscle	0.3155 [-]	0.9625 [-]	0.9161 [+]	0.0406 [-]
Wheel access		0.8374[+]	0.0002 [-]	0.8661 [-]
Linetype x Whlacc		0.4084[+]	0.1969 [+]	0.2700 [-]
S2AGE		0.7683 [+]	0.7388 [-]	
FTIME2		0.3332 [+]	0.0846 [+]	0.4123 [+]

Appendix 2.5 continued P values from ANCOVA for fasting plasma metabolites for Western diet only

	Week 0 *P*	Week 1 *P*	Week 2 *P*	Week 8 *P*
Non HDL-Chol (LDL+VLVL)	(n = 175)	(n = 80)	(n = 83)	(n = 92)
Linetype (HR vs. C)	0.6780 [+]	0.7550 [-]	0.4517 [+]	0.1927 [+]
Wheel access		0.5645[+]	0.0740 [-]	0.1091 [+]
Mini-muscle	0.7626 [-]	0.4950 [+]	0.3246 [-]	0.2261 [+]
Linetype x Whlacc		0.8234[-]	0.8425 [-]	0.4261 [-]
FTIME2		0.9603 [+]	0.5173 [-]	
S2AGE		0.3961 [+]	0.8276 [+]	0.9359 [+]

Appendix 2.6A Body mass with no transform (repeated-measures analysis of covariance, ANCOVA,) without any covariates (four measures, corresponding to when blood samples were taken).

Type 3 Tests of Fixed Effects
n = 786

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	3	18	302.57	<.0001
LINETYPE	1	6	0.78	0.4112
DIET	1	6	19.28	0.0046
WHLACC	1	6	4.89	0.0690
MINI	1	657	2.93	0.0877
REPEATED*LINETYPE	3	18	4.55	0.0153
REPEATED*DIET	3	18	26.76	<.0001
REPEATED*WHLACC	3	18	11.83	0.0002
LINETYPE*DIET	1	6	0.02	0.8991
LINETYPE*WHLACC	1	6	0.14	0.7199
WHLACC*DIET	1	6	5.85	0.0520
REPEATE*LINETYP*DIET	3	18	0.42	0.7407
REPEAT*LINETY*WHLACC	3	18	0.19	0.9019
REPEATED*WHLACC*DIET	3	18	8.73	0.0009
LINETYPE*WHLACC*DIET	1	6	0.03	0.8756
REPE*LINE*WHLAC*DIET	3	18	1.09	0.3774

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	18	130.82	<.0001
sample 1 vs sample 3	1	18	219.77	<.0001
sample 1 vs sample 6	1	18	891.98	<.0001
sample 2 vs sample 3	1	18	13.90	0.0015
sample 2 vs sample 6	1	18	368.07	<.0001
sample 3 vs sample 6	1	18	252.98	<.0001

Appendix 2.6B Non-transformed body mass (repeated-measures analysis of covariance, ANCOVA) without any covariates (all six measures).

Type 3 Tests of Fixed Effects
n = 1183

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	5	30	318.76	<.0001
LINETYPE	1	6	1.50	0.2665
DIET	1	6	19.40	0.0045
WHLACC	1	6	6.95	0.0387
MINI	1	990	2.36	0.1248
REPEATED*LINETYPE	5	30	4.79	0.0025
REPEATED*DIET	5	30	49.84	<.0001
REPEATED*WHLACC	5	30	12.34	<.0001
LINETYPE*DIET	1	6	0.09	0.7726
LINETYPE*WHLACC	1	6	0.14	0.7235
WHLACC*DIET	1	6	7.44	0.0343
REPEATE*LINETYP*DIET	5	30	1.00	0.4330
REPEAT*LINETY*WHLACC	5	30	0.40	0.8419
REPEATED*WHLACC*DIET	5	30	5.63	0.0009
LINETYPE*WHLACC*DIET	1	6	0.05	0.8250
REPE*LINE*WHLAC*DIET	5	30	0.67	0.6509

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	30	186.00	<.0001
sample 1 vs sample 3	1	30	313.20	<.0001
sample 1 vs sample 4	1	30	736.87	<.0001
sample 1 vs sample 5	1	30	1047.45	<.0001
sample 1 vs sample 6	1	30	1197.59	<.0001
sample 2 vs sample 3	1	30	19.83	0.0001
sample 2 vs sample 4	1	30	204.88	<.0001
sample 2 vs sample 5	1	30	391.34	<.0001
sample 2 vs sample 6	1	30	491.94	<.0001
sample 3 vs sample 4	1	30	103.69	<.0001
sample 3 vs sample 5	1	30	249.62	<.0001
sample 3 vs sample 6	1	30	333.29	<.0001
sample 4 vs sample 5	1	30	35.67	<.0001
sample 4 vs sample 6	1	30	74.67	<.0001
sample 5 vs sample 6	1	30	8.20	0.0076

Appendix 2.7 Body mass with no transform (repeated-measures analysis of covariance, ANCOVA) with log body length (crown-rump) as a covariate (four measures, corresponding to when blood samples were taken).

Type 3 Tests of Fixed Effects
n = 784

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	3	18	155.52	<.0001
LINETYPE	1	6	0.75	0.4185
MINI	1	672	3.81	0.0512
WHLACC	1	6	5.44	0.0585
DIET	1	6	21.01	0.0038
LINETYPE*WHLACC	1	6	0.21	0.6643
LINETYPE*DIET	1	6	0.03	0.8667
WHLACC*DIET	1	6	7.38	0.0348
LINETYPE*WHLACC*DIET	1	6	0.06	0.8088
REPEATED*LINETYPE	3	18	3.67	0.0319
REPEATED*WHLACC	3	18	10.82	0.0003
REPEATED*MINI	3	672	0.13	0.9422
REPEAT*LINETY*WHLACC	3	18	0.20	0.8944
REPEATED*WHLACC*DIET	3	672	11.87	<.0001
REPEATED*DIET	3	18	25.21	<.0001
REPEATE*LINETYP*DIET	3	18	0.40	0.7534
TCRLENT	1	672	41.63	<.0001

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	18	55.16	<.0001
sample 1 vs sample 3	1	18	129.59	<.0001
sample 2 vs sample 3	1	18	13.66	0.0017
sample 1 vs sample 6	1	18	434.97	<.0001
sample 2 vs sample 6	1	18	222.53	<.0001
sample 3 vs sample 6	1	18	118.29	<.0001

Appendix 2.8 Body mass with no transform (repeated-measures analysis of covariance, ANCOVA) with log body length (crown-rump) and log food consumption (Kj per day) as covariates (four measures, corresponding to when blood samples were taken).

Type 3 Tests of Fixed Effects
n = 772

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	3	18	202.05	<.0001
LINETYPE	1	6	0.93	0.3719
DIET	1	6	11.29	0.0152
WHLACC	1	6	6.54	0.0430
MINI	1	641	3.87	0.0495
REPEATED*LINETYPE	3	18	4.01	0.0238
REPEATED*DIET	3	18	31.83	<.0001
REPEATED*WHLACC	3	18	11.46	0.0002
LINETYPE*DIET	1	6	0.01	0.9307
LINETYPE*WHLACC	1	6	0.64	0.4530
WHLACC*DIET	1	6	3.96	0.0939
REPEATE*LINETYP*DIET	3	18	0.79	0.5158
REPEAT*LINETY*WHLACC	3	18	0.48	0.6980
REPEATED*WHLACC*DIET	3	18	7.02	0.0025
LINETYPE*WHLACC*DIET	1	6	0.17	0.6981
REPE*LINE*WHLAC*DIET	3	18	1.90	0.1660
CRLENT	1	641	28.01	<.0001
AtePDkj	1	641	26.41	<.0001

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	18	98.74	<.0001
sample 1 vs sample 3	1	18	205.85	<.0001
sample 1 vs sample 6	1	18	581.36	<.0001
sample 2 vs sample 3	1	18	19.11	0.0004
sample 2 vs sample 6	1	18	303.34	<.0001
sample 3 vs sample 6	1	18	176.61	<.0001

Appendix 2.9 Non-transformed mass-adjusted caloric intake (repeated-measures analysis of covariance, ANCOVA) as measured in kJ/day.

Type 3 Tests of Fixed Effects
n = 964

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	4	24	19.07	<.0001
LINETYPE	1	6	2.02	0.2050
MINI	1	826	2.20	0.1387
WHLACC	1	6	16.21	0.0069
DIET	1	6	22.99	0.0030
LINETYPE*WHLACC	1	6	6.82	0.0400
LINETYPE*DIET	1	6	0.59	0.4708
WHLACC*DIET	1	6	13.06	0.0112
LINETYPE*WHLACC*DIET	1	6	4.18	0.0869
REPEATED*LINETYPE	4	24	0.72	0.5869
REPEATED*WHLACC	4	24	10.62	<.0001
REPEATED*MINI	4	826	1.61	0.1710
REPEAT*LINETY*WHLACC	4	24	2.26	0.0924
REPEATED*DIET	4	24	38.06	<.0001
REPEATED*WHLACC*DIET	4	826	1.29	0.2706
REPEATE*LINETYP*DIET	4	24	2.40	0.0780
MASSAVG	1	826	152.25	<.0001

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	24	5.14	0.0327
sample 1 vs sample 3	1	24	0.10	0.7531
sample 2 vs sample 3	1	24	3.78	0.0637
sample 1 vs sample 4	1	24	26.77	<.0001
sample 2 vs sample 4	1	24	18.52	0.0002
sample 3 vs sample 4	1	24	45.47	<.0001
sample 1 vs sample 6	1	24	40.00	<.0001
sample 2 vs sample 6	1	24	32.28	<.0001
sample 3 vs sample 6	1	24	69.29	<.0001
sample 4 vs sample 6	1	24	3.15	0.0885

Appendix 2.10 Non-transformed glucose (repeated-measures analysis of covariance, ANCOVA) with age, fasting time and bleed delay time as covariates.

Type 3 Tests of Fixed Effects
n =700

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	3	18	25.85	<.0001
LINETYPE	1	6	0.03	0.8698
DIET	1	6	4.67	0.0739
WHLACC	1	6	1.10	0.3344
MINI	1	568	0.00	0.9505
REPEATED*LINETYPE	3	18	0.99	0.4213
REPEATED*DIET	3	18	6.04	0.0049
REPEATED*WHLACC	3	18	1.04	0.4002
LINETYPE*DIET	1	6	0.83	0.3983
LINETYPE*WHLACC	1	6	5.66	0.0548
WHLACC*DIET	1	6	4.39	0.0810
REPEATE*LINETYP*DIET	3	18	0.41	0.7507
REPEAT*LINETY*WHLACC	3	18	0.07	0.9775
REPEATED*WHLACC*DIET	3	18	1.39	0.2791
LINETYPE*WHLACC*DIET	1	6	0.04	0.8542
REPE*LINE*WHLAC*DIET	3	18	1.62	0.2195
MASSAGE	1	568	2.27	0.1325
FTIME	1	568	0.14	0.7127
BLEED_D	1	568	2.79	0.0956

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	18	18.44	0.0004
sample 1 vs sample 3	1	18	6.38	0.0212
sample 1 vs sample 6	1	18	4.34	0.0518
sample 2 vs sample 3	1	18	0.36	0.5543
sample 2 vs sample 6	1	18	2.89	0.1062
sample 3 vs sample 6	1	18	3.62	0.0731

Appendix 2.11 Non-transformed total cholesterol (repeated-measures analysis of covariance, ANCOVA) with age and fasting time as covariates.

Type 3 Tests of Fixed Effects
n = 705

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	3	18	26.76	<.0001
LINETYPE	1	6	0.01	0.9434
DIET	1	6	0.55	0.4856
WHLACC	1	6	0.39	0.5575
MINI	1	574	1.58	0.2095
REPEATED*LINETYPE	3	18	0.81	0.5033
REPEATED*DIET	3	18	4.34	0.0181
REPEATED*WHLACC	3	18	0.28	0.8421
LINETYPE*DIET	1	6	0.26	0.6264
LINETYPE*WHLACC	1	6	0.04	0.8556
WHLACC*DIET	1	6	0.83	0.3970
REPEATE*LINETYP*DIET	3	18	0.76	0.5287
REPEAT*LINETY*WHLACC	3	18	0.50	0.6894
REPEATED*WHLACC*DIET	3	18	2.05	0.1435
LINETYPE*WHLACC*DIET	1	6	0.37	0.5647
REPE*LINE*WHLAC*DIET	3	18	0.32	0.8119
MASSAGE	1	574	1.59	0.2085
FTIME	1	574	4.52	0.0339

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	18	8.30	0.0099
sample 1 vs sample 3	1	18	5.24	0.0344
sample 1 vs sample 6	1	18	2.00	0.1747
sample 2 vs sample 3	1	18	2.57	0.1264
sample 2 vs sample 6	1	18	1.36	0.2589
sample 3 vs sample 6	1	18	1.15	0.2970

Appendix 2.12 Non-transformed high density cholesterol (repeated-measures analysis of covariance, ANCOVA) with age and fasting time as covariates.

Type 3 Tests of Fixed Effects

n = 757

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	3	18	48.59	<.0001
LINETYPE	1	6	0.04	0.8489
MINI	1	644	0.00	0.9538
WHLACC	1	6	0.01	0.9397
DIET	1	6	252.77	<.0001
LINETYPE*WHLACC	1	6	1.46	0.2724
LINETYPE*DIET	1	6	1.39	0.2829
WHLACC*DIET	1	6	0.21	0.6642
LINETYPE*WHLACC*DIET	1	6	0.17	0.6958
REPEATED*LINETYPE	3	18	3.83	0.0277
REPEATED*WHLACC	3	18	4.67	0.0139
REPEATED*MINI	3	644	1.54	0.2038
REPEAT*LINETY*WHLACC	3	18	0.33	0.8068
REPEATED*DIET	3	18	54.96	<.0001
REPEATED*WHLACC*DIET	3	644	4.64	0.0032
REPEATE*LINETYP*DIET	3	18	0.25	0.8636
MASSAGE	1	644	0.51	0.4758
FTIME	1	644	11.64	0.0007

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	18	7.90	0.0116
sample 1 vs sample 3	1	18	8.07	0.0109
sample 2 vs sample 3	1	18	7.18	0.0153
sample 1 vs sample 6	1	18	1.54	0.2304
sample 2 vs sample 6	1	18	0.94	0.3449
sample 3 vs sample 6	1	18	0.39	0.5408

Appendix 2.13 Non-transformed non-high density cholesterol (repeated-measures analysis of covariance, ANCOVA) with age and fasting time as covariates.

Type 3 Tests of Fixed Effects

n = 697

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	3	18	3.81	0.0282
LINETYPE	1	6	0.00	0.9984
DIET	1	6	114.27	<.0001
WHLACC	1	6	0.25	0.6380
MINI	1	566	0.00	0.9658
REPEATED*LINETYPE	3	18	2.04	0.1447
REPEATED*DIET	3	18	29.92	<.0001
REPEATED*WHLACC	3	18	3.13	0.0516
LINETYPE*DIET	1	6	0.28	0.6158
LINETYPE*WHLACC	1	6	0.66	0.4489
WHLACC*DIET	1	6	0.12	0.7399
REPEATE*LINETYP*DIET	3	18	0.38	0.7674
REPEAT*LINETY*WHLACC	3	18	0.19	0.8989
REPEATED*WHLACC*DIET	3	18	1.46	0.2599
LINETYPE*WHLACC*DIET	1	6	0.25	0.6380
REPE*LINE*WHLAC*DIET	3	18	0.24	0.8680
MASSAGE	1	566	1.00	0.3189
FTIME	1	566	1.89	0.1692

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	18	0.53	0.4748
sample 1 vs sample 3	1	18	0.15	0.6996
sample 1 vs sample 6	1	18	0.59	0.4515
sample 2 vs sample 3	1	18	0.00	0.9728
sample 2 vs sample 6	1	18	0.60	0.4503
sample 3 vs sample 6	1	18	0.83	0.3745

Appendix 2.14 Non-transformed triglycerides (repeated-measures analysis of covariance, ANCOVA) with age and fasting time as covariates.

Type 3 Tests of Fixed Effects

n = 719

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	3	18	1.25	0.3216
LINETYPE	1	6	0.05	0.8268
DIET	1	6	17.71	0.0056
WHLACC	1	6	1.57	0.2563
MINI	1	588	1.38	0.2402
REPEATED*LINETYPE	3	18	1.36	0.2876
REPEATED*DIET	3	18	2.00	0.1496
REPEATED*WHLACC	3	18	0.33	0.8063
LINETYPE*DIET	1	6	0.02	0.8969
LINETYPE*WHLACC	1	6	1.75	0.2336
WHLACC*DIET	1	6	0.37	0.5664
REPEATE*LINETYP*DIET	3	18	0.09	0.9662
REPEAT*LINETY*WHLACC	3	18	1.06	0.3893
REPEATED*WHLACC*DIET	3	18	1.38	0.2799
LINETYPE*WHLACC*DIET	1	6	0.34	0.5836
REPE*LINE*WHLAC*DIET	3	18	0.89	0.4660
MASSAGE	1	588	0.61	0.4360
FTIME	1	588	0.81	0.3671

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	18	1.01	0.3293
sample 1 vs sample 3	1	18	0.41	0.5276
sample 1 vs sample 6	1	18	0.62	0.4407
sample 2 vs sample 3	1	18	0.06	0.8086
sample 2 vs sample 6	1	18	0.56	0.4645
sample 3 vs sample 6	1	18	0.71	0.4120

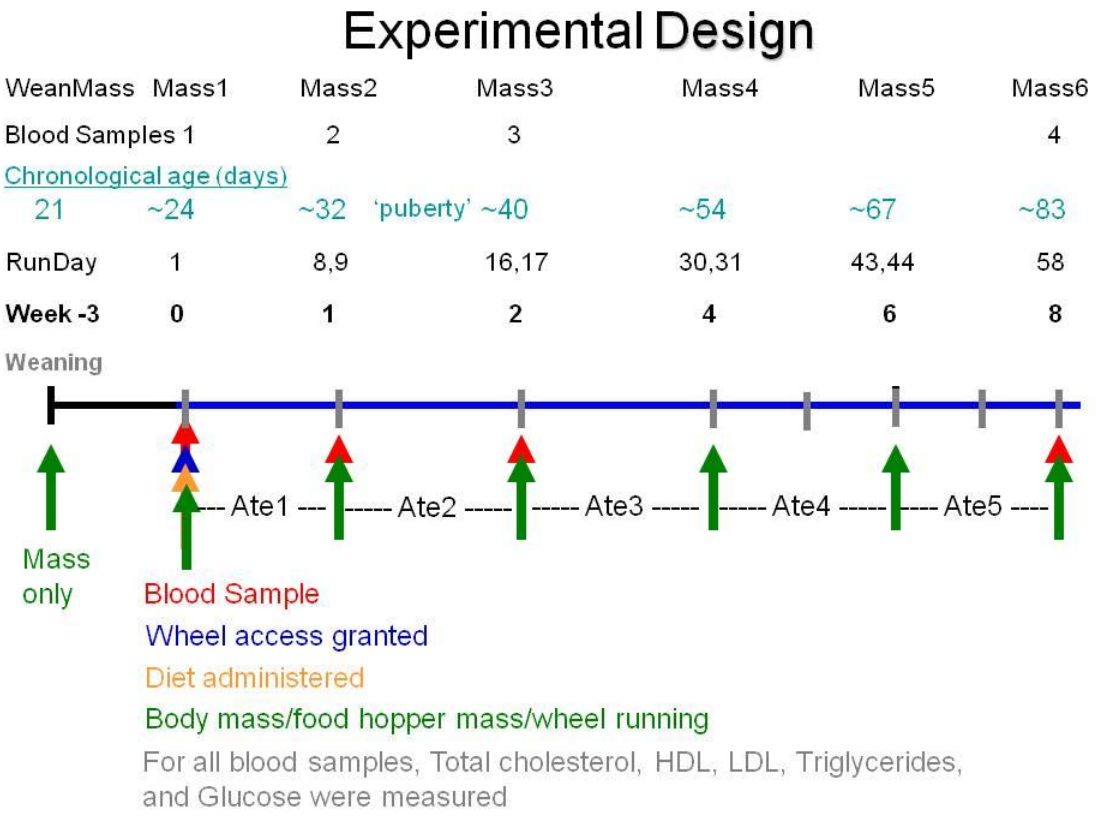
Appendix 2.15 Non-transformed wheel running (repeated-measures analysis of covariance, ANCOVA) binned as in Figure 3 of Meek et al. (2010) with wheel resistance (raised to 0.3 power) as a covariate.

Type 3 Tests of Fixed Effects
n = 492

Effect	Num DF	Den DF	F Value	Pr > F
REPEATED	4	24	80.54	<.0001
LINETYPE	1	6	41.24	0.0007
DIET	1	6	11.57	0.0145
MINI	1	410	0.26	0.6136
LINETYPE*DIET	1	6	10.84	0.0166
REPEATED*LINETYPE	4	24	31.68	<.0001
REPEATED*DIET	4	24	3.78	0.0161
REPEATE*LINETYP*DIET	4	24	2.72	0.0534
TRESall	1	410	0.05	0.8289

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sample 1 vs sample 2	1	24	54.62	<.0001
sample 1 vs sample 3	1	24	268.63	<.0001
sample 1 vs sample 4	1	24	246.32	<.0001
sample 1 vs sample 6	1	24	125.08	<.0001
sample 2 vs sample 3	1	24	180.61	<.0001
sample 2 vs sample 4	1	24	139.12	<.0001
sample 2 vs sample 6	1	24	45.28	<.0001
sample 3 vs sample 4	1	24	2.38	0.1362
sample 3 vs sample 6	1	24	9.14	0.0059
sample 4 vs sample 6	1	24	28.77	<.0001



Appendix 2.16 Experimental design and sampling points for mice from chapters 2, 3, and 4. All mice were weaned at 21 days of age and placed into their respective diet and wheel groups ~24 days old. Running data was recorded daily but analyzed in weekly or bimonthly segments. Mass, blood, and food hopper weight was taken at the end of each "Runday" segment at noon and the new segment started immediately after. For example, Mass2 and blood sample 2 were measured on Runday8 at noon followed by the start of Runday9. The variable "Ate" represents the total food consumption (grams) consumed during the indicated period. For statistical analyses, food consumption was converted to KJoules per day using the metabalizable energy for each diet (see Methods and Meek et al., 2010).

Chapter 3

Western Diet Increases Wheel Running in Mice Selectively Bred for High Voluntary Wheel Running

Thomas H. Meek[1], Joey C. Eisenmann[2], Theodore Garland Jr.[1]

[1]Department of Biology, University of California, Riverside, Riverside, CA 92521, USA

[2]Department of Kinesiology, Michigan State University, East Lansing, MI 48823, USA

Abstract

Objective: Diet and exercise interact with genetic background to produce phenotypes. Mice from a long-term selective breeding experiment for high voluntary wheel running offer a unique model to examine contributions of genetic and environmental factors in determining aspects of behavior and metabolism relevant to body-weight regulation and obesity. Since generation 16 and continuing through 52, mice from the 4 replicate high runner (HR) lines have run 2.5-3-fold more revolutions/day as compared with 4 non-selected control (C) lines, but the nature of this apparent selection limit is not understood.

Methods: Wheel running, food consumption (Teklad Rodent Diet (W) 8604, 14% kJ from fat, or Harlan Teklad TD.88137 Western Diet, 42% kJ from fat), and body mass were measured over 1-2 week intervals in 100 males for two months starting 3 days after weaning.

Results: Western diet (WD) was obesogenic for both HR and C, significantly increasing both body mass and retroperitoneal fat pad mass, the latter even when controlling statistically for wheel-running distance and caloric intake. HR mice had significantly less fat than C, explainable statistically by their greater running distance. Adjusting for body mass, HR had higher caloric intake than C, also explainable by their higher running. Accounting for body mass and running, WD initially caused increased caloric intake in both HR and C, but this effect was reversed during the last 4 weeks of the study. WD had little or no effect on wheel running in C mice, but increased revolutions/day by as much as 75% in HR mice, mainly through increased time spent running.

Conclusion: The remarkable stimulation of wheel running by Western diet in HR mice may involve fuel usage during prolonged endurance exercise and/or direct behavioral effects on motivation. Their unique behavioral responses to Western diet may render HR mice an important model for understanding the control of voluntary activity levels.

Introduction

Understanding the biological basis of voluntary physical activity has been an important goal of both biomedicine (Eisenmann and Wickel, 2009) and evolutionary physiology (Weber, 2009). In addition to biological effects, environmental factors can modulate both physiology and behavior, and effects can be nonadditive, i.e., show genotype-by-environment interaction (Rankinen and Bouchard, 2008). Diet is a highly variable environmental factor (although behavior and physiology affect dietary choices), and elucidating the role that dietary macronutrients play in providing energy during locomotion and other physiological processes should shed light on the current obesity epidemic (Fukuchi et al., 2004; Geiger et al., 2009). As well, comparative, ecological, and evolutionary physiologists are attempting to reveal how dietary physiology may interact with, and even constrain, behavioral ecology and evolution (McClelland, 2004; Karasov and Martinez del Rio, 2007; Weber, 2009).

In the present study, we investigated genotype-by-environmental interactions and potential limitations that diet places on locomotor behavior through use of an artificial selection experiment with laboratory house mice. Starting in 1998, mice were bred for high voluntary wheel-running behavior (a major component of total voluntary locomotor activity when wheels are available), and after 16 generations individuals from the four replicate high runner (HR) lines ran 2.5-3.0-fold as many revolutions per day as compared with those from four non-selected control (C) lines, a differential that has continued through more than 36 subsequent generations of selection (e.g., Keeney et al., 2008; Gomes et al., 2009). Various changes in locomotor performance, behavior, and neurobiology (especially related to motivation for wheel running) have been observed in the HR lines (Rhodes et al., 2005; Belke and Garland, 2007; Swallow et al., 2009). For

instance, treadmill endurance capacity is elevated in HR mice (Meek et al., 2009), as is maximal oxygen consumption (Rezende et al., 2006).

Despite the continued selection since generation 16, HR mice appear to be at a selection limit, the biological causes of which are as yet unclear (Gomes et al., 2009; Rezende et al., 2009). Several pharmacological studies have been performed, and some drugs increase wheel running in the C lines; however, no drug has increased running in the HR lines (Rhodes et al., 2005; Keeney et al., 2008).

One possible factor that could limit further increases in wheel running by the HR lines is the availability of necessary energy substrates. HR mice run for ~6 hours/day, virtually all at night (Rezende et al. 2009), and in mammals, generally, this type of sustained aerobic exercise should be supported primarily by lipid oxidation (Coyle, 1995; McClelland, 2004). At low exercise intensities, lipids, in the form of fatty acids, predominate as the cellular fuel source. As exercise intensity increases, cellular triglycerides, plasma glucose, and cellular glycogen also provide essential energy to maintain exercise performance (Coyle, 1995). In untrained human beings, half the energy used to exercise at 65% of maximal oxygen consumption (VO_2max) comes from lipids in the form of both free fatty acids and muscle triglycerides (Coyle, 1995). In the context of enhancing endurance performance, ingesting carbohydrates during exercise is often emphasized, but diets rich in lipids can also lead to increased performance at low or moderate intensities in rodents (Miller et al, 1984; Simi et al., 1991; Lapachet et al., 1996) and in humans (Horvath et al., 2000; Pendergast et al., 2000, although not always (Erlenbusch et al., 2005).

Recent evidence has shown HR mice do not deplete liver or gastrocnemius muscle glycogen stores any more than controls during nightly wheel running, nor do they have elevated glycogen synthase activity (Gomes et al., 2009). Thus, glycogen depletion

does not appear to limit further increases in running. Previous studies have also found HR mice have less body fat than C, and even a lower % body fat than mice from a separate experiment that selectively bred for low body fat (Nehrenberg et al., 2009). Reanalysis of mice from generation 14 (data from Houle-Leroy et al., 2003) indicates HR males have elevated carnitine palmitoyltransferase compared to C lines, suggesting a higher ability to oxidize fats (Gomes et al., 2009). Therefore, it seems possible that insufficient dietary lipids may be one factor limiting further evolutionary increases in wheel running by the HR lines. As an initial test of this idea, we measured wheel running, food consumption (Teklad Rodent Diet (W) 8604, 14% kJ from fat, or Harlan Teklad TD.88137 Western Diet, 42% kJ from fat), and body mass over 1-2 week intervals in 100 males for two months starting 3 days after weaning (21 days of age). We predicted that HR mice would exhibit elevated wheel running when provided Western diet.

Materials and methods

Experimental animals

Mice were sampled from the 52nd generation of an artificial selection experiment for high voluntary wheel running (reviews in Rhodes et al., 2005; Swallow et al., 2009). The original progenitors of the selection experiment were outbred, genetically variable Hsd:ICR house mice (*Mus domesticus*). After two generations of random mating, 10 pairs of mice were used to create each of eight closed lines (i.e., no interbreeding between lines was allowed). Four lines are bred for high running on wheels (HR lines) and four are bred without regard to wheel running (C lines). In each generation mice are housed individually in standard cages (27 X 17 X 12.5 cm) attached to Wahman-type activity wheels (1.12 m circumference, 35.7 cm diameter, 10-cm-wide running surface) when

they reach 6-8 weeks of age. Wheels are interfaced to a computer and revolutions are recorded in 1-minute intervals, continuously for 6 days; the selection criterion is the number of revolutions run on days five and six. Within each HR family, the highest-running male and female are chosen as breeders to produce the next generation. Within C families, a male and female are chosen without regard for running. Sibling matings are disallowed in all lines. Room temperature is maintained at ~73° Fahrenheit and photoperiod is 12:12, with lights on at 0700 Pacific Time. Water and food (Harlan Teklad Laboratory Rodent Diet [W]-8604) are available ad libitum.

For the present study, 100 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.

Procedures

At an average of 24 ± 0.2 (S.E.) days of age (experimental day 1), mice were housed individually with access to wheels (as described above). We used young mice because this was part of a larger study aimed at examining the ontogeny of voluntary locomotor activity and biomarkers of the metabolic syndrome. Half the mice continued to receive the standard diet (SD) (Harlan Teklad Rodent Diet [W] 8604, 14% kJ from fat) and the other half received Western diet (WD) (Harlan Teklad TD.88137 Western Diet, 42% kJ from fat)(Table 3.1).

Mice and food hoppers were weighed on experimental days 1, 8, 16, 30, 43, and 58. We measured (apparent) food consumption as the difference in hopper mass between two time points, after accounting for any obvious wastage (Koteja et al., 2003). Because the diets differ in mass-specific energy content, we converted food consumption to caloric intake, using the values shown in Table 3.1.

Total wheel running (revolutions) was recorded in one-minute bins for

approximately 23 hours every day. From these records, we computed weekly or bi-weekly averages (to match periods over which food consumption was recorded) 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).

After 8 weeks of the experimental protocol, mice were weighed and body length was measured as nose to rump length on live mice prior to sacrifice by decapitation. The retroperitoneal fat pad was then dissected and weighed.

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, and the linetype X diet interaction were tested relative to the variance among replicate lines, and degrees of freedom were always 1 and 6. Covariates depended on the trait analyzed and included age, body length or body mass, wheel freeness, and total wheel running.

One unexpected feature of the selection experiment has been the increase in frequency in two of the four HR lines of a small-muscle phenotype, termed mini-muscle and characterized by an approximately 50% reduction in triceps surae and whole-hindlimb muscle mass (Houle-Leroy et al., 2003 and references therein). Pleiotropic effects of this Mendelian recessive allele are many and varied, and include alterations in muscle fiber type composition (fewer type IIb fibers) (Bilodeau et al., 2009), contractile properties (Syme et al., 2005), a doubling of mass-specific aerobic capacity and hexokinase activity (Houle-Leroy et al., 2003; Rezende et al., 2006), increased glycogen concentration in gastrocnemius (Gomes et al., 2009), increased myoglobin concentration

in medial gastrocnemius (Rezende et al., 2006), and increased heart ventricle mass (Meek et al., 2009). Mini-muscle individuals run faster on wheels, and run more total revolutions/day under some conditions (Syme et al., 2005; Gomes et al., 2009). Thus, we included mini-muscle status as an independent variable in all analyses that involved the HR lines.

As we found significant linetype X diet interactions for several traits, separate analyses of HR and C lines were also performed. In these analyses, line was treated as a fixed effect. Although differences among the replicate lines (or line X diet interactions) were found for several traits, they are not the focus of the present report and so are not discussed here, and we simply report the effects of diet.

Results

Body mass and retroperitoneal fad pad mass

Analysis of body length indicated no statistical effect of linetype, diet, their interaction or mini-muscle status (all $P > 0.43$). HR mice tended to be lighter in body mass than C, especially during the later weeks of the experiment and regardless of diet (Figure 3.1), although the difference was not statistically significant at any measurement time (results not shown). The linetype X diet interaction was never close to significant (all $P > 0.73$), and Western diet increased body mass (Figure 3.1), the effect reaching statistical significance ($P < 0.05$) at weeks 2 and 8.

With log body length as a covariate, WD significantly increased retroperitoneal fad pad mass, and HR mice were leaner than C, with no linetype X diet interaction (Table 3.2). When wheel running was included in the model as an additional covariate, the effect of diet remained, but the difference in body fat between HR and C mice was eliminated (Table 3.2). Thus, the reduced fat pad mass of HR mice can be explained, at

least in statistical terms, by the intermediate phenotype of increased wheel running. Finally, we added caloric intake (log transformed) as an additional covariate. In this analysis, the effects of wheel running and diet remained highly significant (Table 3.2). Thus, Western diet per se increased fat pad mass in all mice, even after adjusting for caloric intake. In all three models, mini-muscle individuals had significantly larger fat pads (Table 3.2), despite tending to have reduced total body mass (results not shown).

Caloric intake

As shown in Table 3, body mass was always a highly significant, positive predictor of caloric intake. Controlling for body mass, food consumption (kilocalories/day) was influenced by both linetype and diet, but the effects changed over the course of the experimental period (Figure 3.2A, Table 3.3). During the first week, Western diet increased caloric intake of all mice by approximately 23%. By week 2 and continuing through the end of the experiment, the interaction between linetype and diet was significant, such that HR mice on WD were consuming more calories than all other groups, while C mice on WD reduced their caloric intake (Figure 3.2A). Mini-muscle individuals had significantly higher caloric intake during weeks 2-4 (Table 3.4).

When amount of wheel running (average revolutions/day) was included as a covariate, it always had a positive effect on caloric intake (as did body mass), and this effect was highly significant across weeks 3-8 (Table 3.4). In addition, amount of wheel running had substantial effects on the significance levels for some main effects during some measurement periods. In particular, during weeks 5-8 inclusion of wheel running as a covariate eliminated the significantly higher caloric intake of HR mice relative to C, made the diet effect significantly negative, and reduced the significance of the linetype X diet interaction (Table 3.3, Figure 3.2B). Thus, after adjusting for both variation in body mass and amount of wheel running on an individual basis, caloric intake was reduced on

WD for all mice during weeks 5-8.

For analyses of food consumption in grams/day (not shown), *P* values were identical to those shown in Table 3.3 for each measurement period, with the exception of diet, which was highly significant at all weeks (all $P < 0.0001$), with or without wheel running as a covariate. In all cases WD, decreased food consumption measured as grams/day.

Voluntary wheel running

The effect of Western diet on wheel running (revolutions/day) differed greatly between HR and C mice (Figure 3.3), and the linetype X diet interaction was statistically significant or nearly so across weeks 2-6 (Table 3.4). As expected from many previous studies of HR and C mice, in this sample the daily wheel-running distance of HR mice was much higher than for C when on standard diet, with the fold difference ranging from 2.2 to 4.0 (Figure 3.3B). Western diet increased this differential during all measurement periods, with the fold difference reaching as high as 5.7 during weeks 5-6 (Figure 3.3B).

As shown in Figure 3.3C, the effect of WD on wheel running was much greater in absolute terms (revolutions/day) in HR as compared with C lines. Separate analyses of the HR and C lines (not shown) indicated that the revolutions/day of HR mice was significantly (2-tailed $P < 0.05$) increased by WD during all time periods, except weeks 7-8 ($P = 0.0878$). In contrast, WD had no statistical effect on running by C mice, except during the first week ($P = 0.0438$). Based on analyses of the HR lines, the stimulation of daily running distance was primarily a function of time spent running, which was increased by 66%, 49%, 34%, 16%, and 11%, respectively, across the five time periods ($P < 0.0001$, $P < 0.0001$, $P = 0.0009$, $P = 0.0877$, and $P = 0.2408$, respectively). Average running speeds also tended to be increased, with corresponding values of -1%, 20%, 17%, 11%, and 6% ($P = 0.8940$, $P = 0.0200$, $P = 0.0326$, $P = 0.1122$, and $P = 0.2998$).

To determine when the stimulation of running first occurred in HR mice, we analyzed individual days. Revolutions/day were increased by 58%, 39%, 58%, 57%, 70%, 60%, and 56% on days 1-7, respectively ($P = 0.0321$, $P = 0.0832$, $P = 0.0160$, $P = 0.0086$, $P = 0.0014$, $P = 0.0073$, $P = 0.0168$ respectively).

Discussion

Variation in physical activity levels may reflect underlying differences in both motivation for being active and physical abilities to engage in locomotor activity of particular intensities or durations. Here, we investigated the effect of a Western diet (WD; 42.0% of kJ from fat, plus added sucrose) on voluntary wheel-running behavior by lines of mice (HR) that have been selectively bred for high wheel running. Since approximately generation 16, mice from the four replicate HR lines have run 2.5-3-fold more on a daily basis as compared with four replicate non-selected control (C) lines. In spite of continued selective breeding, running by the HR lines has not increased appreciably since generation 16 (Gomes et al., 2009). In this context, our most dramatic finding is that Western diet caused a large increase in daily wheel running of HR mice, with little or no effect in C (Figure 3.3). No other pharmacological or environmental agent has had this dramatic, positive effect on running by HR mice. The reason for this remarkable difference in response to WD between HR and C mice is unclear, but may involve WD's role in providing necessary lipids to help sustain prolonged submaximal exercise and/or stimulating areas in the brain involved in motivation for, or reward received from, wheel running.

To our knowledge, no other studies have examined the effect of this particular "Western diet" (Table 3.1) on any aspect of voluntary locomotor activity in mice. However, studies examining voluntary activity in animals fed high-fat diet show a variety

of responses. In a study using mice bred for low (L) or high (F) % body fat, Simoncic et al. (2008) reported that L % fat mice did not change their total running distance when eating high-fat diet. The F % fat mice increased wheel running compared to F % fat mice on regular chow, but running distances did not exceed values of L % fat animals on either diet (see their Figure 3.3). Cheng et al. (1997) reported that male Long-Evans rats fed high-fat diet for up to 6 weeks did not show any significant differences in voluntary wheel-running distance compared to rats fed standard diet, despite the fact rats administered the high-fat diet showed increased activities of carnitine acyltransferase (also called carnitine palmitoyltransferase) and β-hydroxy-acyl-CoA dehydrogenase in soleus muscle. Other studies have reported no change in home-cage activity levels when on high-fat diet or a high-fat + high-sucrose diet for male C57Bl/6J and A/J mice (Brownlow et al., 1996). In a study of one HR and one C line (i.e., a subset of the lines that we studied), Vaanholt et al. (2008) found that high-fat diet did not statistically increase home-cage activity in males or females that were housed without wheel access. Bjursell et al. (2008) used a diet very similar (high fat, high sucrose, 19.6kJ/g) to that used in the present study and found a decrease in home-cage activity for inbred C57Bl/6J mice. Thus, the stimulation of wheel running in HR mice fed Western diet is a novel observation.

Trained endurance athletes (human and rodent) are able to oxidize more free fatty acids for fuel during exercise than untrained counterparts (Hurley et al., 1986; Cheng et al., 1997; Phillips et al., 1996; Lee et al., 2001; Phillips 2006). As compared with C mice, HR mice exhibit several differences that are similar to those observed in trained athletes, including higher endurance (Meek et al., 2009) and aerobic capacity (Rezende et al., 2006), increased insulin-stimulated glucose uptake in isolated extensor digitorum longus muscle (Dumke et al., 2001), and higher carnitine palmitoyltransferase activity in

mixed hindlimb muscle (Gomes et al., 2009). WD may have affected the HR mice in the present study so rapidly (within 1-3 days) because they are already primed to oxidize lipids. Moreover, as compared with C mice, HR mice show a much greater upregulation of GLUT-4 in gastrocnemius muscle within only 5 days of wheel access (Gomes et al. 2009), and it is possible this occurs within 1-3 days. The foregoing characteristics suggest that HR mice, even in the untrained state, may be relying mostly on lipids during submaximal exercise, although previous respirometry studies have not reported significant differences in respiratory exchange ratio during rest or at maximal oxygen consumption during voluntary running (Rezende et al., 2009).

Even if HR mice are not primed to oxidize fats, if motivation is not limiting, then additional dietary lipids may lead to increases in running because diets high in fat have many physiological effects on cellular mechanisms that act to promote endurance performance capacity which would go unutilized by C animals. A high-fat diet and elevated levels of plasma fatty acids lead to increases in the activity of citrate synthase, 3-hydroxyacyl-CoA dehydrogenase, and carnitine palmitoyl-transferase (Miller et al., 1984; Cheng et al., 1997; Lee et al., 2001; Turner et al., 2007), and induce increased biogenesis of mitochondria in skeletal muscle (Garcia-Roves et al., 2007; Hancock et al., 2008), all of which would act to increase fatty-acid metabolism. These effects can appear in only a matter of days, and in some cases reach their maximum levels within a week (Miller et al., 1984; Lapachet et al., 1996). On the other hand, high-fat diets can also lead to higher levels of mitochondrial uncoupling protein (UCP3), which reduces the efficiency of energy production and therefore can impair exercise capacity (Murray et al., 2009).

Although the precise limitation(s) on endurance exercise performance depend on the intensity and/or duration of the activity, the depletion of energy substrates is a commonly considered cause of fatigue. The ingesting, loading, or utilization of particular

dietary macronutrients is important in maintaining muscular activity and delaying fatigue during exercise. The alteration in the rate of use or balance of carbohydrates and lipids, often through training or diet, can lead to increased performance. For instance, Simi et al. (1991) found high-fat diet acts in an additive fashion with endurance training, leading to increased VO_{2max} and submaximal endurance in rats. High-fat diet can serve to increase the duration of moderate physical activity by sparing glycogen, the depletion of which is considered to be a cause of fatigue (Hickson et al., 1977). However, as HR mice do not appear to deplete liver or muscle glycogen to any greater extent than C mice when fed standard rodent chow (Gomes et al., 2009), this explanation seems unlikely for the present wheel-running results. Nor does the Western diets added sucrose appear to have any exercise stimulating effects as HR and C mice, when administered sucrose drinking solutions, drank considerably more (compared to water) but showed no change in wheel running (E. M. Kolb and T. Garland, *unpublished results*).

The physiological basis for the increased wheel running in HR lines appears to be different from mechanisms involved in the semipalmated sandpiper (*Calidris pusilla*), where natural diets rich in n-3 polyunsaturated eicosapentaenoic acid (20:5, EPA) and n-3 docosahexaenoic acid (22:6, DHA) appear to increase the bird's oxidative capacity (Maillet and Weber, 2007). Both fatty acids lead to an increase cellular membrane fluidity and serve as ligands for peroxisome proliferators-activated receptors (PPARs) (Nagahuedi et al., 2009), which are involved in regulation of lipid metabolism. Different types of fatty acids, however, are not identical in their effects on performance (Ayre and Hulbert, 1997) or health (Vessby et al., 2002; Fukuchi et al., 2004). There appear to be differences between polyunsaturated verses monounsaturated and saturated fats, as well as differences among types of polyunsaturated fats (Ayre and Hulbert, 1997; Helge et al., 1998). In our study, only 4% of the fat in the WD was polyunsaturated, with less that

0.5% coming from EPA and DHA, whereas 64% was saturated fat, with 10.3%, 29.4%, 12.6% of total fatty acids coming from saturated fatty acid chains of 14, 16, and 18 carbons long, respectively. Therefore, if the running stimulation observed in HR mice occurs through direct or indirect effects on muscle physiology, then it seems likely other fatty acid types besides polyunsaturated fats are playing key roles.

In addition to the many effects high-fat diet has on muscles, it has been shown to stimulate certain areas (e.g., hypothalamus) and neurotransmitters (e.g., dopamine) in the brain, putatively involved in reward (Davis et al., 2008; Geiger et al., 2008; South and Huang, 2008; Stice et al., 2008; Geiger et al., 2009) and possibly involved with the 'activitystat' (Rowland, 1998). Voluntary wheel running is also well known for its impact on the central nervous system and is considered to be a classic self-rewarding behavior. Previous studies demonstrate that the reward circuitry, including dopaminergic and endocannabinoid pathways, have been altered in HR mice (e.g., see Rhodes et al., 2005; Belke and Garland, 2007; Keeney et al., 2008). Therefore, it is possible that the motivation for wheel running and sensitivity of the 'activitystat' responds differently to a Western diet in HR as compared with C mice.

Alternatively, the increased wheel running in HR mice on WD may represent part of a homeostatic mechanism that defends against the development of obesity. Initially, both linetypes consume more energy on WD (Figure 3.2), but after the first week C mice exhibit an ontogenetic switch and start to eat less WD, particularly in comparison with C mice on regular chow. HR mice, however, continue to eat the most kJ/day on WD. Recent research supports the presence of a neuro-hormonal system that maintains energetic balance and regulates total body weight (Speakman et al., 2004; Wilkin et al., 2006). Studies have reported that both mice and human beings exhibit "compensatory mechanisms" to deal with excess caloric intake, including decreased food intake,

increased metabolic rate, and increased non-exercise activity thermogenesis (NEAT) (Leibel et al., 1995; Speakman, 2004; Levine, 2005; Simoncic et al., 2008; Vaanholt et al., 2008; but see Westerterp, 2008). The high levels of wheel running by HR mice may represent an alternative compensatory mechanisms, but it is unclear if they are a cause or a consequence of the extra caloric intake.

As expected, both diet and exercise had effects on body mass. WD (which has a high amount of saturated fats) led to increased adiposity (Table 3.2), despite decreased caloric intake during the latter part of the study (Table 3.3). The high palatability of fatty diets often leads to hyperphagia and thus weight gain, but not always (West and York, 1998). Weight gain can occur in spite of decreased food consumption when on a high-fat diet, perhaps due to differences in the efficiency of storage or thermic effects of digestion (Lapachet et al., 1996; West and York, 1998; Astrup et al., 2001; Bjursell et al., 2008).

The WD used in the present study has very high levels of saturated fats and added sucrose, both of which are considered unhealthy if ingested frequently because they can contribute to the metabolic syndrome and other chronic diseases. The impact this diet would have on health-related traits in these mice has not yet been explored, but the elevated running in HR mice may serve to ameliorate the other negative effects of such a diet, much like wheel running reduced body fat in the present study.

In closing, we would argue that an experimental evolution approach offers a powerful alternative to other models for studying the evolution of lipid metabolism (review in McClelland 2004). The present results make clear that genetic selection history has a large impact on such complex traits as locomotor behavior, susceptibility to weight gain, and possibly central nervous system reward generated from exercise or eating. A better understanding of these traits is imperative for addressing obesity and the development of the metabolic syndrome (Noland et al. 2007). Moreover, our results

emphasize the importance of considering gene-by-environment interactions when studying the limits to sustained, relatively high-speed, voluntary locomotion. Further work will be required to determine the mechanism behind WD's unprecedented stimulatory effect on wheel running in the HR mice.

Chapter 3 Acknowledgements

We would like to thank Brooke K. Keeney for assistance. All animal procedures are in compliance with the UCR Institutional Animal Care and Use Committee and U.S. laws. This study was supported by Michigan State University Seed Grant to JCE and NSF IOB-0543429 to TG.

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Table 3.1 Composition of the diets

	Standard diet (8604)		Western diet TD.88137	
	g/kg	%KJ	g/kg	%KJ
Protein	245	33	173	15.2
Added casein			195*	0
Fat	45	14	212	42.0
Saturated	10		133	
Monounsaturated	11		59	
Polyunsaturated	21		9	
Carbohydrates	409	53	485	42.7
Added sucrose			341	
Energy content	13.0 KJ/g		19.0 KJ/g	

Diet composition of both standard and Western diets. Percent kilojoules of total protein, fat, and carbohydrates are provided for each diet and grams/kilogram are shown with additional nutritional information of dietary components.

* Added casein in g/kg is greater then the total protein content of the diet because casein itself is only ~87% protein. The remaining content composing casein comes from fat (~1%), ash (~1%), and moisture (~11%).

Table 3.2 *P* values from four alternate ANCOVAs of retroperitoneal fat pad mass (log transformed) with covariates

	P	*P*	*P*	*P*
HR vs. C	**0.0283** [-]	**0.0263** [-]	0.6646 [-]	0.7542 [-]
Diet	**0.0007** [+]	**0.0007** [+]	**0.0005** [+]	**0.0001** [+]
Linetype X Diet	0.8222	0.7830	0.7388	0.8993
Mini-muscle	**0.0052** [+]	**0.0048** [+]	**0.0055** [+]	**0.0045** [+]
log Body Length	**0.0078** [+]	**0.0133** [+]	**0.0425** [+]	0.2788 [+]
Wheel Running			**<0.0001** [-]	**<0.0001** [-]
log Caloric Intake		0.7249 [+]		**0.0100** [+]
AIC	-31.6	-31.5	-35.0	-40.6

Results are from 2-way nested analysis of covariance models in SAS Procedure Mixed with categorical factors of linetype (HR vs. C), diet, and mini-muscle status, and covariates of body length, wheel running, and/or caloric intake. Replicate line was included as a random effect in all analyses. N = 99. Wheel running represents mean revolutions/day over the two weeks prior to sacrifice. + indicates direction of effect, including HR > C, Western diet > Standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (HR vs. C), diet, and their interaction. For tests of mini muscle, log body length, wheel running, and caloric intake, d.f. are 1 and 81, 80, or 79. All *P* values are 2 tailed. Significant values ($P < 0.05$) are in bold. AIC is Akaike Information Criterion, with smaller values indicating better-fitting overall model.

Table 3.3 *P* values from ANCOVA for caloric intake (kJoule/day)

	Week 1 (n = 92)		Week 2 (n = 99)		Weeks 3 & 4 (n = 98)	
	P	*P*	*P*	*P*	*P*	*P*
HR vs. C	0.4836 [+]	0.8699 [+]	0.2275 [+]	0.4531 [+]	0.0866 [+]	0.8238 [+]
Diet	**<0.0001** [+]	**<0.0001** [+]	**0.0101** [+]	**0.0228** [+]	0.8856 [+]	0.2834 [-]
Linetype X Diet	0.9527	0.7966	**0.0475**	0.0907	**0.0359**	0.0734
Mini-muscle	0.1784 [+]	0.1583 [+]	**0.0020** [+]	**0.0028** [+]	**0.0120** [+]	**0.0083** [+]
log Body mass	<**0.0001** [+]	<**0.0001** [+]	<**0.0001** [+]	<**0.0001** [+]	<**0.0001** [+]	<**0.0001** [+]
Wheel running		0.2124 [+]		0.1980 [+]		**<0.0001** [+]

Results from 2-way nested ANCOVA in SAS Procedure Mixed with categorical factors of linetype (HR vs. C), diet, and mini-muscle status, and covariates of body mass and wheel running. Replicate line was included as a random effect in all analyses. Food consumption analyzed as log (kilojoules per day). Log body mass was a covariate in all analyses, and age was a covariate in weeks 1, 2, 3, and 4 (results not shown). Models were analyzed both without and with wheel running (mean revolutions/day) as an additional covariate. + indicates direction HR > C, Western diet > Standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (HR vs. C), diet, and their interaction. For tests of mini muscle, log-transformed body mass, and wheel running, d.f. are 1 and approximately 78. All *P* values are 2 tailed. Two outliers were removed from week 1 analysis and a single outlier was removed from all other weeks. Significant values ($P < 0.05$) are in bold. For analyses of food consumption in grams/day (not shown), *P* values were identical to those shown above for each measurement period, with the exception of diet, which was highly significant at all weeks (all $P < 0.0001$), with or without wheel running as a covariate. In all cases WD, decreased food consumption measured as grams/day.

Table 3.3 continued. *P* values from ANCOVA for caloric intake (kJoule/day)

	Weeks 5 & 6 (n = 98)		Weeks 7 & 8 (n = 98)	
	P	*P*	*P*	*P*
HR vs. C	**0.0195** [+]	0.7614 [-]	**0.0077** [+]	0.8990 [-]
Diet	0.4178 [-]	**0.0088** [-]	0.3905 [-]	**0.0216** [-]
Linetype X Diet	**0.0212**	0.0517	0.0672	0.2219
Mini-muscle	0.3361 [+]	**0.0111** [+]	0.6812 [+]	0.1225 [+]
log Body mass	<**0.0001** [+]	<**0.0001** [+]	<**0.0001** [+]	<**0.0001** [+]
Wheel running		**<0.0001** [+]		**<0.0001** [+]

Results from 2-way nested ANCOVA in SAS Procedure Mixed with categorical factors of linetype (HR vs. C), diet, and mini-muscle status, and covariates of body mass and wheel running. Replicate line was included as a random effect in all analyses. Food consumption analyzed as log (kilojoules per day). Log body mass was a covariate in all analyses, and age was a covariate in weeks 1, 2, 3, and 4 (results not shown). Models were analyzed both without and with wheel running (mean revolutions/day) as an additional covariate. + indicates direction HR > C, Western diet > Standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for linetype (HR vs. C), diet, and their interaction. For tests of mini muscle, log-transformed body mass, and wheel running, d.f. are 1 and approximately 78. All *P* values are 2 tailed. Two outliers were removed from week 1 analysis and a single outlier was removed from all other weeks. Significant values ($P < 0.05$) are in bold. For analyses of food consumption in grams/day (not shown), *P* values were identical to those shown above for each measurement period, with the exception of diet, which was highly significant at all weeks (all $P < 0.0001$), with or without wheel running as a covariate. In all cases WD, decreased food consumption measured as grams/day.

Table 3.4 *P* values from ANCOVA for wheel running (revolutions/day)

	Week 1 (n = 96) *P*	Week 2 (n = 100) *P*	Weeks 3 & 4 (n = 100) *P*	Weeks 5 & 6 (n = 99) *P*	Weeks 7 & 8 (n = 99) *P*
HR vs. C	**0.0010** [+]	**0.0038** [+]	**0.0012** [+]	**0.0012** [+]	**0.0057** [+]
Diet	**0.0266** [+]	**0.0039** [+]	**0.0343** [+]	0.1072 [+]	0.3195 [+]
Linetype X Diet	0.1281	**0.0106**	**0.0301**	0.0590	0.0709
Mini-muscle	0.8736 [-]	0.4441 [+]	0.9039 [+]	0.2090 [-]	0.5694 [-]

Results from 2-way nested ANCOVAs in SAS Procedure Mixed. Replicate line was included as a random effect in all analyses, and wheel freeness was used as a covariate (results not shown). Age was an additional covariate in weeks 1, 2, 3, and 4 (results not shown). + indicates direction HR > C, Western diet > Standard diet, mini-muscle > normal. Degrees of freedom are 1 and 6 for all tests, except for mini-muscle, where d.f. were 1 and 77-81, depending on week. All *P* values are 2 tailed. Significant values ($P < 0.05$) are in bold.

Figure 3.1

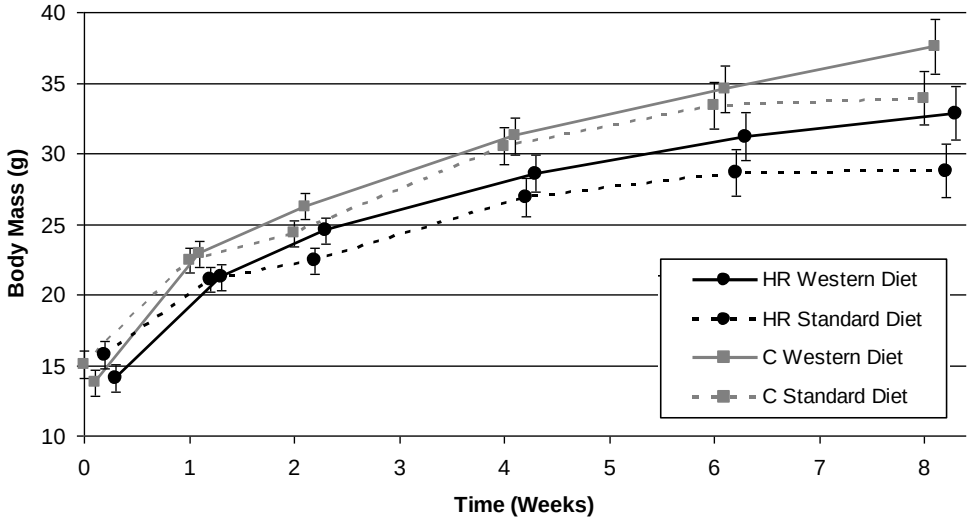

Figure 3.1 Least squares means and standard errors for body mass of high runner (HR) and control (C) mice housed with access to wheels. At week 0 mice were 24 days old, given access to wheels and separated into diet groups. Points are staggered along the X-axis for visual clarity of standard errors.

Figure 3.2 Panel A

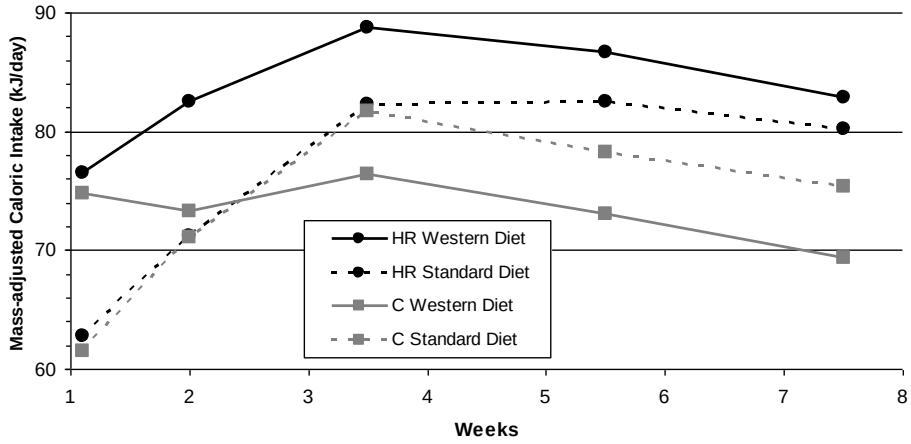

Figure 3.2 Panel B

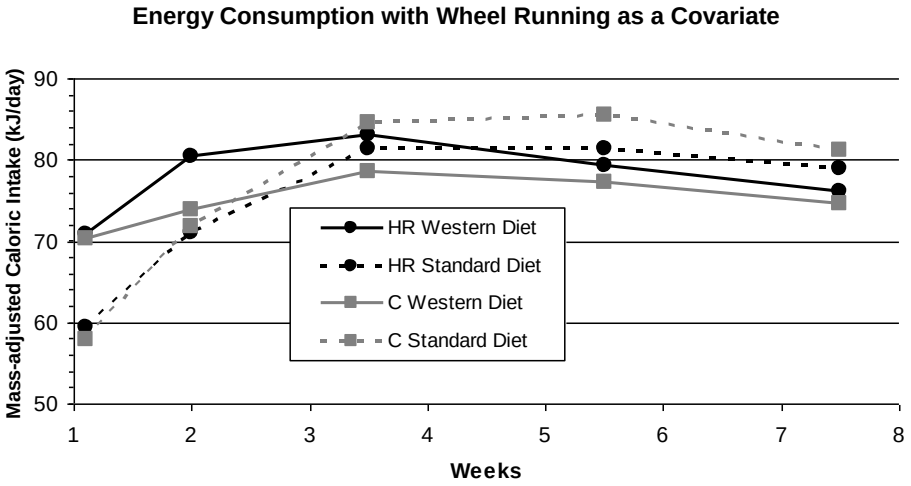

Figure 3.2 Mass-adjusted caloric intake (ANCOVA) as measured in kJ per day. Values are least squares means, back transformed from log scale. Two outliers were removed from week 1 analysis and a single outlier was removed from all other weeks. In bottom panel B, amount of wheel running was included as an additional covariate. Standard errors are not presented as back-transformed values require separate upper and lower confidence limits, rather than standard errors, which encumbers the visual presentation. See Table 3.3 for statistical results.

Figure 3.3 Panel A

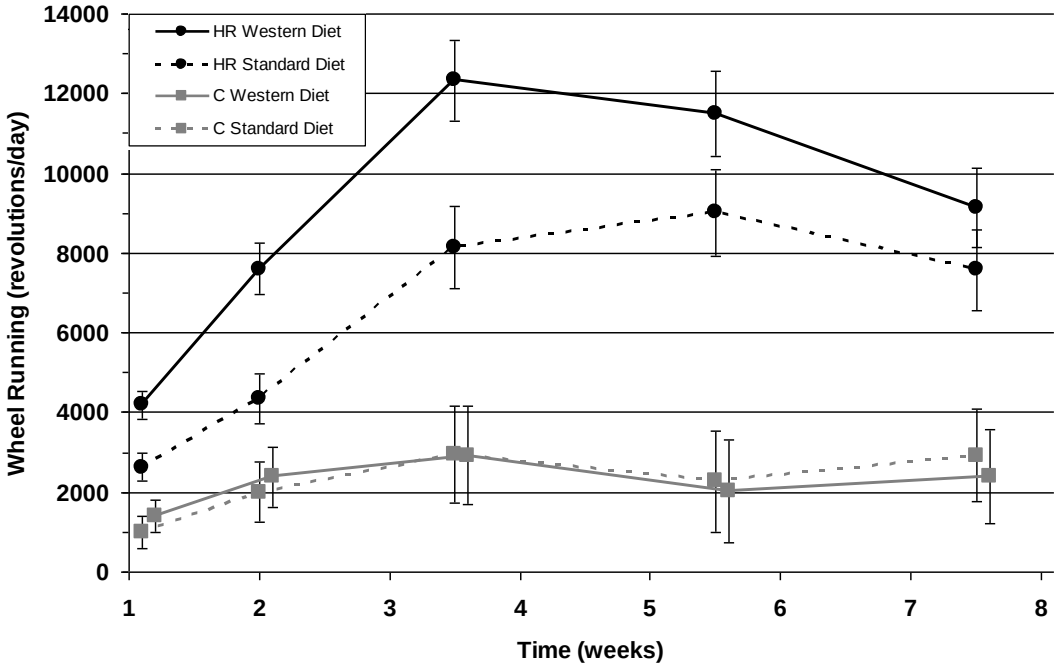

Figure 3.3 Panel B

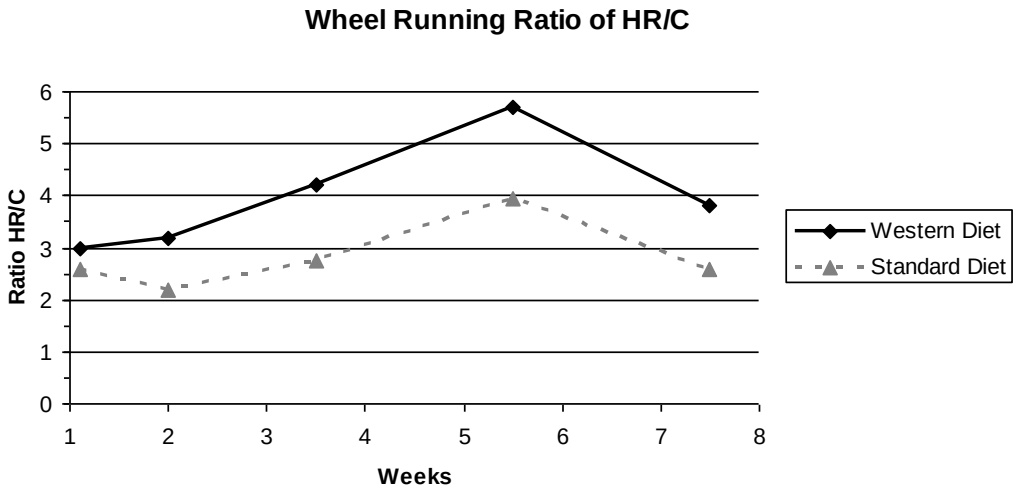

Figure 3.3 Panel C

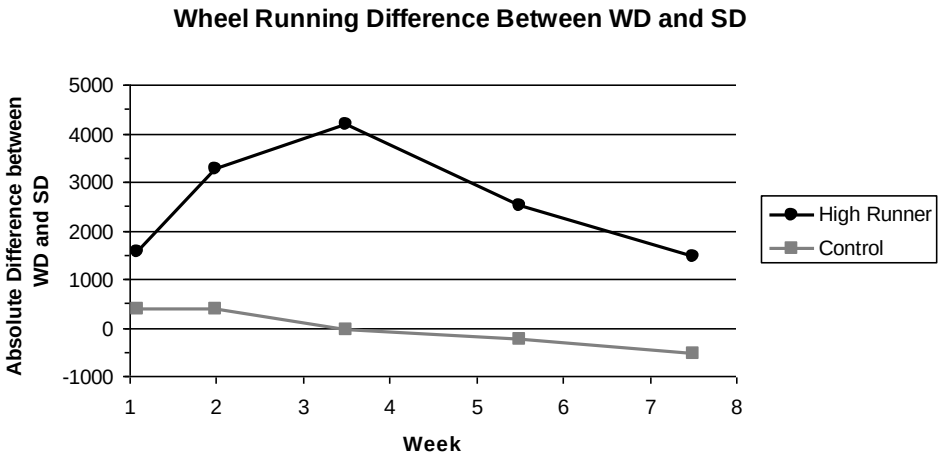

Figure 3.3 Least squares means and standard errors for wheel running for both C (points staggered along the X-axis for clarity) and HR mice on both diets. HR mice ran ~3 fold more than C mice when on standard diet, but this differential was greatly increased on Western diet.

Chapter 4

Effects of voluntary exercise on skeletal muscle metabolic capacities differ in mice selectively bred for wheel running, and are not modulated by Western diet

Thomas H. Meek[1], Mallory R. Marshall[2], Lemar I. Smith[3], Kim Vu[1], Andrew T. Ludlow[2], Sabrina Aviani-Cerveny[1], Joey C. Eisenmann[4], Stephen M. Roth[2], John Y.-J. Shyy[3], Theodore Garland, Jr.[1]

[1]Department of Biology, University of California, Riverside, Riverside, CA 92521, USA

[2]Department of Kinesiology, University of Maryland, College Park, MD 20742, USA

[3]School of Biomedical Sciences, University of California, Riverside, Riverside, CA 92521, USA

[4]Department of Kinesiology, Michigan State University, East Lansing, MI 48823, USA

Abstract

Previously, we found Western diet (WD) greatly increased voluntary wheel running (up to 75%) in mice from four selectively bred High Runner (HR) lines, but had little effect in four non-selected control lines. To test whether the increased running in HR mice was paralleled by enhanced maximal activities of metabolic enzymes, males were given WD or standard chow and further split by wheel access or sedentary housing, beginning at ~24 days of age. After 8 weeks, triceps surae muscles were assayed at 37^{o}C for Vmax of hexokinase (HK), citrate synthase (CS), carnitine palmitoyl-transferase (CPT), and β-hydroxyacyl-CoA dehydrogenase (HAD). Wheel access increased heart ventricle mass in both HR and control lines, but only HR showed increased enzyme activities. Analysis of wheel-access groups indicated the greater training effect in HR could be explained statistically by their greater wheel running for ventricle mass, CS, and HAD, but for HK and CPT, HR mice had greater adaptive plasticity of the response to voluntary exercise. Thus, despite voluntary exercise, mice from control lines did not run at levels sufficient to exhibit many metabolic training responses observed in HR. Larger exercise effects were also observed in HR mice for phosphorylation of 5'-AMP-activated protein kinase (AMPK). WD groups showed higher HAD and telomere length, but no effects of linetype or wheel access were observed. As WD did not differentially affect HR and control for any trait measured, its stimulatory effect on wheel running in HR must involve factors other than maximum enzymatic capacity of skeletal muscles.
Keywords: behavior; experimental evolution; plasticity; telomere length; voluntary exercise.

Introduction

Skeletal muscle is necessary for locomotion and is the major site of energy use during exercise. As such, metabolic changes that occur in muscle during exercise likely play a critical role in fatigue. Endurance training is known to increase reliance on lipid oxidation during submaximal exercise, allowing for glycogen-sparing effects and increased performance (Hurley et al., 1986; Phillips, 2006 pp 187-213). Diet also has the potential to affect exercise performance. In particular, the consumption of a high-fat diet increases fat oxidation, the ability to oxidize fat, and mitochondrial biogenesis (Miller et al., 1984; Cheng et al., 1997; Garcia-Roves et al., 2007; Hancock et al., 2008; Fillmore et al., 2010).

The changes that a high-fat diet prompts in skeletal muscle translate into increased whole-animal maximal oxygen consumption (VO_2max) and endurance performance, even in the untrained state, for rodents (Miller et al., 1984; Simi et al., 1991; Lapachet et al., 1996; Lee et al., 2001) and sometimes humans (Pendergast et al., 2000; Erlenbusch et al., 2005). However, despite the increase in performance ability when eating high-fat diets, previous studies of rodents have generally not reported any concomitant increase in voluntary locomotor activity as measured in home cages or via running wheels (Pitts 1984; Brownlow et al., 1996; Cheng et al., 1997; Bjursell et al., 2007).

Recently, we fed mice selectively bred for high voluntary wheel running a Western diet (high in saturated fats + extra sucrose) and observed an up to 75% increase in voluntary running, depending on the age (Meek et al., 2010). This response is even

more interesting given the fact that mice from these four replicate High Runner (HR) lines have been at an apparent selection limit for more than 40 generations, typically running ~2.5-3 fold more revolutions per day than those from four non-selected control (C) lines (Swallow et al., 2009; Kolb et al., 2010; Garland et al., 2011a). No other environmental or pharmacological agent has had this substantial, positive effect on running in HR mice (Rhodes et al., 2005; Keeney et al., 2008; Kolb et al., 2010).

As the duration of physical activity increases, which necessarily entails a decrease in the average intensity of work if fatigue is to be avoided, submaximal exercise is increasingly supported by intracellular lipids and free fatty acids (Coyle, 1995; McClelland 2004). Mice from HR lines run greater total distances than C, which is accomplished mainly by increased average running speeds (and increased intermittency: Girard et al., 2001), but also an increased duration of running (up to ~8 hours per night), especially in males (Garland et al., 2011a). Mice from HR lines also have lower body fat than C (Swallow et al., 2001; Nehrenberg et al., 2009). Glycogen depletion, a common cause of fatigue during prolonged exercise (Hickson et al., 1977), does not appear to be constraining further evolutionary increases in running by HR mice, as liver and gastrocnemius glycogen are not diminished any more than in C after nightly wheel running, nor is initial glycogen content or glycogen synthase activity different in HR mice (Gomes et al., 2009). Maximal oxygen consumption per se does not appear to be limiting voluntary wheel running, as increases in VO_2max caused by administration of an erythropoietin analog do not lead to increases in wheel running in either HR or C lines (Kolb et al., 2010).

Given the foregoing differences from C lines and HR's dramatic and differential wheel-running response to high-fat diet (Meek et al., 2010), we hypothesized that HR have greater capacity to utilize oxidative metabolic pathways, which could contribute to their enhanced running. The upregulation of enzymes directly involved in metabolism is often used to demonstrate the relevance of particular pathways or training regimens. The down- or up-regulation of enzyme abundance (as determined by maximal, in vitro catalytic rates, Vmax) can provide an indirect measure of the reliance on that pathway. Previous work on male HR mice from generation 14 found no effect of selection on enzyme activity (Houle-Leroy et al., 2000). However, this work was before the selection plateau, and when HR mice were running ~2 rather than ~3 times as far as C mice. It is not known if the ongoing selection and subsequent increase in running distance since that time has been sufficient to tax muscle metabolic capacities. Additionally, what role cellular metabolism plays in HR's response to high-fat diet remains an unanswered question.

Training-induced and diet-induced increases in oxidative enzyme capacity have been shown to act in an additive fashion in rats (Simi et al., 1991). High-fat diets independent effect of increasing oxidative enzyme capacity in skeletal muscle may enable HR mice to sustain prolonged sub-maximal running and overcome the physiological factors that limit individual voluntary wheel running. Given that the expression of oxidative metabolic enzymes largely determines the oxidative capacity of skeletal muscle (McGee and Hargreaves, 2010), we measured skeletal muscle enzyme

activity in HR and C mice after 8 weeks of a standard or Western diet while mice were either sedentary (housed in standard cages) or had access to a running wheel.

Methods

Animals. We studied male mice from the 52nd generation of an ongoing artificial selection experiment for high voluntary wheel running (Swallow et al., 1998a). The starting population for this experiment were outbred, genetically variable (Carter et al., 1999) mice of the Hsd:ICR strain, which has been the subject of numerous studies in exercise physiology and genetics (e.g., Dohm et al., 1994; Dohm et al., 2001; Nehrenberg et al., 2009). Since approximately generation 16, mice from the 4 replicate High Runner (HR) lines have been running ~2.5-3 fold more revolutions per day as compared with 4 non-selected control (C) lines (e.g., Keeney, Kolb et al., 2010; Garland et al., 2011a). Mice from within each of the 8 lines were separated into experimental groups starting at ~24 days of age. Animals were housed singly either with or without access to wheels (1.12 m circumference: with wheel = W; no wheel = NW) and further separated within these groups to receive a standard diet (SD, 14% kcal from fat [W] 8604 Harlan Teklad) or a Western diet (WD, 42% kcal from fat plus added sucrose, TD.88137 Harlan Teklad; see Meek et al., 2010 for dietary composition). Room temperature was maintained at ~72° F and on a photoperiod of 12:12 h. Water and food were available *ad libitum*. After 8 weeks, mice were sacrificed by decapitation and the heart ventricles were dissected free, blotted, and weighed. Triceps surea muscles were dissected, weighed, and frozen on dry ice.

Enzyme assays. The proximal half of the triceps surae was homogenized 1:10 (wt/vol) on ice in 100mM phosphate buffer (K_2HPO_4/KH_2PO_4) with 0.1% Triton X-100, 5mM EDTA, and 1mM DTT, pH 7.2 using a hand held tissue-tearor (Biospec Products, Bartsville, OK). After homogenization, samples were centrifuged (Allied Fisher Scientific micro-centrifuge model 235C) for 10 minutes at 12,400 rpm in an incubator set at 1.5^{o}C (Precision low temperature illuminated incubator model 818). After further dilutions, supernatant was used for assays at 37^{o}C in triplicate using saturating concentrations of substrates and cofactors, closely following the protocol of Houle-Leroy et al. (2000).

For citrate synthase (EC 4.1.3.7) assay, 100mM Tris-HCl, 0.2mM acetyl-CoA, 0.1mM DTNB, and 1mM oxaloacetate (omitted for controls), pH 8.0 were used with homogenate that had gone through 2 freeze thaw cycles to further break up the mitochondria. Hexokinase (EC 2.7.1.1) assay, (50mM triethanolamine-HCl, 8mM $MgCl_2$, 0.5mM NADP, 8mM ATP, 4U of G6PDH (in excess) and 4mM glucose (omitted for controls), pH 7.6. β-hydroxyacyl CoA dehydrogenase (EC 1.1.1.211) 100mM triethanolamine-HCl, 5mM EDTA, 0.28mM NADH, 0.1mM acetoacetyl-CoA (omitted for controls), pH 7.0. For carnitine palmitoyl-transferase (EC 2.3.1.21) assay, 75mM Tris-HCl,1.5mM EDTA, 0.05mM palmitoyl CoA, 0.2 mM DTNB, and 2mM carnitine (omitted for controls), pH 8.0 were used.

CS and CPT were measured at 412 nm using the extinction coefficient of 13.6 $ml \cdot cm^{-1} \cdot \mu mol^{-1}$ for DTNB. HAD and HK were measured at 340 nm using extinction coefficient 6.22 $ml \cdot cm^{-1} \cdot \mu mol^{-1}$ for NAD(P)H. The use of Triton x-100 increases

solubilization of mitochondrial enzymes but may also inhibit CPT1 activity (Woeltje et al.,1987) so our reported CPT activity likely represents only CPT2 (Suarez et al., 2009). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme activities were measured using a Spectramax Plus microplate reader (Molecular devices Sunnyvale, CA USA) with temperature-controlled plate holder. Absorbance was measured for each sample every 5 seconds with Vmax determined from the slope of the 20 steepest data points. Values for control samples were then subtracted from the average Vmax obtained from the 3 triplicates. Data were collected using Softmax PRO data acquisition software (4.3.1, 2003, Molecular Devices).

Immunoblotting assays. Polyclonal, anti– phosphorylation- 5'-AMP-activated protein kinase Thr172 (detects both α-1 and α-2 isoforms of the catalytic subunit), was from Cell Signaling Technology (Beverly, Mass). Frozen triceps surea muscle samples at -80^{o} C were homogenized in lysis buffer containing 10 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L, NaF, 20 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol,1 mmol/L phenylmethanesulfonyl fluoride and one tablet per 10ml of Complete mini protease inhibitor cocktail (Roche Diagnostics Indianapolis, IN). Equal amounts of protein extracts were separated by using 8% SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc., Hercules, CA). After blocking for 1 hour in a Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk, the membrane was probed with various primary

antibodies, followed by secondary antibodies conjugated to horseradish peroxidase. The immunoreactivity was revealed by use of an ECL kit (Amersham Biosciences Co., Piscataway, NJ), and the intensities of the protein bands were quantified by ImageJ software (National Institutes of Health, USA).

Gene expression assays. The left triceps surae muscle group was powdered with mortar and pestle while frozen in a bath of liquid nitrogen to ensure that fast and slow twitch fibers from the gastrocnemius and soleus muscles were evenly distributed in each sample of muscle homogenate. RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA) and quantified by spectrophotometry. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed (Applied Biosystems, Carlsbad, CA) and primers were designed to evaluate the levels of mRNA of PGC1-α and telomere-related genes Trf1, Trf2, Pot1a, Pot1b, and p53 (as a marker of apoptosis), with mRNA levels of Gapdh measured as a positive control. PCR was performed and products were run on two percent agarose gels stained with ethidium bromide. Gels were analyzed by semi-quantitative densitometry and assessed using ImageJ software (National Institutes of Health, USA).

Telomere assays. DNA was isolated from powdered triceps surae muscle using a PureGene DNA isolation system (Gentra Systems, Minneapolis, MN). Relative telomere length (Telomere PCR to Single-copy gene PCR, or T/S ratio) was measured via a real-time PCR assay previously described (Cawthon, 2002; Callicott and Womack, 2006) but

modified slightly for our particular samples. All telomere length assays were performed on an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). For both T and S PCRs, 5.83 uL of 10 ng/uL DNA was added to a mastermix of SYBR green dye, primers, and distilled water. Primers and cycling conditions are available by request. T/S ratios are proportional to average telomere length (Cawthon 2002) and were derived from the critical threshold (Ct) values generated by fluorescence of the SYBR green dye that reflects the level of expression of the gene of interest.

Statistical analyses. Analyses for enzyme activities were performed using REML estimation in the Mixed Procedure of SAS 9.1.3 (SAS Institute, Cary, NC, USA) to apply analysis of covariance models with Type III tests of fixed effects. Four-way ANOVA were performed with linteype (HR vs. C), wheel access (W vs. NW), diet (WD vs. SD), and presence of the mini-muscle phenotype (see below) as the main effects. Replicate line was a random effect nested within linetype. Degrees of freedom for testing the effects of linetype, wheel access, diet, linetype X wheel access interaction, linetype X diet interaction, wheel access X diet interaction, and the linetype X wheel access X diet interactions were always 1 and 6. For testing the effect of mini-muscle, degrees of freedom were 1 and ~151 when all mice were analyzed, or 1 and ~76 when only the wheel-access groups were considered (see tables). When linetype by wheel access interactions were statistically significant ($P < 0.05$), differences of least squares means from the same analyses are reported across diets for both C and HR lines.

Data on wheel running, food consumption, body mass, and retroperitoneal fat pad mass for the wheel access group were presented in Meek et al. (2010). For analyses of wheel running (total revolutions per day, number of intervals with at least one revolution, mean revolutions per minute for the active intervals), we analyzed mean values from either the final two weeks (days 44-58; shown previously) or cumulative running over the entire experiment (excluding the first three days; days 4-58, not shown previously). The only covariate for analysis of wheel-running data was wheel freeness, which is a measure of how many revolutions a wheel spins following acceleration to a standard velocity (e.g., Kolb et al., 2010; Garland et al., 2011a).

In analyses of wheel-access groups, up to three models were compared: one without the amount of wheel running as a covariate, the second with the final two weeks of running, and the third with the cumulative running over days 4-58. Twice the difference in log maximum likelihoods between the models is assumed to be distributed (asymptotically) as a χ^2 with degrees of freedom equal to the number of parameters that have been added. Models estimated by maximum likelihood were used for these χ^2 calculations, whereas models estimated by REML were used to generate *F* statistics and *P* values (Garland and Kelly, 2006), as indicated above.

Within 2 of the 4 HR lines and 1 of the 4 C lines, a small-muscle phenotype has been observed (Garland et al., 2002; Hannon et al., 2008). These so-called mini-muscle individuals are homozygous for a Mendelian recessive allele that causes a 50% reduction in triceps surae (and hindlimb) muscle mass (Houle-Leroy et al., 2003). Many pleiotropic effects have been observed, including alterations in contractile properties

(Syme et al., 2005), larger livers (Garland et al., 2002; Kolb et al., 2010), and larger ventricles (Hannon et al., 2008; Meek et al., 2009). Mini muscle individuals also show differences in wheel-running behavior under some conditions (Syme et al., 2005).

Analysis of p-AMPK (Thr172)/t-AMPK was also done with SAS Procedure Mixed, but owing to low sample sizes in some cells, line was not nested within linetype for these analyses. Several gels were used to gather the information, so gel number was included as a random effect in these analyses. All other main effects and interactions remained the same, except mini-muscle individuals were not included so it was not incorporated as a factor in the model. Data are presented as least squares means ± S.E. from SAS Procedure Mixed.

No main effects or interactions were found to be statistically significant ($P < 0.05$) for the housekeeping gene GAPDH, so analyses for gene expression data were performed without using ratios of each gene to GAPDH. Data were collected across several gels, but care was taken to include samples from all groups on each gel. Differences among gels were tested by including gel as a random effect in the analysis of GAPDH. Gel was non-significant and explained only 2.8% of the variance, so it was removed and not used for any of the analyses presented.

Data are presented as least squares means ± associated S.E. from SAS Procedure Mixed.

Results

Voluntary wheel running. Considering total revolutions per day averaged over experimental days 4-58, the interaction between linetype and diet was statistically significant ($P = 0.0232$; Table 4.1). Inspection of the least squares means, as shown in Figure 4.1, indicates that Western diet had essentially no effect on running in C lines (-3%) but increased it by 38% in HR lines. On standard chow, HR mice ran 2.90-fold more than C (typical of many previous reports on these lines, e.g., Swallow et al., 2009; Kolb et al., 2010; Garland et al., 2011a), while on Western diet HR ran an average of 4.13-fold more than C. The higher running by HR animals on Western diet versus standard chow was attributable to a longer running duration (+30%) and a more modest change in speed (+13%), as compared with HR mice on standard chow. Results were similar for wheel running during the final two weeks of the experiment (Table 4.1).

Ventricle mass. When housed without wheels, HR and C mice did not differ in ventricle mass adjusted for body mass (differences of least squares means from SAS Proc Mixed, $P = 0.8700$). Both HR and C lines exhibited a classic training response -- an increased ventricle mass -- when given access to wheels (Figure 4.2; differences of least squares means: Control $P = 0.0328$, HR $P = 0.0005$). However, the training response was greater in HR mice (linetype X wheel access $P = 0.0237$), which can be explained statistically by their greater running (see Table 4.1 supplement). Specifically, the difference between the linetypes loses significance when either the amount of running over days 4-58 or only during the final two weeks of running is included as an additional

covariate in analyses of the mice with wheel access. Interestingly, the amount of running during the final two weeks was a somewhat better predictor of ventricle mass as compared with running across the entire experimental period (Table 4.1 supplement).

Enzyme activities. All four enzyme activities showed a significant linetype by wheel access interaction (Table 4.2, Figure 4.3). Pooling over diet treatments, differences of least squares means from the analyses shown in Table 4.2 indicate that the interactions are driven by HR's significant induction of enzyme activity when given access to wheels (CS $P = 0.0017$, HK $P = 0.0043$, CPT $P = 0.0065$, HAD $P = 0.0032$), as compared with a lack of a response by C mice (CS $P = 0.1091$, HK $P = 0.6492$, CPT $P = 0.7122$, HAD $P = 0.6797$).

Analysis of only wheel-access groups indicates the greater training effect in HR for CS and HAD can be explained statistically by their greater amount of wheel running during the final two weeks. For HK and CPT, however, the higher running does not explain the greater enzyme activity; rather, HR mice have greater plasticity in response to voluntary exercise, as shown by the difference in the ln maximum likelihoods (Table 4.2B supplement). Similar analyses but using the amount of wheel running over experimental days 4-58 always yielded lower likelihoods for the models, thus indicating that running during the final two weeks is a better predictor of final enzyme activities (Table 4.2B supplement).

Western diet significantly increased HAD activity (Figure 4.3, Table 4.2) and did not statistically interact with either linetype or wheel access. In addition, Western diet

increased the HAD/CS ratio indicating that fatty acid oxidation capacity was elevated compared to total aerobic metabolic capacity (Table 4.2 [Pette & Dolken, 1975]). This was not the case for HK/CS ratio, where the relative capacity of anaerobic vs. aerobic metabolism remained unchanged by diet (or wheel access). Mini-muscle mice had a significantly greater HAD/CS ratio and much higher activity of all enzymes except CPT (Table 4.2, Figure 4.4), likely due to inherent differences in muscle mass and fiber-type composition that favors oxidative ability (see Discussion).

Molecular data. In the analyses of all groups only the linetype by wheel access interaction was significant for p-AMPK/t-AMPK ($P = 0.0170$, Table 4.3). The direction of the interaction is similar to the enzyme results, whereby HR mice show a greater increase than C when given access to wheels. This increase is primarily attributable to the higher running observed in these lines (results not shown). Additionally, differences in least squares means from SAS Proc Mixed revealed that wheel access significantly increased p-AMPK/t-AMPK in HR mice ($P = 0.0432$), but not in C mice ($P = 0.1850$) across both diets. Western-diet effects were non-significant.

Telomere-related data. Telomere length was significantly greater in mice fed the Western diet compared with those on regular chow ($P = 0.0020$; Figure 4.6). Back-transformed least squares means for WD (0.825 ± 0.308, 0.167; mean with asymmetrical 95% CI) was 34% greater than for normal chow (0.542 ± 0.301, 0.140). No other statistical effects on telomere length were observed (all $P > 0.29$). No differences were

found for P53 or PGC1-α. There were no statistical differences in mRNA levels of the telomere-protecting shelterin components Trf1, Trf2, Pot1a or Pot1b for diet, wheel access or linetype (all $P > 0.2425$). Mini-muscle mice had higher Trf2 ($P = 0.0119$) by approximately one third compared to other mice (2,083 ± 106 and 1,457 ± 230, respectively; least squares means ± S.E.), but no other differences were observed.

Discussion

Lipids predominate as the cellular fuel source during low-to-moderate-intensity, sustained aerobic exercise, particularly in trained individuals, for both humans and rodents (Coyle, 1995; Wasserman and Cherrington, 1996; Brooks, 1998; McClelland, 2004; Weber, 2011). Male mice from four replicate lines that have been selectively bred for more than 50 generations for high voluntary wheel running (HR lines) show a distinctive locomotor response to high-fat diet. Whereas most rodents, including those from the corresponding non-selected control (C) lines, decrease or do not change locomotor activity when administered high-fat diet, male HR mice significantly increase their wheel running (Meek et al., 2010; Figure 1). Considering HR's running ~8 h/day, their low body fat (Swallow et al., 2001; Nehrenberg et al., 2009), and similar liver and muscle glycogen depletion during wheel running as compared with C mice, lipids likely serve as a critical substrate during their wheel running. However, Western diet did not differentially affect mice from HR and C lines for any trait measured in the present study, which suggests that its stimulatory effect on wheel running in HR mice involves cellular factors other than maximum enzymatic capacities in triceps surae muscle.

Western diet increased the absolute capacity to use lipids and the relative potential for lipid oxidation as compared with overall oxidative metabolism (HAD and HAD/CS results: Table 4.2). However, we did not observe any linetype by diet interactions, which implies that HR's skeletal muscle response to the Western diet was not statistically different from that of mice from C lines, and hence not responsible for HR's increased running. Therefore, the remarkable differential response in wheel running between the linetypes (Figure 4.1, Table 4.1; Meek et al., 2010) is not directly attributable to skeletal muscle lipid oxidation capacity.

Our results for enzymatic and molecular data show a general training effect when all mice are pooled, and statistically greater up-regulation in HR mice for CS, HK, HAD, CPT (as evident by the linetype by wheel access interactions shown in Table 4.2). Mice from this study were "self trained" by voluntary running on wheels, rather than through a forced-exercise paradigm. Therefore, the baseline difference in wheel running between C and HR mice potentially complicates interpretation of a training response. One possible explanation for HR mice having greater enzyme induction is simply through greater training volume. Alternatively, HR mice could show greater plasticity than C mice (Swallow et al., 2005; Garland and Kelly, 2006). In other words even at the same training amount, HR mice could have greater enzyme up-regulation. This latter possibility has been confirmed previously for HR lines with respect to GLUT4 up-regulation in females after five days of wheel access (Gomes et al., 2009), as well as hematocrit, hemoglobin content in the blood, cytochrome c oxidase, and pyruvate dehydrogenase following eight weeks of wheel access (Garland and Kelly, 2006). To test

between these alternative hypotheses, we compared models using maximum likelihood ratio tests (supplemental Table 4.2B). The linetype by wheel access interaction for CS and HAD is driven by HR's greater daily running. In essence, greater training volume results in greater enzyme up-regulation. Higher activity of HK and CPT in HR mice with wheel access, however, cannot be fully explained by the amount of wheel running. Instead, HR mice appear to have evolved greater plasticity that may be beneficial for wheel running (i.e., adaptive plasticity: Garland and Kelly, 2006).

Inspection of 'Differences of least squares means' from SAS Procedure Mixed reveals that C mice showed a statistically significant training response only for ventricle mass and not for any enzyme or molecule studied ($P > 0.10$ for all). As suggested in previous studies (Houle-Leroy et al., 2000) C mice simply may not run enough to elicit a training response for some traits. This implies, at least for C mice, that typical voluntary running distances are not limited by maximal skeletal muscle metabolic properties; instead, "motivation" may limit wheel running in C mice (see also Rhodes et al., 2005; Kolb et al., 2010; Garland et al., 2011b). As also noted previously (Houle-Leroy et al., 2000), the evolution of increased voluntary exercise levels clearly has not blunted the training responses of HR mice.

Other multi-week training studies with voluntary wheel running have not always shown an enzymatic training response in rats (Bagby et al., 1986; Podolin et al., 1999) or mice (MacNeil and Hoffman-Goetz, 1993; Landisch et al., 2008). MacNeil and Hoffman-Goetz (1993) did not find any difference in citrate synthase activity from the soleus of C3H/He male mice after 9 weeks of wheel access, even though mice were

running more than twice as far per day as our Control animals. However, caution needs to be taken when comparing running distances among studies that differ in wheel size and construction. Landisch et al. (2008) found that wheel running of 7.5 km/day did not increase CS in the soleus or extensor digitorum longus but did in the tibialis anterior muscle of mice. Cytochrome c oxidase failed to increase in the soleus or tibialis anterior muscle but did in the extensor digitorum longus (Landisch et al., 2008).

Rockl et al. (2007) reported increases of citrate synthase, hexokinase II, and PGC-1α, but not GLUT-4 in wildtype (FVB background) and α2iTG mice running voluntarily 4.5 km/day. In other wildtype and transgenic (γ2iTG) groups, running 2 km/day, hexokinase II and GLUT-4 did not significantly train, although CS did for wildtype mice. Running ~3 km/day (as in Control mice from the present study) has been shown to elicit a training response in female C57BL/6 mice for CS and cytochrome c oxidase in plantaris and soleus muscle after 16 weeks of wheel access compared to sedentary mice (Hoffman-Goetz et al., 2009).

In a separate study using female C57BL/6 mice, whole-organism traits, such as time to exhaustion during a treadmill test and VO_2max, showed training improvements also after 16 weeks of voluntary wheel running (Davidson et al., 2006). From the same study, activities of the enzymes CS, succinate dehydrogenase, and phosphofructose kinase were all elevated in at least some tissues composing the triceps surae. CS activity increased with wheel running by 9%, 16%, 18%, and 26% in the white gastrocnemius, soleus, red gastrocnemius, and plantaris respectively, with only the white gastrocnemius being statistically non-significant. Our results for CS show a non-significant increase of

13% in control mice given wheel access and standard diet (vs. +31% in HR mice on standard diet). Although the percent increase in CS for wheel-running control mice is non-significant in the present study, it is nonetheless very similar to the increase reported by Davidson et al. (2006), despite our mice being on wheels half as long (8 vs. 16 weeks), a different sex (males), and measured from homogenate of all the muscles of the triceps surae combined.

Treadmill exercise consistently results in training responses for most exercise-related enzyme activities in mice. For example, one hour of forced exercise training at different intensities (20 or 25 m/min) and inclines (0^o or 8^o), 5 days a week for 4 weeks, raised activities for CS, cytochrome c oxidase, lactate dehydrogenase, and malate dehydrogenase in the rectus femoris of female C57BL/6 mice (Vihko et al., 1978). Even lower speeds and shorter daily durations of treadmill training (15 m/min for 30 min/day for 9 weeks) are sufficient to elicit an increase in soleus CS of mice, although 5 m/min for 5 minutes a day was not (MacNeil and Hoffman-Goet, 1993) nor is less than ten days of training in eiher sex (Brown et al., 2007). Mehl et al. (2005) report increases in gastrocnemius CS activity after treadmill training (55 min at 18 m/min and 5% grade for 9 weeks) from male $APC^{Min/+}$ mice (C57BL/6 background). However, unlike MacNeil and Hoffman-Goet (1993), continuous wheel access over 9 weeks induced a greater enzymatic response than treadmill running.

Overall, the present and previous studies demonstrate that voluntary wheel running can certainly result in enzymatic up-regulation in rodent muscle, but not always. Difficulties comparing responses to voluntary exercise may exist because total distance is

not necessarily obtained by the same style of running. Both running speed and the duration of running can vary widely in rodents (e.g., see Lightfoot et al., 2004; Nehrenberg et al., 2009), as can the intermittency of running. For instance, HR mice run more intermittently than C mice (Girard et al., 2001), which may or may not have an effect on training responses. Treadmill training often produces an enzymatic training effect despite being shorter in daily duration and sometimes at lower speeds. Often, the total distance covered in the daily treadmill training regimen is 75% less that the distance voluntarily run on wheels (Landisch et al., 2008). Strong training similarities exist between the two conditions, but the differences may arise because treadmill training does not reflect typical running behavior of mice. Continuous, steady-state running for many minutes during treadmill tests is distinctly different from intermittent running at varying speeds (and perhaps gaits), which is characteristic of voluntary wheel exercise in laboratory mice (MacNeil and Hoffman-Goetz, 1993; Girard et al., 2001). Interestingly, physical activity in children tends to occur in an intermittent fashion, whereas continuous steady physical activity predominates as the prescribed treatment for metabolic diseases (Ekkekakis, 2009).

Western diet increased HAD activity and acted in an additive fashion with wheel running (Table 4.2, Figure 4.3). Similarly, in rats, Simi et al. (1991) found that high-fat diet increased VO_2max additively with training-induced increases. Lee et al. (2001) showed that high-fat diet increases endurance (as measured by time to exhaustion during forced treadmill running) even in untrained rats. Furthermore, they showed that concomitant treadmill training and high-fat feeding for 8 weeks resulted in increased

endurance over carbohydrate feed rats. Results from our study, with respect to HAD, along with those of Simi et al. (1991), support Lee et al's. (2001) conclusion that exercise-induced and diet-induced training adaptations are under at least partly independent control.

The higher oxidative capacity (Table 4.2, Figure 4.4) in mini-muscle triceps surae is likely attributable to reduced muscle mass accompanied by increased relative proportion of oxidative fiber types. The triceps surae of mini-muscle mice show a near 50% reduction in total mass, but the soleus is almost double in mass (Syme et al., 2005; McGillivray et al., 2009; Audet et al., in press). The mouse soleus is comprised of mostly (oxidative) type I myosin heavy chain fibers, while the remaining portions of the triceps surae (gastrocnemius and plantaris) contain relatively more glycolytic isoforms. The gastrocnemius and plantaris of mini-muscle mice have a large reduction of type II_b myosin heavy chain isoform and a shift towards a more oxidative phenotype (types I, II_a, II_x myosin) (McGillivray et al., 2009).

AMPK is a major regulator of cellular energy balance and coordinates lipid flux into the mitochondrial through phosphorylation (and inhibition) of Acetyl-CoA carboxylase. Very similar to the linetype by wheel access interaction observed with the enzymes, p-AMPK/t-AMPK was largely increased in HR mice when given access to wheels as compared with C mice (compare Figs. 3 and 5). The greater magnitude can be accounted for by greater running distances in HR mice.

Phosphorylation is a much more sensitive and ephemeral cellular marker of energy balance/flux than enzymatic activities. AMPK phosphoylation, due to an acute

bout of exercise, can be detected in as little as 20 minutes in murine gastrocnemius (Tadaishi et al., 2011), and the signal is quickly diminished once cellular energy equilibrium is approached. An hour after a 90-minute bout of treadmill running, α-AMPK phosphorylation already approaches baseline levels in mouse quadriceps muscle (Jørgensen et al., 2005). The differences in voluntary running style (speed, duration, intermittency: Girard et al., 2001; Garland et al., 2011a), % of VO_2max at which they run (Rezende et al., 2005), and amount of running immediately preceding the measurement time point is likely to influence the level of phosphorylation. Muscle samples were obtained during the diurnal period, after wheels had been blocked and mice fasted for 4-6 hours. Understanding how AMPK oscillates over a 24 hour period in response to food consumption and/or exercise would provide an indication as to the possible differences in cellular energy stress among our experimental groups.

Unexpectedly, skeletal muscle telomere length was significantly longer in Western diet-fed mice compared to those fed standard chow. Poor diet is thought of as a risk factor for cellular aging, but here Western diet appears to positively affect telomere length. The Western diet may have resulted in elevated oxidative stress, inflammation, etc., and over a lifetime this would result in telomere shortening. However, in young animals, the effects of diet may be manifested not as short telomeres but instead dysfunction may be revealed as telomeres that are actually longer than those of their counterparts fed a regular diet. In a human study of leukocyte telomere length in patients with coronary artery disease, also considered a risk factor for cellular aging, telomere length increased in some individuals, remained constant in others, and in still others,

decreased after a five year follow-up (Farzaneh-Far et al., 2010). The individuals with the shortest telomeres at baseline tended to have the longest telomeres after 5 years, and vice versa. In another study with HR mice (generation 52), Western diet increased wheel running and caloric intake compared to controls on Western diet (Meek et al., 2010). We suspect that a reversal of telomere length would result had the mice been allowed to age.

We observed no statistical effect of voluntary exercise on telomere length. The mice may have been too young (approximately 12 weeks of age at sacrifice) and/or the exercise stimulus too short (8 weeks) to result in changes in telomere length as a result of the exercise intervention. In previous animal studies, neither an exercise stimulus of 3 weeks nor 6 months resulted in telomere shortening in myocardium (Werner et al., 2008) or aorta (Werner et al., 2009), although telomerase activity was higher in the exercised mice and mRNA levels of telomere-regulatory proteins were altered as a result of the exercise training (Werner et al., 2008). One other possibility is that the mice from c lines had low physical activity levels and the HR mice exercised at very high levels, resulting in a similar "inverted U" shape to that seen by Ludlow and colleagues (Ludlow et al., 2008), where both low and high physical activity levels were associated with shorter telomeres compared to moderately active individuals. The large gap in average distance run between HR and C mice (Figure 1) is consistent with this hypothesis.

Breeding for increased wheel running has taxed the oxygen transport and delivery system, as evidenced by elevated treadmill endurance and VO_2max in untrained mice (Swallow et al., 1998b; Rezende et al., 2006; Meek et al., 2009; Kolb et al., 2010). Studies of rats have shown that high-fat diet can increase VO_2max and endurance even in

the absence of exercise (Miller et al., 1984; Simi et al., 1991; Lapuchet et al., 1996; Lee et al., 2001). But these effects, if occurring in HR mice, likely do not play an immediate role in the Western diet-induced increase in wheel running. Kolb et al. (Kolb et al., 2010) treated HR and C mice with a synthetic analog of erythropoietin (EPO), successfully elevating hemoglobin and VO_2max, with no observed increase in wheel running in either linetype.

Lipids are also known to have direct and indirect effects on the brain (Geiger et al., 2008; South and Huang, 2008; Stice et al., 2008). Leptin and/or adiponectin, both released from adipocytes, help coordinate whole-body energy balance, of which locomotion can be a major component (Garland et al., 2011b). Some studies have shown that HR mice have low circulating levels of leptin and high levels of adiponectin, even after taking into account body adiposity as a statistical covariate (Girard et al., 2007; Vaanholt et al., 2008). These hormones are known to bind to receptors in the brain and, in principle, could be acting in a feedback manner to influence motivation for voluntary exercise (Kubota et al., 2007). Increased adiposity associated with eating a Western diet leads to an increase in circulating leptin concentrations in HR mice (Vannholt et al., 2008), and perhaps the changes in hormonal concentrations or sensitivity are responsible for changes in locomotor activity.

The biological basis of variation in voluntary physical activity has become a critical topic for both biomedical researchers and evolutionary physiologists (Garland et al., 2011b). HR mice, but not C mice, responded to high-fat diet consumption by substantially increasing wheel running (Meek et al., 2010). Given the present results

showing an absence of differential enzymatic up-regulation in HR as compared with C, high-fat diets stimulatory effect does not appear to be governed by the capacity of skeletal muscles to metabolize lipids. Lipids have diverse effects on a variety of tissues and the mechanism responsible for the increased wheel running in HR mice remains unknown. A more thorough understanding of the role of diet in modulating physical activity and thereby indirectly influencing food consumption, body composition, and metabolic traits continues to be an essential line of research.

Chapter 4 Acknowledgments

We would like to thank Brooke K. Keeney, Robert M. Hannon, and Sarah L. Frank for assistance. All animal procedures are in compliance with the UCR Institutional Animal Care and Use Committee and U.S. laws.

Grants

This study was supported by Michigan State University Seed Grant to JCE; NSF IOB-0543429 and IOS-1121273 to TG; and the University of Maryland Department of Kinesiology's Graduate Research Initiative Fund to MRM.

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Table 4.1 Analysis of covariance for wheel running for experimental days 4-58 and 44-58 of wheel access.

	Revs/Day	Minutes/Day	Mean Speed
Days 4-58			
Linetype (HR vs. C)	**$F_{1,6}$ = 29.61 + P = 0.0016**	**$F_{1,6}$ = 6.75 + P = 0.0387**	**$F_{1,6}$ = 60.03 + P = 0.0002**
Diet (Std. Vs. Western)	**$F_{1,6}$ = 8.02 + P = 0.0299**	**$F_{1,6}$ = 8.29 + P = 0.0281**	$F_{1,6}$ = 1.41 + P = 0.2795
Linetype X Diet	**$F_{1,6}$ = 9.15 P = 0.0232**	**$F_{1,6}$ = 7.27 P = 0.0357**	$F_{1,6}$ = 1.67 P = 0.2438
Mini-muscle	$F_{1,82}$ = 0.15 - P = 0.7017	$F_{1,81}$ = 0.62 - P = 0.4330	$F_{1,82}$ = 0.17 + P = 0.6777
Days 44-58			
Linetype (HR vs. C)	**$F_{1,6}$ = 19.82 + P = 0.0043**	$F_{1,6}$ = 5.38+ P = 0.0595	**$F_{1,6}$ = 32.23 + P = 0.0013**
Diet (Std. Vs. Western)	$F_{1,6}$ = 2.14 + P = 0.1935	$F_{1,6}$ = 0.17 + P = 0.6930	$F_{1,6}$ = 0.33 + P = 0.5878
Linetype X Diet	**$F_{1,6}$ = 6.88 P = 0.0394**	$F_{1,6}$ = 4.98 P = 0.0672	$F_{1,6}$ = 0.77 P = 0.4153
Mini-muscle	$F_{1,80}$ = 0.20 - P = 0.6582	$F_{1,80}$ = 0.56 - P = 0.4574	$F_{1,80}$ = 0.00 + P = 0.9785

Results from nested ANCOVAs in SAS Procedure Mixed. Replicate line nested within linetype was included as a random effect in all analyses, and wheel freeness was used as a covariate (results not shown). + indicates direction HR > C, Western diet > standard diet, mini-muscle > normal. See Figure 4.1 for corresponding least squares means and standard errors.
All P-values are two-tailed. Significant values ($P < 0.05$) are in bold. N = 100 (one outlier was deleted from analysis of days 44-58).

Table 4.2 Values for F tests and corresponding P values from three-way ANOVAs of enzyme activities from triceps surae muscle

	CS	*HK*	*CPT*	*HAD*	*HAD/CS*	*HK/CS*
Linetype (HR vs. C)	$F_{1,6}$ = 0.42+ P = 0.5410	$F_{1,6}$ = 0.73+ P = 0.4244	$F_{1,6}$ = 0.79+ P = 0.4091	$F_{1,6}$ = 0.06+ P = 0.8075	$F_{1,6}$ = 0.02- P = 0.8988	$F_{1,6}$ = 0.06+ P = 0.8185
Wheel Access (WhlAcc)	**$F_{1,6}$ = 26.63+ P = 0.0021**	**$F_{1,6}$ = 12.42+ P = 0.0125**	**$F_{1,6}$ = 10.06+ P = 0.0193**	**$F_{1,6}$ = 13.61+ P = 0.0102**	$F_{1,6}$ = 1.16- P = 0.3229	$F_{1,6}$ = 3.57- P = 0.1077
Diet (Std. Vs. Western)	$F_{1,6}$ = 0.02+ P = 0.8944	$F_{1,6}$ = 0.80+ P = 0.4045	$F_{1,6}$ = 0.89+ P = 0.3810	**$F_{1,6}$ = 8.16+ P = 0.0290**	**$F_{1,6}$ = 7.96+ P = 0.0303**	$F_{1,6}$ = 0.45+ P = 0.5292
Linetype X WhlAcc	**$F_{1,6}$ = 6.36 P = 0.0452**	**$F_{1,6}$ = 8.15 P = 0.0290**	**$F_{1,6}$ = 6.91 P = 0.0392**	**$F_{1,6}$ = 9.50 P = 0.0216**	$F_{1,6}$ = 0.55 P = 0.4850	$F_{1,6}$ = 0.12 P = 0.7392
Linetype X Diet	$F_{1,6}$ = 0.57 P = 0.4798	$F_{1,6}$ = 0.99 P = 0.3576	$F_{1,6}$ = 0.02 P = 0.8894	$F_{1,6}$ = 1.31 P = 0.2942	$F_{1,6}$ = 0.00 P = 0.9941	$F_{1,6}$ = 0.06 P = 0.8100
WhlAcc X Diet	$F_{1,6}$ = 0.00 P = 0.9926	$F_{1,6}$ = 0.73 P = 0.4266	$F_{1,6}$ = 0.00 P = 0.9871	$F_{1,6}$ = 0.70 P = 0.4352	$F_{1,6}$ = 0.95 P = 0.3678	$F_{1,6}$ = 0.01 P = 0.9402
Linetype X WhlAcc X Diet	$F_{1,6}$ = 1.02 P = 0.3521	$F_{1,6}$ = 0.60 P = 0.4680	$F_{1,6}$ = 0.31 P = 0.6000	$F_{1,6}$ = 0.67 P = 0.4449	$F_{1,6}$ = 0.03 P = 0.8723	$F_{1,6}$ = 0.55 P = 0.4870
Mini-muscle	**$F_{1,151}$ = 11.15+ P = 0.0011**	**$F_{1,151}$ = 14.98+ P = 0.0002**	$F_{1,148}$ = 0.66+ P = 0.4193	**$F_{1,148}$ = 52.34+ P = <0.0001**	**$F_{1,147}$ = 12.77+ P = 0.0005**	$F_{1,150}$ = 3.74+ P = 0.0549

Values for F tests and degrees of freedom for enzymes citrate synthase (CS), hexokinase (HK), carnitine palmitoyl-transferase (CPT), 3-hydroxyacyl-CoA dehydrogenase (HAD) and molecules p-AMPK and p-ACC. Bold indicates significance $P < 0.05$. + indicates HR > control, wheel access > sedentary, Western diet > standard chow, mini-muscle > other HR mice. See Figure 4.3 for average enzyme activity within each group.

Table 4.3 F tests and likelihood models, for mice with wheel access (active group).

	CS		*HK*		*CPT*		*HAD*	
Linetype (HR vs. C)	$F_{1,6}$ = 1.45+	$F_{1,6}$ = 0.24-	$F_{1,6}$ = 3.94+	$F_{1,6}$ = 0.72+	$F_{1,6}$ = 2.45+	$F_{1,6}$ = 0.08+	$F_{1,6}$ = 3.84+	$F_{1,6}$ = 0.01+
	P = 0.2743	P = 0.6390	P = 0.0945	P = 0.4286	P = 0.1682	P = 0.7904	P = 0.0977	P = 0.9169
Diet (Std. Vs. Western)	$F_{1,6}$ = 0.00+	$F_{1,6}$ = 0.04-	$F_{1,6}$ = 1.32+	$F_{1,6}$ = 1.03+	$F_{1,6}$ = 0.63+	$F_{1,6}$ = 0.26+	$F_{1,6}$ = 1.48+	$F_{1,6}$ = 1.04+
	P = 0.9830	P = 0.8504	P = 0.2950	P = 0.3504	P = 0.4575	P = 0.6273	P = 0.2694	P = 0.3467
Linetype X Diet	$F_{1,6}$ = 1.07	$F_{1,6}$ = 0.51	$F_{1,6}$ = 1.36	$F_{1,6}$ = 0.80	$F_{1,6}$ = 0.10	$F_{1,6}$ = 0.03	$F_{1,6}$ = 1.37	$F_{1,6}$ = 0.70
	P = 0.3398	P = 0.5026	P = 0.2873	P = 0.4042	P = 0.7639	P = 0.8608	P = 0.2862	P = 0.4340
Mini-muscle	**$F_{1,74}$ = 17.80+**	**$F_{1,72}$ = 20.08+**	**$F_{1,74}$ = 5.43+**	**$F_{1,72}$ = 7.54+**	**$F_{1,71}$ = 4.74+**	**$F_{1,69}$ = 7.05+**	**$F_{1,73}$ = 23.71+**	**$F_{1,71}$ = 26.16+**
	P = <0.0001	P = <0.0001	P = 0.0225	P = 0.0076	P = 0.0328	P = 0.0098	P = <0.0001	P = <0.0001
Running		$F_{1,72}$ = 3.78+		$F_{1,72}$ = 1.49+		$F_{1,69}$ = 1.93+		$F_{1,71}$ = 3.56+
Average over days 44-58		P = 0.0557		P = 0.2268		P = 0.1689		P = 0.0632
ln maximum likelihood	-354.1	-348.6	-108.0	-106.3	42.1	43.2	-291.3	-286.6
Twice the difference		**11.09***		3.44		2.33		**9.30***

Values for *F* tests and degrees of freedom for enzymes citrate synthase (CS), hexokinase (HK), carnitine palmitoyl-transferase (CPT), β-hydroxyacyl-CoA dehydrogenase (HAD) for wheel access mice only, with and without previous 2 weeks of wheel running (mean revolutions/day) as a covariate. Bold indicates significance $P < 0.05$. + indicates HR > control, Western diet > standard chow, mini-muscle > other HR mice.

* indicates the model that includes a measure of wheel running as a covariate has a significantly higher likelihood ($P < 0.05$) as compared with the model that does not include wheel running (twice the difference between the models is assumed to be distributed asymptotically as a χ^2 with 1 degree of freedom).

Table 4.3 continued.

	CS	*HK*	*CPT*	*HAD*
Linetype (HR vs. C)	$F_{1,6}$ = 0.19-	$F_{1,6}$ = 1.40+	$F_{1,6}$ = 0.074-	$F_{1,6}$ = 0.04-
	P = 0.6747	P = 0.2813	P = 0.8050	P = 0.8465
Diet (Std. Vs. Western)	$F_{1,6}$ = 0.15-	$F_{1,6}$ = 0.98+	$F_{1,6}$ = 0.09+	$F_{1,6}$ = 0.47+
	P = 0.7087	P = 0.3598	P = 0.7721	P = 0.5187
Linetype X Diet	$F_{1,6}$ = 0.31	$F_{1,6}$ = 0.99	$F_{1,6}$ = 0.03	$F_{1,6}$ = 0.37
	P = 0.5989	P = 0.3577	P = 0.8765	P = 0.5654
Mini-muscle	**$F_{1,73}$ = 19.95+**	**$F_{1,73}$ = 5.81+**	**$F_{1,70}$ = 11.77+**	**$F_{1,72}$ = 27.05+**
	P = <0.0001	P = 0.0185	P = 0.0010	P = <0.0001
Running	$F_{1,73}$ = 2.41+	$F_{1,73}$ = 0.14+	$F_{1,70}$ = 2.93+	$F_{1,72}$ = 3.50
Average over days 4-58	P = 0.1253	P = 0.7125	P = 0.0916	P = 0.0653
ln maximum likelihood	-352.9	-107.8	43.6	-289.4
Twice the difference	2.54	0.36	3.08	3.68

Values for *F* tests and degrees of freedom for enzymes citrate synthase (CS), hexokinase (HK), carnitine palmitoyl-transferase (CPT), β-hydroxyacyl-CoA dehydrogenase (HAD) for wheel access mice only, with and without previous 2 weeks of wheel running (mean revolutions/day) as a covariate. Bold indicates significance $P < 0.05$. + indicates HR > control, Western diet > standard chow, mini-muscle > other HR mice.

* indicates the model that includes a measure of wheel running as a covariate has a significantly higher likelihood ($P < 0.05$) as compared with the model that does not include wheel running (twice the difference between the models is assumed to be distributed asymptotically as a χ^2 with 1 degree of freedom).

Table 4.4 Values for F tests and corresponding *P* values from three-way ANOVA for p-AMPK/t-AMPK from triceps surae muscle

Effect	
Linetype (HR vs. C)	$F_{1,16} = 3.36+$
	$P = 0.0845$
Wheel Access (WhlAcc)	$F_{1,16} = 0.68+$
	$P = 0.4201$
Diet (Std. Vs. Western)	$F_{1,16} = 1.61-$
	$P = 0.2232$
Linetype X WhlAcc	$F_{1,16} = 7.09$
	$P = 0.0170$
Linetype X Diet	$F_{1, 16} = 1.95$
	$P = 0.1817$
WhlAcc X Diet	$F_{1,16} = 0.04$
	$P = 0.8519$
Linetype X WhlAcc X Diet	$F_{1,16} = 0.04$
	$P = 0.4316$

Values for F tests, degrees of freedom, and corresponding *P* values for the ratio of phosphorylated (Thr172) to total AMPK. + indicates HR > control, wheel access > sedentary, Western diet > standard chow. N = 28. See Figure 4.5 for least squares means for each group.

Table 4.1 supplement. F tests, corresponding *P* values, and model likelihoods for ventricle mass from mice with wheel access

	Ventricle Mass		
Linetype (HR vs. C)	$\boldsymbol{F_{1,6} = 7.10+}$ **P = 0.0373**	$F_{1,6} = 0.01-$ $P = 0.9146$	$F_{1,6} = 0.00-$ $P = 0.9773$
Diet (Std. Vs. Western)	$F_{1,6} = 1.02+$ $P = 0.3523$	$F_{1,6} = 0.00-$ $P = 0.9576$	$F_{1,6} = 0.20-$ $P = 0.6680$
Linetype X Diet	$F_{1,6} = 2.98$ $P = 0.1352$	$F_{1,6} = 1.45$ $P = 0.2742$	$F_{1,6} = 1.01$ $P = 0.3543$
Mini-muscle	$F_{1,81} = 0.00+$ $P = 0.9681$	$F_{1,80} = 0.38+$ $P = 0.5375$	$F_{1,80} = 0.20+$ $P = 0.6582$
Running Average over days 44-58		$F_{1,80} = 24.28+$ $P = <0.0001$	
Running Average over days 4-58			$F_{1,80} = 13.10+$ $P = 0.0005$
ln maximum likelihood	171.1	180.6	176.2
Twice the difference		**18.87***	**10.22***

Values for *F* tests and degrees of freedom for ventricle mass with log body mass as a covariate for wheel access mice only. Models including previous 2 weeks of wheel running (mean revolutions/day) or all but the first three days of running (4-58) as a covariate. N = 99 for all models. Bold indicates significance $P < 0.05$. + indicates HR > control, Western diet > standard chow, mini-muscle > other HR mice.
* indicates the model that includes a measure of wheel running as a covariate has a significantly higher likelihood ($P < 0.05$) as compared with the model that does not include wheel running (twice the difference between the models is assumed to be distributed asymptotically as a χ^2 with 1 degree of freedom).

Table 4.2A supplement. F tests and corresponding *P* values for mice without wheel access (sedentary group).

	CS	*HK*	*CPT*	*HAD*
Linetype (HR vs. C)	$F_{1,6}$ = 0.54-	$F_{1,6}$ = 2.08-	$F_{1,6}$ = 1.20-	$F_{1,6}$ = 2.19-
	P = 0.4898	*P* = 0.1995	*P* = 0.3150	*P* = 0.1896
Diet (Std. Vs. Western)	$F_{1,6}$ = 0.03+	$F_{1,6}$ = 0.00-	$F_{1,6}$ = 1.05+	**$F_{1,6}$ = 9.25+**
	P = 0.8782	*P* = 0.9919	*P* =0.3442	***P* = 0.0228**
Linetype X Diet	$F_{1,6}$ = 0.01	$F_{1,6}$ = 0.00	$F_{1,6}$ = 0.09	$F_{1,6}$ = 0.05
	P = 0.9082	*P* = 0.9486	*P* = 0.7773	*P* = 0.8249
Mini-muscle	$F_{1,76}$ = 1.90+	**$F_{1,76}$ = 29.22+**	$F_{1,76}$ = 0.01+	**$F_{1,74}$ = 24.35+**
	P = 0.1724	***P* = <0.0001**	*P* = 0.9417	***P* = <0.0001**

Values for *F* tests and degrees of freedom for enzymes citrate synthase (CS), hexokinase (HK), carnitine palmitoyl-transferase (CPT), β-hydroxyacyl-CoA dehydrogenase (HAD) for sedentary mice only. Bold indicates significance at $P < 0.05$. + indicates HR > control, Western diet > standard chow, mini-muscle > other HR mice.

Table 4.2B supplement. F tests and likelihood models, for mice with wheel access (active group).

	CS		*HK*		*CPT*		*HAD*	
Linetype (HR vs. C)	$F_{1,6}$ = 1.45+	$F_{1,6}$ = 0.24-	$F_{1,6}$ = 3.94+	$F_{1,6}$ = 0.72+	$F_{1,6}$ = 2.45+	$F_{1,6}$ = 0.08+	$F_{1,6}$ = 3.84+	$F_{1,6}$ = 0.01+
	P = 0.2743	P = 0.6390	P = 0.0945	P = 0.4286	P = 0.1682	P = 0.7904	P = 0.0977	P = 0.9169
Diet (Std. Vs. Western)	$F_{1,6}$ = 0.00+	$F_{1,6}$ = 0.04-	$F_{1,6}$ = 1.32+	$F_{1,6}$ = 1.03+	$F_{1,6}$ = 0.63+	$F_{1,6}$ = 0.26+	$F_{1,6}$ = 1.48+	$F_{1,6}$ = 1.04+
	P = 0.9830	P = 0.8504	P = 0.2950	P = 0.3504	P = 0.4575	P = 0.6273	P = 0.2694	P = 0.3467
Linetype X Diet	$F_{1,6}$ = 1.07	$F_{1,6}$ = 0.51	$F_{1,6}$ = 1.36	$F_{1,6}$ = 0.80	$F_{1,6}$ = 0.10	$F_{1,6}$ = 0.03	$F_{1,6}$ = 1.37	$F_{1,6}$ = 0.70
	P = 0.3398	P = 0.5026	P = 0.2873	P = 0.4042	P = 0.7639	P = 0.8608	P = 0.2862	P = 0.4340
Mini-muscle	**$F_{1,74}$ = 17.80+**	**$F_{1,72}$ = 20.08+**	**$F_{1,74}$ = 5.43+**	**$F_{1,72}$ = 7.54+**	**$F_{1,71}$ = 4.74+**	**$F_{1,69}$ = 7.05+**	**$F_{1,73}$ = 23.71+**	**$F_{1,71}$ = 26.16+**
	P = <0.0001	P = <0.0001	P = 0.0225	P = 0.0076	P = 0.0328	P = 0.0098	P = <0.0001	P = <0.0001
Running		$F_{1,72}$ = 3.78+		$F_{1,72}$ = 1.49+		$F_{1,69}$ = 1.93+		$F_{1,71}$ = 3.56+
Average over days 44-58		P = 0.0557		P = 0.2268		P = 0.1689		P = 0.0632
ln maximum likelihood	-354.1	-348.6	-108.0	-106.3	42.1	43.2	-291.3	-286.6
Twice the difference		**11.09***		3.44		2.33		**9.30***

Values for *F* tests and degrees of freedom for enzymes citrate synthase (CS), hexokinase (HK), carnitine palmitoyl-transferase (CPT), β-hydroxyacyl-CoA dehydrogenase (HAD) for wheel access mice only, with and without previous 2 weeks of wheel running (mean revolutions/day) as a covariate. Bold indicates significance $P < 0.05$. + indicates HR > control, Western diet > standard chow, mini-muscle > other HR mice.

* indicates the model that includes a measure of wheel running as a covariate has a significantly higher likelihood ($P < 0.05$) as compared with the model that does not include wheel running (twice the difference between the models is assumed to be distributed asymptotically as a χ^2 with 1 degree of freedom).

Table 4.2B supplement continued.

	CS	*HK*	*CPT*	*HAD*
Linetype (HR vs. C)	$F_{1,6}$ = 0.19-	$F_{1,6}$ = 1.40+	$F_{1,6}$ = 0.074-	$F_{1,6}$ = 0.04-
	P = 0.6747	P = 0.2813	P = 0.8050	P = 0.8465
Diet (Std. Vs. Western)	$F_{1,6}$ = 0.15-	$F_{1,6}$ = 0.98+	$F_{1,6}$ = 0.09+	$F_{1,6}$ = 0.47+
	P = 0.7087	P = 0.3598	P = 0.7721	P = 0.5187
Linetype X Diet	$F_{1,6}$ = 0.31	$F_{1,6}$ = 0.99	$F_{1,6}$ = 0.03	$F_{1,6}$ = 0.37
	P = 0.5989	P = 0.3577	P = 0.8765	P = 0.5654
Mini-muscle	**$F_{1,73}$ = 19.95+**	**$F_{1,73}$ = 5.81+**	**$F_{1,70}$ = 11.77+**	**$F_{1,72}$ = 27.05+**
	P = <0.0001	P = 0.0185	P = 0.0010	P = <0.0001
Running	$F_{1,73}$ = 2.41+	$F_{1,73}$ = 0.14+	$F_{1,70}$ = 2.93+	$F_{1,72}$ = 3.50
Average over days 4-58	P = 0.1253	P = 0.7125	P = 0.0916	P = 0.0653
ln maximum likelihood	-352.9	-107.8	43.6	-289.4
Twice the difference	2.54	0.36	3.08	3.68

Figure 4.1

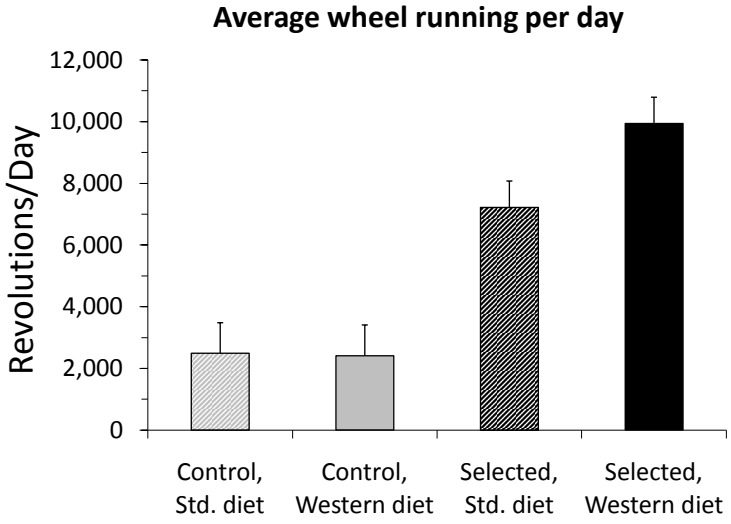
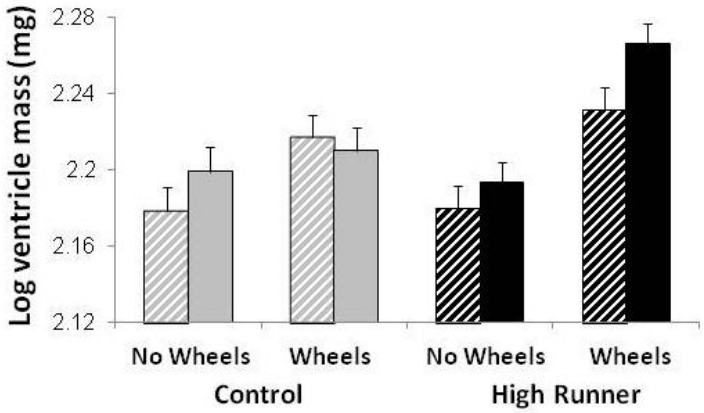


Figure 4.1 Wheel running revolutions for HR and control mice on different diets averaged for days 4-58 of wheel access. Values are least squares means + S.E. from nested analysis of covariance in SAS Procedure Mixed (see Table 4.1 for statistical results). Mice from HR lines showed a significant increase in revolutions/day when administered the Western diet, but those from Control lines did not (see Results).

Figure 4.2



Effect	*P*
Linetype (HR vs. C)	0.1790+
Wheel access (WhlAcc)	**0.0006+**
Diet	0.0841+
Linetype X WhlAcc	**0.0237**
Linetype X Diet	0.2173
Wheel access X Diet	0.8034
Linetype X WhlAcc X Diet	0.0944
Mini-muscle	0.5648+
Log Body mass	**<0.0001+**

Figure 4.2 Log_{10} ventricle mass (mg) for all 8 groups, adjusted for variation in log_{10} body mass (N = 194). Hatched bars represent standard diet, solid bars represent Western diet groups. Values are least squares means and associated standard errors from ANCOVA with line as a random effect nested within linetype (SAS Procedure Mixed), as shown in tabular entries (sign after *P* value indicates direction of effect). Wheel access had a statistically significant positive effect and the linetype by wheel access interaction was also significant.

Figure 4.3

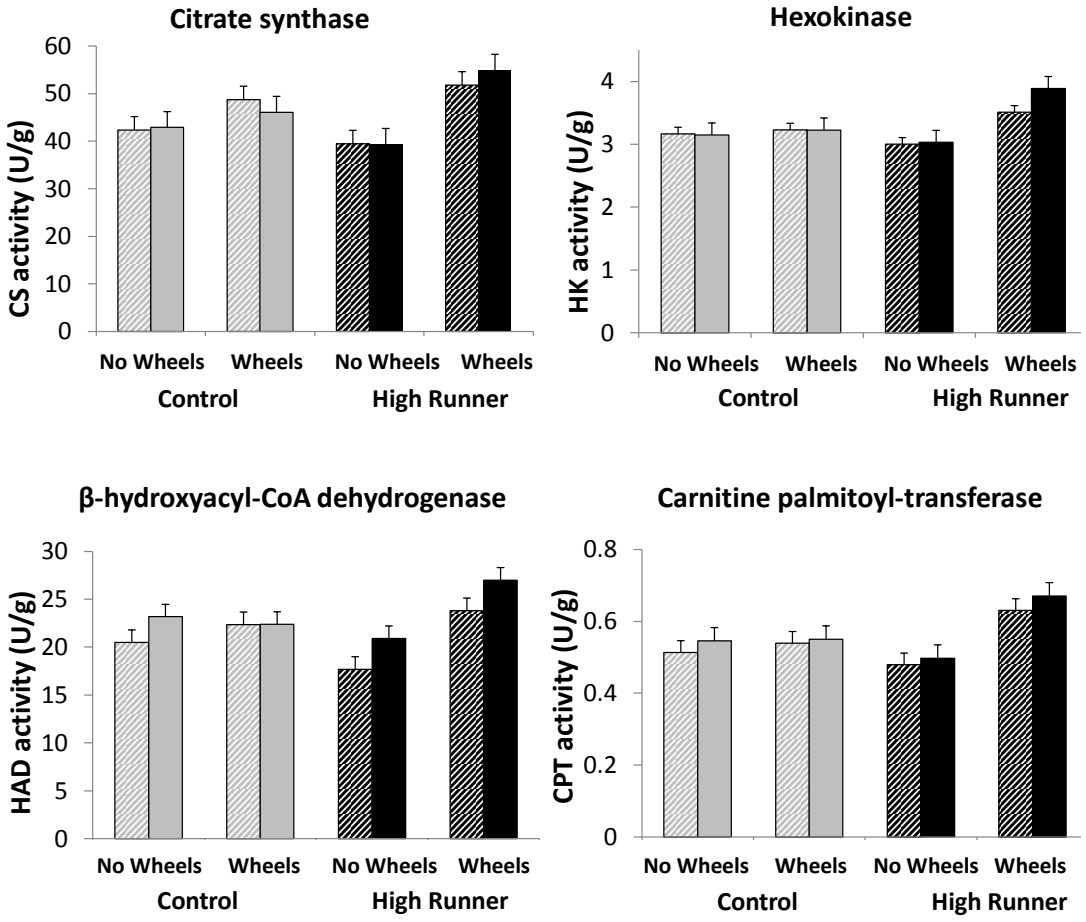


Figure 4.3 Enzyme activity + S.E. of citrate synthase, hexokinase, β-hydroxyacyl-CoA dehydrogenase, and carnitine plamitoyl-transerase. Hatched bars represent standard diet, solid bars represent Western diet groups. Each graph shows enzyme activity for each linetype, diet, and wheel access. For all enzymes, wheel access had a significant positive effect and the linetype by wheel assess interaction was also statistically significant (Table 4.2). Diet had a significant effect only on HAD, increasing its activity, and had no significant interactive effects (Table 4.2).

Figure 4.4

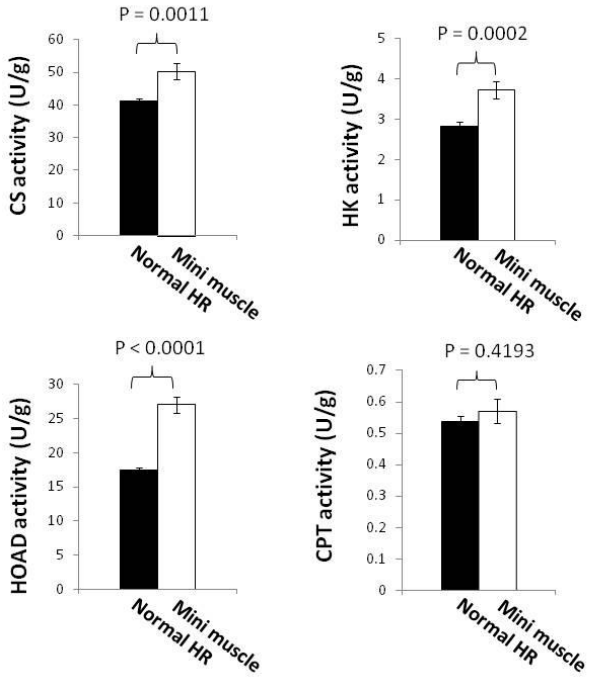


Figure 4.4 Enzyme activity + S.E. of citrate synthase, hexokinase, β-hydroxyacyl-CaA dehydrogenase, and carnitine plamitoyl-transerase for mini-muscle mice relative to normal individuals. All values are least squares means from ANOVAs with line as a random effect nested within linetype (SAS Procedure Mixed; see results in Table 4.2). For all enzymes except CPT, mini-muscle mice show statistically higher activities (Table 4.2).

Figure 4.5

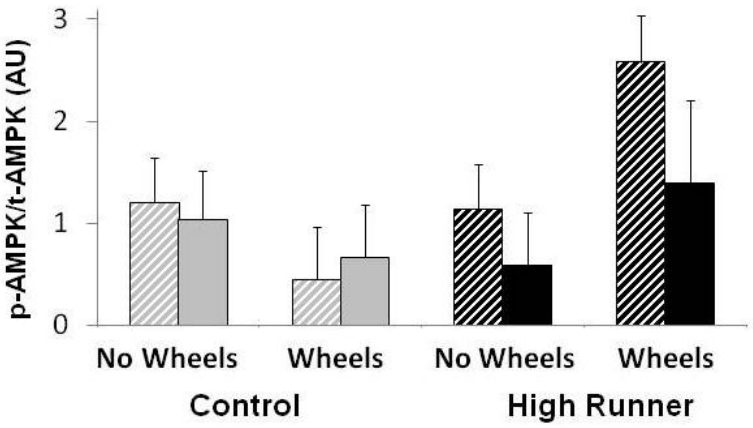


Figure 4.5 p-AMPK(Thr 172)/t-AMPK ratio for all groups. Values are least squares means with associated standard errors from SAS Procedure Mixed. Only the linetype by wheel access interaction is significant (P = 0.0170), with no other statistical differences among groups (see Table 4.4).

Figure 4.6

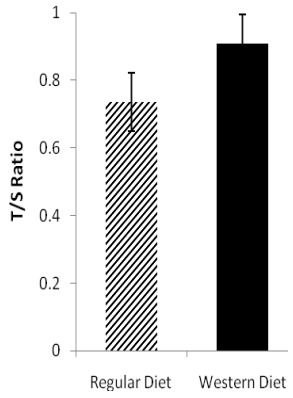


Figure 4.6 Telomere length as measured by T/S ratio. Raw data were transformed by raising them to the 0.3 power prior to statistical analyses. All values are least squares means with associated standard errors from SAS Procedure Mixed. Western diet mice had significantly higher T/S ratio than mice fed standard diet, with no other statistical differences among any groups (see Results).

Chapter 5

Effects of leptin treatment and Western diet on wheel running in high runner mice

Thomas H. Meek, Elizabeth M. Dlugosz, Kim T. Vu, Theodore Garland, Jr*

Department of Biology, University of California, Riverside, Riverside, CA 92521, USA

Abstract

The role leptin has in regulating physical activity is varied. The behavioral effects of leptin signaling depend on the type of activity and the animal's physiological state. We used mice from lines selectively bred for high voluntary wheel running to further study how leptin regulates volitional exercise. Mice from four replicate high runner (HR) lines typically run ~3-fold more revolutions per day than those from four non-selected control (C) lines. HR mice have altered dopamine function and differences from C in brain regions known to be important in leptin-mediated behavior. Furthermore, male HR mice have been found to dramatically increase running when administered Western diet, an effect possibly mediated through leptin signaling. Male mice from generation 61 were given wheel access at 24 days of age and given either Western diet (high in fat and with added sucrose) or standard chow. Western diet significantly increased running in HR mice and, after four weeks, significantly increased circulating leptin, insulin, C-peptide, gastric inhibitory polypeptide, and inflammatory hormone resistin concentrations. During the fifth week, all mice received two days of intra-peritoneal sham injections (physiological saline) followed by three days of recombinant leptin injections, and then another six days of sham injections. Leptin treatment significantly decreased food consumption and body mass in all groups. Wheel running significantly increased with leptin injections only in HR mice (fed Western or standard diet). Whether Western diet and leptin treatment stimulate wheel running through the same physiological pathways awaits future study. These results have implications for understanding the neural and endocrine systems that control locomotor activity, food consumption, and body weight.

Introduction

Behaviors such as eating and engaging in volitional activity are regulated by a myriad of physiological and neurological interactions (Garland et al., 2011a). These two behaviors also interact through their effects on homeostasis and by various direct mechanisms. In rodents, wheel access has been shown many times to increase food consumption (e.g., Tokuyama et al., 1981; Bell et al., 1997; Koteja et al., 1999b; Swallow et al., 2001), although the effect does not always occur (Jung et al., 2010; Jung and Luthin, 2010). Severe underfeeding or food deprivation can result in substantial increases in locomotor activity, postulated to represent an increase in motivation for foraging behavior in mice and rats (Overton and Williams, 2004; Adan et al., 2010).

Different diets can have diverse effects on locomotor activity in rodents. However, determining to what extent variable effects are caused by different macronutrient compositions, the amount of calories ingested, differences among strains of rodents, changes in body mass or composition, or the type of activity measured has been challenging (Brownlow et al., 1996; Bell et al., 1997; Novak et al., 2006; Simoncic et al., 2008; Bjursell et al., 2008).

The role of leptin as an endocrine signaling molecule in both the periphery and central nervous system has become a well-appreciated (Baile et al., 2000; Karsenty, 2006; Morton et al., 2006). Many studies have focused on leptin's role in food consumption, thermoregulation, metabolic rate, and corresponding changes in body mass. Fewer studies have examined leptin's effect on physical activity, particularly voluntary exercise (Choi et al., 2005; Morton et al., 2011). Both spontaneous physical activity (also

known as non-exercise activity thermogenesis [NEAT]) and voluntary exercise can have large impacts on body mass (Levine, 2004; Levine et al., 2005; Nehrenberg et al., 2009). These forms of activity can function as electively modifiable components of total energy expenditure, and thus serve as a major option for the treatment of many metabolic diseases.

We studied mice from lines that have been selectively bred for high voluntary wheel running to shed light on the role leptin may play in regulating activity levels. Mice from the four replicate high runner (HR) lines typically run ~3-fold more revolutions/day per day than those from four non-selected control lines, and have evolved lower body size and lower body fat (Swallow et al., 2005; Garland et al., 2011b). Circulating leptin concentrations are also lower in HR mice and, interestingly, leptin is lower than predicted based on fat mass, at least in young adult females (Girard et al., 2007). As a corollary, HR mice also have elevated food consumption (Swallow et al., 2001), but still remain leaner than controls (Meek et al., 2010). It is unknown whether depressed leptin concentrations play a part in the motivation for increased wheel running, are a result of high activity, or are a correlated response to selection that is unrelated to activity levels per se.

The heightened wheel running of HR mice involves neurochemical changes affecting motivation and reward. Dopamine transporter blockers (Ritalin, cocaine, GBR 12909), which increase function of dopamine, reverse hyperactivity on wheels in HR mice, with an increase or no change in C lines. This suggests reduced functionality of the dopamine receptors in HR mice. Specifically, HR mice are more sensitive to blocking

D1-like receptors rather than blocking D2-like receptors (Rhodes et al., 2001; Rhodes and Garland, 2003, Rhodes et al., 2005). When wheel access is blocked, the caudate-putamen complex, prefrontal cortex, nucleus accumbens, and lateral hypothalamus have differential activity in HR as compared with control mice (measured immunohistochemically using c-Fos) (Rhodes et al., 2003). Not only are these brain regions involved in voluntary locomotion and/or motivation, but the latter two, along with the mesolimbic dopaminergic system, are known to be important in leptin-mediated behavior (Baile et al., 2000; Hommel et al., 2006).

None of a number of previously-tested pharmacological agents increased running in HR mice (Rhodes et al., 2005; Keeney et al., 2008), but administration of a Western diet increased daily wheel running in HR mice up to 75%, with no change in control mice (Meek et al., 2010). In that study, both control and HR mice gained substantial fat mass when fed Western diet, and presumably circulating leptin concentrations rose as well. Given that HR mice have changes in brain regions involved in leptin signaling, differences in baseline circulating leptin concentrations, and respond uniquely to Western diet, we investigated leptin's role in modulating wheel running, and determined if leptin's effects were consistent between different diets.

Materials and methods

Subjects

Mice from generation 61 of an ongoing selection experiment for high voluntary wheel running were used. The original progenitors of the colony were outbred,

genetically variable Harlan Sprague Dawley mice: Institute for Cancer Research strain (Indianapolis, Indiana, USA). Eight closed lines were formed, four selected for high voluntary wheel running (based on days 5 and 6 of a 6-day test) and four bred without regard to running (Swallow et al., 1998).

58 male mice from generation 61 were weaned at 21 days of age and housed with access to Harlan Teklad Laboratory Rodent Diet [W]-8604 until they reached 24 days of age. One control line and three of the four selected HR lines were represented in this study as our focus was on elucidating the HR phenotype. The excluded HR line (lab designation #6) was polymorphic for the mini-muscle phenotype (see below). Room temperature was maintained at ~73° Fahrenheit and photoperiod was 12:12, with lights on at 0700 Pacific Time.

Experimental Groups

At 24 days of age, all mice were placed onto Wahman-type wheels (1.12 m circumference, 35.7 cm diameter, 10 cm-wide running surface) attached to standard cages (27 X 17 X 12.5 cm). Wheel running was recorded for 23 hours each day. Half the mice received standard diet (SD) (Harlan Teklad Rodent Diet [W] 8604, 14% kJ from fat) and the other half received Western diet (WD) (Harlan Teklad TD.88137 Western Diet, 42% kJ from fat with added sucrose; see Meek et al. [2010] for details of diet composition). Every six days mice were weighed, body length (tip of snout to base of tail) was taken, while the mouse was held behind the neck, and apparent food consumption measured. Food consumption was determined as the difference in hopper mass between two time points, after accounting for any obvious wastage (Koteja et al., 2003). Because the diets

differ in mass-specific energy content, we converted food consumption from grams to caloric intake, using total kJ of metabolizable energy of 12.98 and 19.01 per gram of wet mass for SD and WD, respectively (Meek et al., 2010).

Blood Sample

A 130 µl blood sample was taken after mice had been in experimental groups for two weeks (38 days of age). Blood was acquired through the orbital sinus under isoflurane anesthesia seven days before the first sham injection. Blood was collected in non-heparinized microcapillary tubes. 1.1 ul of dipeptidyl peptidase IV inhibitor (EC 3.4.14.5, Millipore MO, USA), 2.5 ul of 0.05M phenylmethanesulfonylfluoride dissolved in methanol, and 10 ul of Roche mini Complete serine protease inhibitor cocktail (Roche Diagnostics Mannheim, Germany) were added to whole blood and mixed thoroughly. Serum was collected after blood was centrifuged (Sorvall Legend Micro 17R) at 13,000 rpm for 10 minutes at 4^{o}C.

Hormones were assayed using a Milliplex Mouse Metabolic Magnetic Bead Panel MMHMAG-44K-14 (Millipore MO, USA) in a Luminex 200. Standards were plotted and concentrations determined using Milliplex Analyst software version 3.5.5. Due to the limited number of wells, and to ensure adequate sample sizes in HR lines, only the three HR lines had blood assayed (blood samples from control mice were not assayed).

Injections

Starting at 24 days of age, mice were given 3 weeks of uninterrupted wheel access to allow daily wheel running to plateau before injections began. Recombinant mouse leptin (R&D Systems, Inc.) was prepared by dissolving in physiological saline

immediately prior to use. Mice were given 2 µg/g body mass two hours before lights off via intraperitoneal (i.p.) injections. Each mouse received two consecutive days of sham (physiological saline) followed by three consecutive days of leptin treatment and six more days of sham injections. The mass used to adjust injection volume was measured during the initial two days of sham injection. Accordingly, even though leptin treatment changed body mass, injection volumes of leptin did not change over the course of the experiment. Injection volumes ranged from 0.12 mL to 0.17 mL.

Dissections

Mice were dissected one day after the final sham injection (26 days after the blood sample). Body mass, body length, and food consumption were recorded. The mouse was skinned and its pelt weighed. The ventricles, liver, and triceps surae were then dissected and weighed. Different fat pad masses were also dissected and weighed, including the sub-scapular brown fat with the sub-scapular adipose tissue, epididymal fat, and retroperitoneal fat (Cinti, 2005). For analyses, "total fat" refers to the sum of all 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. Line and diet were main effects. Covariates depended on the trait analyzed and included body length, body mass, fat mass, wheel freeness, and/or total wheel running (revolutions). To compare values for food consumption, body mass, and wheel running, during the initial sham (2 days), leptin (3 days), and final sham injections

(5 days), we analyzed the average values for each of these segments. *A priori* contrasts were used in SAS Procedure Mixed for a repeated-measures ANCOVA with covariates of body mass or wheel freeness and AR(1) rather than compound symmetry was used for covariate structure.

One of the three HR lines studied here (lab designation #3) is fixed for a Mendelian recessive allele that causes 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 allele include alterations in muscle fiber type composition (especially reduced type IIB fibers) (Bilodeau et al., 2009), contractile properties favoring stamina at the expense of speed (Syme et al., 2005), and a doubling of mass-specific aerobic capacity and hexokinase activity (Houle-Leroy et al., 2003). Mini-muscle animals showed similar increases in wheel running compared to normal muscle HR mice when given Western diet, so they are not distinguished in the results.

Results

Blood Samples

As expected, plasma leptin concentrations were higher in mice fed Western diet (Table 5.1, Figure 5.1). When total fat mass was included in the analysis as a covariate, it was highly significant and diet lost its significance. A similar pattern was observed for insulin. WD also increased C-peptide, which remained significantly elevated when fat mass was included in the model as a covariate. Resistin and gastric inhibitory

polypeptide (GIP) were both higher in WD-fed mice, but monocyte chemotactic protein-1 (MCP-1) was not (Table 5.1, Figure 5.1). The significant line effect for several hormones reveals baseline differences among the high runner lines. Despite these differences, their responses to Western diet are similar, as there is no significant diet x line interaction for any hormone.

Food Consumption

In the repeated-measures analysis (with body mass as a covariate), leptin treatment decreased food consumption (a priori contrast of initial sham vs. leptin injection period, $P < 0.0001$, Supplemental table 5.1, Figure 5.2). Once leptin injections stopped, food consumption increased but did not quite reach baseline levels (a priori contrast of initial sham vs. final sham, $P = 0.0104$, Supplemental table 5.1). Both diet groups responded similarly to leptin injections (Diet X Time interaction $P = 0.1488$). The lines were different from each other ($P = 0.0084$), with the control line eating less overall, and no statistical interactions with the line effect (Supplemental table 5.1).

Body Mass

Repeated-measures analysis showed that leptin treatment decreased body mass ($P < 0.0001$, Supplemental table 5.2, Figure 5.3). After leptin injections stopped, body mass increased to levels statistically indistinguishable from baseline values ($P = 0.8786$). Western diet increased body mass ($P = 0.0370$) of all lines (no line X diet interaction). Both diet groups responded to leptin injections in a similar way (no time X diet interaction). Lines differed significantly in body mass ($P = 0.0146$), with all three HR lines weighing less than the one control line (results not shown; averaged values shown in

Figure 5.3). The response to leptin was similar among lines (P = 0.2316 for time x line interaction, Supplemental table 5.2).

Wheel Running

Before the start of sham injections, mice from HR lines ran ~3.3 times further per day relative to control mice (day 35 in Figure 5.7). HR mice ran more on Western diet (+16%), which is comparable to our previous study when mice were this age (Meek et al., 2010).

With the control line included in the analyses, repeated-measures analyses indicated significant differences across the time points (P < 0.0001). Leptin treatment increased total running when compared to either pre- or post-sham injections (P = 0.0038, P < 0.0001, respectively). Additionally, the time x line interaction was significant (P = 0.0019), so we analyzed control and high runner lines separately.

In the control line, neither time nor diet, nor their interaction were statistically significant (P = 0.1896, 0.0671, 0.1434, respectively), although the WD-fed mice tended to run less (Figure 5.4).

In HR lines, time was highly significant (P < 0.0001, and contrasts showed that leptin increased running (Supplemental table 5.3: P = 0.0035, P < 0.0001 compared to pre-leptin sham and post-leptin sham, respectively). Mice ran less during the post-leptin sham than during the pre-leptin sham (P = 0.0388, Figure 5.4). The time x line interaction was significant (P = 0.0308, Supplemental table 5.3), thus indicating somewhat different responses among the three HR lines. However, inspection of the Least Squares Means from SAS showed that all three lines followed the same general

pattern of increasing wheel running during leptin injections, followed by a decrease in running after injections stopped (results not shown).

Discussion

Evidence of leptin's effect on physical activity has been varied. Leptin injections (I.P.) in mice lacking a functional leptin gene (ob/ob mice) can lead to increases in activity in a novel cage over 15 minutes (Pelleymounter et al., 1995) and in wheel running over a daily cycle (Morton et al., 2011). Intracerebroventricular leptin-injected rats increase spontaneous physical activity during the initial 20 hours after injections (Ainslie et al., 2001), as well as throughout 5 days of consecutive treatment (Choi et al., 2008). Leptin treatment in fed wildtype C57Bl/6 mice via intraperitoneal injections or subcutaneous osmotic pumps produced no change in activity in a new cage environment as measured by human observation over 15 minutes (Pelleymounter et al., 1995) or wheel running during 4-6 days (Morton et al., 2011). Conversely, leptin treatment via minipumps can reduce fasting-induced hyperactivity in Winstar rats and C57Bl/6 mice (Exner et al., 2000; Morton et al., 2011).

Several lines of evidence suggest a link between voluntary wheel running, diet, and leptin in HR mice. Unlike all pharmacological agents tested thus far in our HR mice (Rhodes et al., 2001; Rhodes and Garland, 2003, Li et al., 2004; Rhodes et al., 2005; Keeney et al., 2008), WD substantially increases wheel running (Meek et al., 2010). In the present study, WD increased leptin concentrations nearly 5-fold after only 18 days in HR mice (Figure 1) (control lines not assayed). Additionally, acute leptin injections also

increased wheel running in HR mice (Figures 4). From this, it is tempting to deduce that Western diet stimulates wheel running in HR lines at least partly through leptin signaling.

Accordingly, leptin injections in HR mice on standard diet produced a larger increase in running (1,446 additional revolutions per day; Figure 4) than in animals on WD (500 additional revolutions per day). However, we did not observe a statistically significant time by diet interaction in analysis of HR mice, so it is difficult to conclude leptin injections differentially affected wheel running in the different diet groups. Given the nature of our findings, more data are needed to resolve the discrepancy and establish if HR mice on WD respond the same as standard-fed HRs. The use of a leptin antagonist would help clarify the issue (e.g. Elinav et al., 2009), and provide evidence as to what, if any, leptin-independent effects WD has.

Similar to effects observed in inbred strains of rodents on ad lib food (Morton et al., 2011), leptin injections did not increase running in our control line. However, HR mice on the standard diet (and WD) increased wheel running with leptin treatment. Thus, a fundamental difference may have evolved between the control and HR lines in this respect. The increased running in HRs due to leptin treatment begs the question as to why HR mice have evolved lower leptin levels in the face of continuous selective breeding for high voluntary wheel running (while they have ad lib access to food). Fasting in rodents can result in substantial increases in locomotor activity. During fasting, leptin drops precipitously, contributing to the increase in activity (Exner et al, 2000; Morton et al., 2011). It seems unlikely that HR mice are running more because their innately low leptin contributes a starvation signal that results in greater motivation

for foraging (Exner et al, 2000). If this was the case, then the increased leptin in mice on WD should have decreased running.

Several other explanations are possible for the evolution of low leptin in HR mice, including pleiotropy, gene linkage, or reward substitution. In regards to reward substitution, the low body fat (and leptin) in HR mice may result from neural reward generated by high activity substituting for reward generated by eating. Reward substitution has been observed in rats, where a preference for high fat diet (over standard chow) in sedentary conditions is eliminated when wheel access is granted (Scarpace et al., 2010). A similar scenario has also been found with nicotine and food consumption in mice. Nicotine activation of pro-opiomelanocortin neurons leads to reduced food intake and subsequently reduced body mass (Mineur et al., 2011). If there is a parallel relationship in HR mice between exercise and food consumption, then the independent evolution of a smaller body size in all four HR lines could, at least in part, be a consequence of pleasure generated from running. Alternatively, it has been suggested that the reduced body size of HR mice may be related to their elevated circulating corticosterone levels (Girard and Garland, 2001; Malisch et al., 2007). An additional alternative, suggested by Plomin et al. (1990, pp. 261-262 and their Figure 10.1), is that perturbations in tyrosine hydroxylase or dopa decarboxylase enzymes could affect dopamine and/or norepinephrine simultaneously and result in more wheel running and less eating.

Recent evidence supports this view, as HR mice, when switched from tap water to a non-caloric sweet drink (Sweet ‘N Low, Equal or Splenda) do not increase consumption

to the same extent as controls (Kolb, 2010). "This alteration in incentive salience for a competing reward is characteristic of reward substitution, where a highly valued reward (wheel running) substitutes or obscures the effect of a competing reward (sweet taste)..."(Kolb, 2010, p.13).

With the exception of monocyte chemotactic protein-1 (MCP-1), involved in inflammation, WD increased all the measured hormone concentrations. Interestingly, body fat, which was measured one month after the blood samples were taken, was still correlated with several hormone concentrations in the ANCOVA models (Table 5.1). Both gastric inhibitory polypeptide (GIP) and bioactive molecule C-peptide are involved in insulin secretion and are elevated, along with insulin, in mice fed WD. There is also evidence of greater systemic inflammation, as resistin concentrations rose 2.4-fold in the WD group (although MCP-1 did not significantly increase).

It is well known that high leptin concentrations can cause leptin resistance (El-Haschimi et al., 2000; Lin et al., 2000). Lin et al. (2000) showed that one week of WD did not affect peripheral leptin sensitivity but eight weeks of WD in sedentary conditions was enough to induce resistance to exogenous leptin administration. In our study, animals were on Western diet for 38 days before leptin injections began. WD mice gained considerable fat mass and had corresponding high leptin. But, WD mice do not appear to have developed leptin resistance. In terms of food consumption and body mass changes, all groups responded to leptin treatment to a similar degree. It is possible not enough time elapsed for mice to develop leptin resistance. Importantly, all of the mice in

our study had access to wheels, which could be helping animals maintain leptin sensitivity (Scarpace et al., 2010).

Over the course of 61 generations of selective breeding for high voluntary wheel running, the response to Western diet as well as to direct leptin treatment has been altered. We do not yet know what physiological mechanism account for these differences, but understanding the changes will provide insight into the evolution of voluntary behaviors and overall energetic homeostasis.

Chapter 5 Acknowledgements

Supported by University of California, Riverside Undergraduate Research Award to KTV and NSF grant IOS-1121273 to TG.

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Table 5.1 *P* values from ANOVA and ANCOVA for plasma hormones

	Leptin (n = 33)		Insulin (n = 38)		C-peptide (n = 38)		Resistin (n = 38)	GIP (n = 38)	MCP-1 (n = 21)
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Diet	**0.0031+**	0.5448+	**0.0002+**	0.0726+	**<0.0001+**	**0.0010+**	**<0.0001+**	**0.0012+**	0.7025+
Line	**0.0466**	0.2266	**0.0267**	0.3436	**0.0368**	0.2935	**0.0386**	**0.0456**	0.9291
Diet x Line	0.620	0.9981	0.1797	0.8325	0.3588	0.1851	0.2106	0.3309	0.5885
Fat mass		**<0.0001**		**0.0002**		**0.0010**			

Results from ANOVA for circulating hormone levels with categorical factors of diet (Western vs. standard) or line (3 separate HR lines), and covariate of total body fat (see Methods) measured in grams at the end of the experiment. + indicates direction Western > Standard diet. Monocyte chemotactic protein-1 (MCP-1), Gastric inhibitory polypeptide (GIP). All *P* values are 2-tailed. Significant main effect values ($P < 0.05$) are in bold.

Table 5.2 *P* values from ANCOVA for total fat and pelt mass

	Total Fat (n = 51)				Pelt (n = 52)			
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Line	**<0.0001**	**<0.0001**	**<0.0001**	**0.0018**	**0.0133**	**0.0203**	**0.0159**	0.1179
Diet	**<0.0001+**	**<0.0001+**	**<0.0001+**	**<0.0001+**	**0.0001+**	**0.0001+**	**0.0003+**	**0.0005+**
Line X diet	0.1696	0.1816	0.5060	0.3260	0.6506	0.7020	0.5622	0.6836
Body length		0.6651+	0.4443+	0.4986-		0.2036+	0.3284+	0.7521-
Food consumption			0.2064-	0.9012+			0.3706+	**0.0258+**
Running				**0.0378-**				**0.0060-**

Results from ANCOVA with categorical factors of line, standard or Western diet, and covariates of body length (crown to rump length), food consumption (in kJ/day over the final six days of the experiment), and running (distance run per day over the final six days of the experiment). + indicates direction Western diet > standard diet, and direction of covariate effect. All *P* values are 2-tailed. Significant values ($P < 0.05$) are in bold.

Table 5.2 continued. *P* values from ANCOVA for organ masses

	log Epididymal fat (n = 52)				log Retroperitoneal fat (n = 51)				log Brown fat + subscapular fat (n = 52)			
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Line	**<0.0001**	**<0.0001**	**<0.0001**	**0.0035**	**<.0001**	**<.0001**	**<.0001**	**0.0019**	**0.0078**	**0.0062**	**0.0095**	**0.0406**
Diet	**0.0003+**	**0.0003+**	**0.0002+**	**0.0002+**	**0.0002+**	**0.0002+**	**0.0001+**	**0.0005+**	**0.0034+**	**0.0037+**	**0.0031+**	**0.0105**
Line X diet	0.2487	0.2622	0.6444	0.2570	0.4919	0.4927	0.7307	0.5122	0.6292	0.7026	0.8538	0.8752
Body length		0.8969 +	0.6294+	0.3013-		0.6158+	0.4639+	0.6949-		0.2159+	0.1681+	0.4513+
Food consumption			0.1990-	0.8553-			0.3680-	0.6387+			0.4653-	0.8068+
Running				0.1854-				**0.0126-**				0.0999-

Results from ANCOVA with categorical factors of line, standard or Western diet, and covariates of body length (crown to rump length), food consumption (in kJ/day over the final six days of the experiment), and running (distance run per day over the final six days of the experiment). + indicates direction Western diet > standard diet, and direction of covariate effect. All *P* values are 2-tailed. Significant values ($P < 0.05$) are in bold.

Table 5.2 continued. *P* values from ANCOVA for organ masses

	log Liver (n = 52)				log Ventricles (n = 52)				Tricepts Surae (n = 52)			
	P	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*	*P*
Line	**<0.0001**	**<0.0001**	**<0.0001**	**0.0033**	0.1626	0.0825	0.1791	0.2275	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Diet	**<0.0001+**	**<0.0001+**	**<0.0001+**	**0.0001+**	**0.0476+**	**0.0426+**	0.1056+	0.1656+	**0.0253+**	**0.0273+**	0.0502+	0.1079+
Line X diet	0.2243	0.1848	0.1937	0.1850	0.1777	0.1425	0.3033	0.2842	0.9788	0.9801	0.9599	0.1659
Body length		0.2722+	0.3378+	0.8113+		**0.0112+**	0.0522+	0.5763+		0.2953+	0.4800+	0.7258-
Food consumption			0.7500+	0.0910+			**0.0135**	**0.0013+**			0.2690+	**0.0368+**
Running				**0.0167-**				0.1115-				0.2299-

Results from ANCOVA with categorical factors of line, standard or Western diet, and covariates of body length (crown to rump length), food consumption (in kJ/day over the final six days of the experiment), and running (distance run per day over the final six days of the experiment). + indicates direction Western diet > standard diet, and direction of covariate effect. All *P* values are 2 tailed. Significant values ($P < 0.05$) are in bold.

Table 5.3 Least-squares (adjusted) means and 95% CI corresponding to tests in table 2 with body length as a covariate.

Linetype		Std. Diet	Western diet
Total fat (g)	HR	0.8856 (0.0210)	0.9520 (0.0193)
	C	0.9155 (0.0269)	0.9941 (0.0265)
Epididymal fat (g)	HR	0.2012 (0.0857, 0.1495)	0.5181 (0.2148, 0.3689)
	C	0.2775 (0.1425, 0.2928)	0.6934 (0.3521, 0.7152)
Retroperitoneal fat (g)	HR	0.0283 (0.012, 0.0230)	0.0730 (0.0320, 0.0573)
	C	0.0399 (0.0214, 0.0462)	0.1117 (0.0593, 0.1266)
Brown fat	HR	0.0658 (0.0207, 0.0303)	0.1064 (0.0313,0.0446)
+ subscapular fat (g)	C	0.0899 (0.0344, 0.0556)	0.1259 (0.0475, 0.0763)
Triceps surae (g)	HR	0.1132 (0.0062)	0.1229 (0.0058)
	C	0.1489 (0.0082)	0.1618 (0.0080)
Ventricles (g)	HR	0.1487 (0.0129, 0.0142)	0.1666 (0.0137, 0.0149)
	C	0.1510 (0.0170, 0.0192)	0.1419 (0.0158, 0.0177)
Liver (g)	HR	1.6445 (0.1489. 0.1637)	1.8400 (0.1579, 0.1729)
	C	1.7697 (0.2083, 0.2361)	2.3950 (0.2778, 0.3142)
Liver (g)	HR	1.6445 (0.1489. 0.1637)	1.8400 (0.1579, 0.1729)
	C	1.7697 (0.2083, 0.2361)	2.3950 (0.2778, 0.3142)
Pelt (g)	HR	3.4669 (0.2012)	4.1578 (0.5800)
	C	4.0500 (0.2648)	4.5750 (0.2648)

Least Squares means adjusted for body length. All results correspond to *P* values from table 5.2. Values presented are means back transformed from log with asymmetric 95% confidence intervals, except for triceps surae and total fat mass which were not transformed and show standard errors.

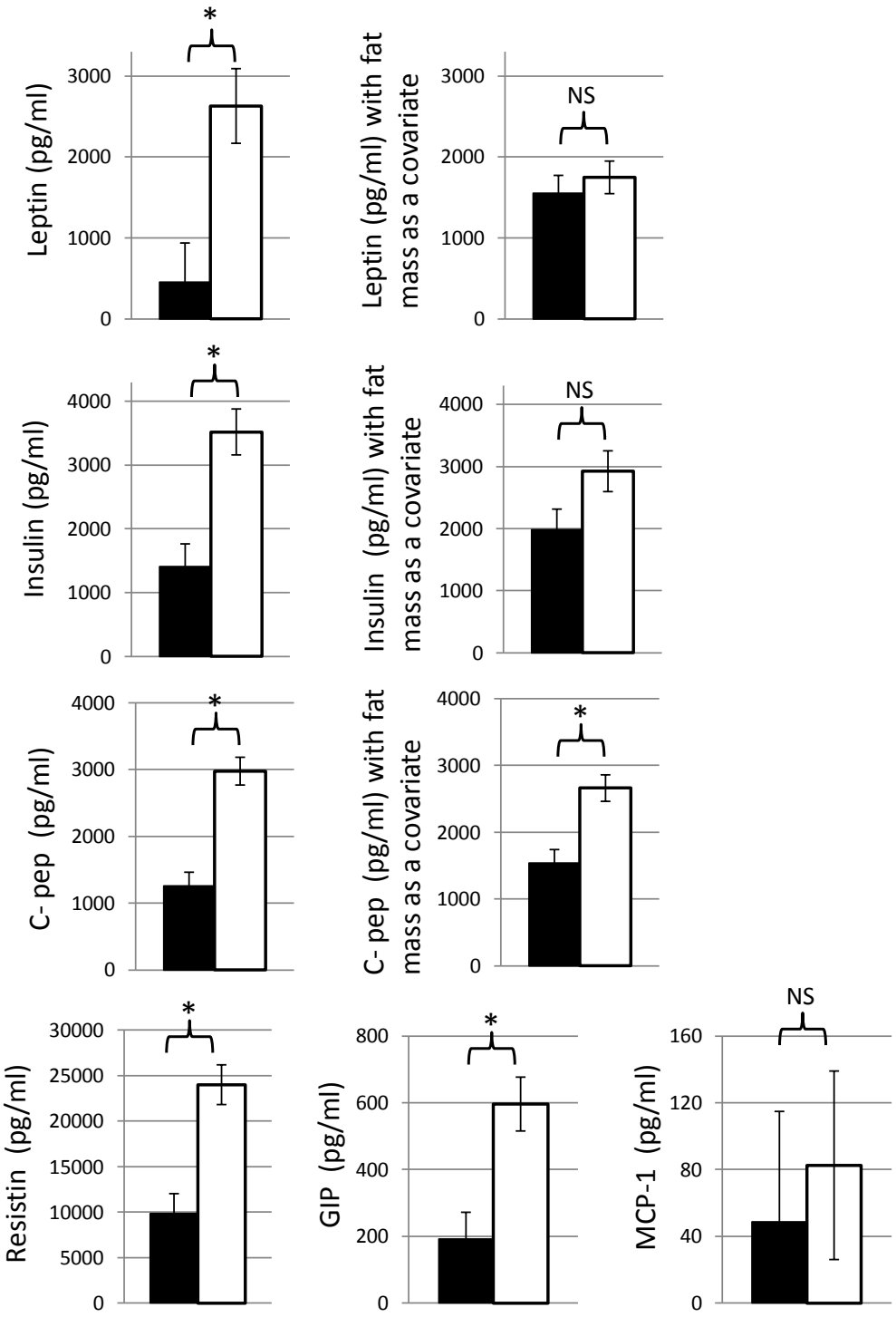

Figure 5.1 Least Squares Means ± S.E. for circulating hormone concentrations (pg/ml). Solid bars represent mice fed standard diet and open bars represent mice fed Western diet. Blood samples for the control line were not run, so all data are averages of the three HR lines. * indicates $P < 0.05$. See Table 5.1 for additional statistical results.

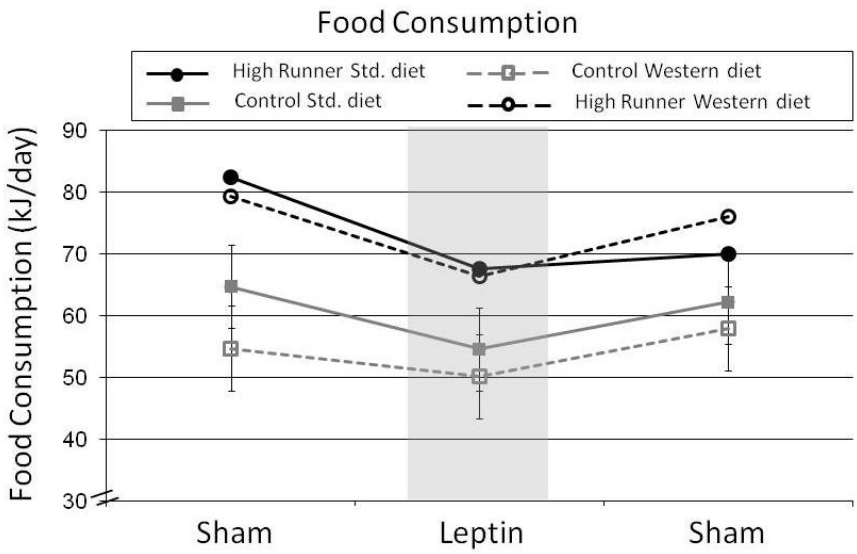

Figure 5.2 Least Squares Means for food consumption from repeated-measures analysis of covariance in SAS. Body mass was used as a covariate. Pre-leptin sham is the average food consumption for the two days of sham injections preceding leptin treatment. Leptin is the average food consumption for the three leptin injection days, and post-leptin sham is the average food consumption for the final five days of sham injections. The three HR lines were averaged from the analysis with line as a main affect and therefore do not have S.E. bars.

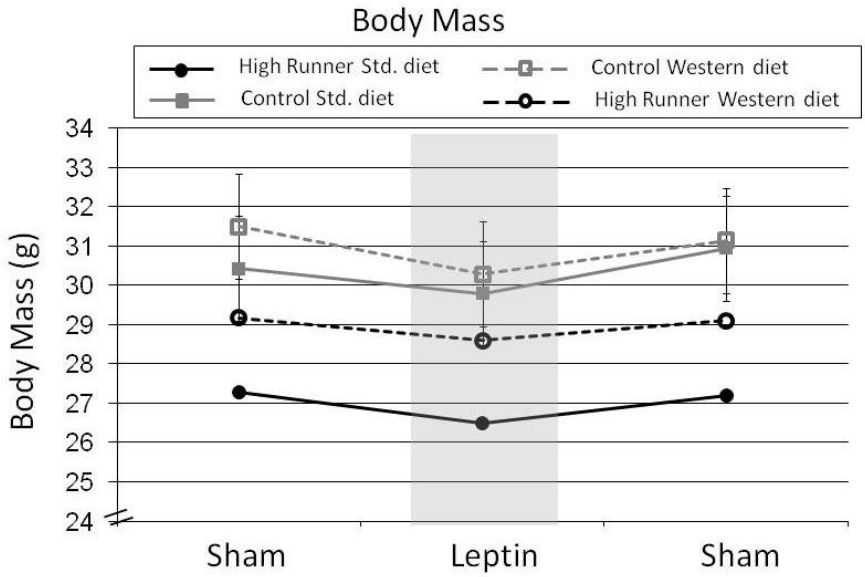

Figure 5.3 Least Squares Means for body mass from repeated-measures analysis in SAS. Pre-leptin sham is the average body mass for the two days of sham injections preceding leptin treatment. Leptin is the average body mass for the three leptin injection days, and post-leptin sham is the average body mass for the final five days of sham injections. The three HR lines were averaged from the analysis with line as a main affect and therefore do not have S.E. bars.

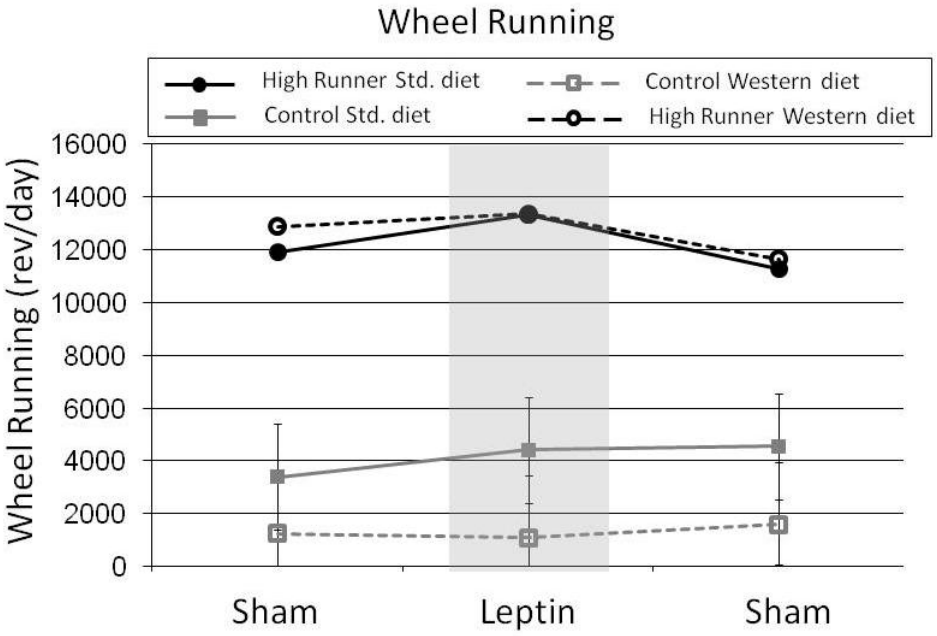

Figure 5.4 Least Squares Means for wheel running from repeated-measures analysis in SAS. Pre-leptin sham is the average wheel running for the two days of sham injections preceding leptin treatment. Leptin is the average wheel running for the three leptin injection days, and post-leptin sham is the average wheel running for the final five days of sham injections (although an additional day was excluded due to data corruption). The three HR lines were averaged from the analysis with line as a main affect and therefore do not have S.E. bars.

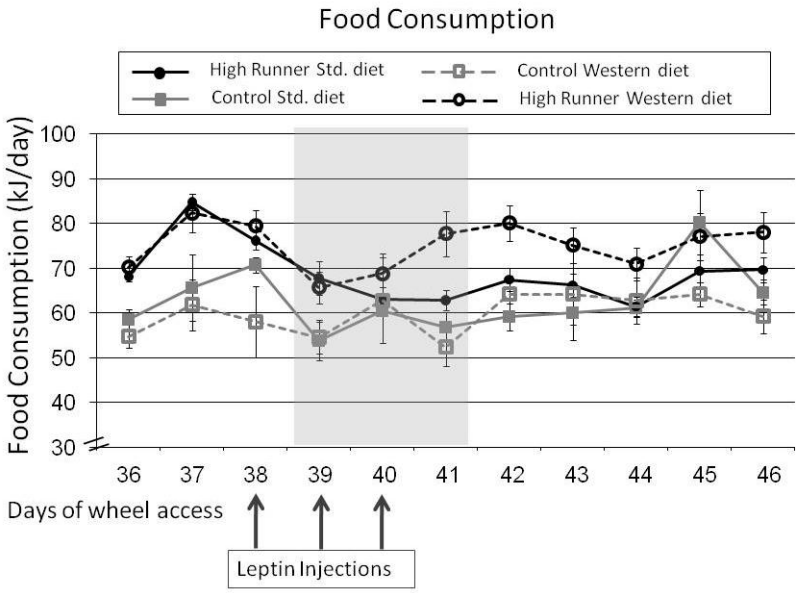

Figure 5.5 Simple means ± S.E. for food consumption in kJ/day, plotted every day for all four groups. Leptin injections occurred on days 38, 39, and 40, but the physiological effects on food consumption occur the following night. The grey shaded area on the graph represents this time frame.

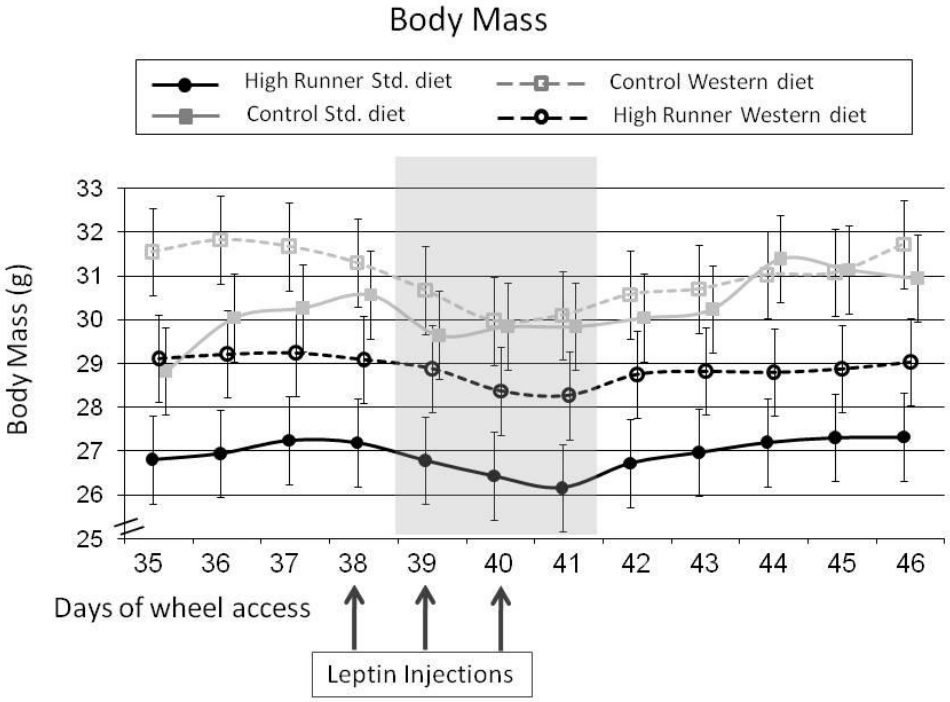

Figure 5.6 Simple means and standard errors for body mass plotted every day for all four groups. Leptin injections occurred on days 38, 39, and 40, but the physiological effects on body mass are not observed until one day later. The grey shaded area on the graph represents this time frame.

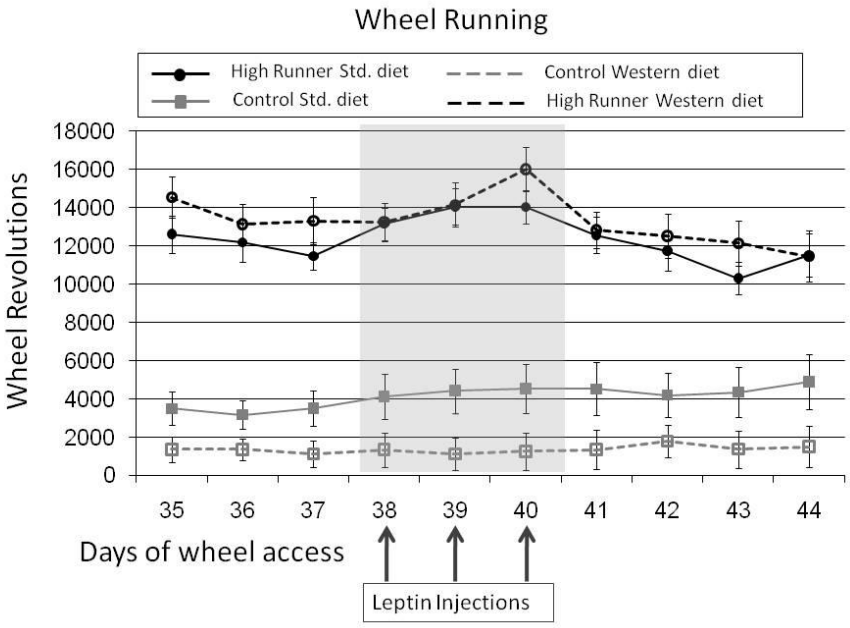

Figure 5.7 Simple means ± S.E. for wheel running plotted every day for all four groups.

Supplemental table 5.1 Repeated-measures ANCOVA for food consumption (KJ/day) with body mass as a covariate.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
REPEAT	2	83	26.11	<.0001
DIET	1	44	0.11	0.7408
LINE	3	44	4.42	0.0084
DIET*REPEAT	2	83	1.95	0.1488
REPEAT*LINE	6	83	1.85	0.1001
DIET*LINE	3	44	1.07	0.3715
DIET*REPEAT*LINE	6	83	0.47	0.8265
MASS	1	83	7.99	0.0059

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sham1 vs leptin	1	83	48.15	<.0001
sham2 vs leptin	1	83	12.86	0.0006
sham1 vs sham2	1	83	6.87	0.0104

Sham1 represents the average food consumption for the two days of sham injections preceding leptin treatment. Leptin is the average food consumption for the three leptin injection days, and sham 2 is the average food consumption for the final five days of sham injections. The sham injection immediately following leptin injections was not included in any of the time periods. For contrasts, + = sham>leptin, sham1>sham 2.

Supplemental table 5.2 Repeated-measures ANOVA for body mass (g).

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
REPEAT	2	84	32.37	<.0001
DIET	1	44	4.63	0.0370
LINE	3	44	3.91	0.0146
DIET*REPEAT	2	84	0.35	0.7084
REPEAT*LINE	6	84	1.38	0.2316
DIET*LINE	3	44	0.16	0.9200
DIET*REPEAT*LINE	6	84	0.83	0.5506

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sham1 vs leptin	1	84	35.44	<.0001
sham2 vs leptin	1	84	30.90	<.0001
sham1 vs sham2	1	84	0.02	0.8786

Sham1 represents pre-leptin sham which is the average body mass for the two days of sham injections preceding leptin treatment. Leptin is the average body mass for the three leptin injection days, and sham2 is the average body mass for the final five days of sham injections. The sham injection immediately following leptin injections was not included in any of the time periods. For contrasts, + = sham>leptin, sham1>sham 2.

Supplemental table 5.3 Repeated-measures ANCOVA for wheel running of HR mice only.

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
REPEAT	2	67	19.53	<.0001
DIET	1	40	0.11	0.7409
LINE	2	40	0.34	0.7121
DIET*REPEAT	2	67	1.23	0.2977
REPEAT*LINE	4	67	2.84	0.0308
DIET*LINE	2	40	0.63	0.5395
DIET*REPEAT*LINE	4	67	1.32	0.2725
RESALL	1	40	0.12	0.7302

Contrasts

Label	Num DF	Den DF	F Value	Pr > F
sham1 vs leptin	1	67	9.14	0.0035
sham2 vs leptin	1	67	32.57	<.0001
sham1 vs sham2	1	67	4.44	0.0388

Sham1 represents pre-leptin sham which is the average wheel running for the two days of sham injections preceding leptin treatment. Leptin is the average wheel running for the three leptin injection days, and post-leptin sham is the average wheel running for the final five days of sham injections (although an additional day was excluded due to data corruption). The sham injection immediately following leptin injections was not included in any of the time periods. For contrasts, + = sham>leptin, sham1>sham 2.

Concluding Remarks

The evolution of complex traits has been at the forefront of scientific research since at least the time of Charles Darwin (1868), and gained renewed interest with the emergence of evolutionary physiology (Garland and Carter, 1994; Feder et al., 2000; Swallow and Garland, 2005; Garland and Kelly, 2006; Mykles et al., 2010). Complex traits are composed of many subordinate traits at lower levels of biological organization. Higher-order traits, such as behavior, putatively require controlling elements of both motivation and ability. This dissertation provides evidence that both motivation and ability have evolved over the course of selection directed at voluntary wheel-running behavior. The array of correlated responses to selection for high levels of voluntary exercise has enabled the study of limits to sustained exercise as well as particular traits relevant to human health. Endurance, a direct measure of aerobic running ability, is significantly improved in mice from the selectively bred high runner (HR) lines of mice (chapter 1), but not nearly to the extent of wheel running. A similar relationship has been observed in rats bred for high endurance capacity. The high-endurance rats show large increases in endurance, the trait under selection, with a more modest increase in voluntary wheel running (Waters et al., 2008).

Both physical activity and the ability to engage in physical activity are known to be important for offsetting various human diseases (Blair et al., 1995; LaMonte et al., 2005; Church, 2010). Mice from the high runner lines do not however show any statistically significant differences from their non-selected control counterparts in any of several health-related markers, whether housed under "standard" conditions (without

wheel access, standard rodent chow), nor when challenged with a high fat diet and housed under standard conditions (chapter 2). However, when housed with access to wheels, their higher wheel running (further stimulated by high fat diet) does confer resistance to diet-induced obesity (chapter 2). Interestingly, high runner mice significantly increase wheel running when given high fat diet, after only a matter of days, and continue running more revolutions per day for 8 weeks (chapter 3). This response is not observed in control mice or in any other strain of mice that has been reported in the literature.

I hypothesized that the unique response to high fat diet observed in mice from the high runner lines was a direct response to greater circulating lipid availability to fuel long-duration, aerobic exercise. High runner mice did display classic training responses for all enzymes measured, and for ventricle mass, but no statistical interaction with diet was observed for any trait (chapter 4). These results do not support the hypothesis that high runner mice are lipid-limited during wheel running. Therefore, the stimulatory effect of Western diet on wheel running in HR mice likely involves cellular factors other than enzymatic capacity or direct lipid utilization. Alternatively, and not mutually exclusively, it may involve stimulation of the "motivation" for wheel running.

Lipids are known to have both direct and indirect effects on the brain (Geiger et al., 2008; South and Huang, 2008; Stice et al., 2008). One indirect mechanism know to partially control food intake, physical activity, and total energy expenditure involves leptin signaling in the hypothalamus. Some studies of HR mice have observed low circulating leptin concentrations, even after taking into account their reduced body

adiposity (Girard et al., 2007). The increase in adiposity typically associated with eating a Western diet leads to a proportional increase in circulating leptin concentrations (Vaanholt et al., 2008) and perhaps removal of the inhibitory signal for locomotor activity in HR mice. My results (chapter 5) implicate leptin as an important hormone in not only energy balance but specifically wheel running. The behavioral response to leptin may depend on the preexisting physiological state of the animal (Morton et al., 2011). It will be important to determine why leptin has evolved to be lower in HR mice, including elucidation of whether this evolutionary change is adaptive, non-adaptive or possibly even maladaptive (see also Malisch et al. [2007] on the evolution of high circulating corticosterone levels in HR mice).

The findings of this dissertation provide evidence of the often unpredictable nature of evolution, and how appreciation for genotype-by-environmental interactions are essential in understanding, and eventually treating, metabolic diseases. My results also underscore the utility of selection experiments in both evolutionary biology and the biomedical sciences (Garland and Rose, 2009).

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