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The Genetic Architecture of High Voluntary Wheel Running in House Mice

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of the requirements for the degree of

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by

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ABSTRACT OF THE DISSERTATION

The Genetic Architecture of High Voluntary Wheel Running in House Mice

by

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Doctor of Philosophy, Graduate Program in Genetics, Genomics, and Bioinformatics
University of California, Riverside, December 2010
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The voluntary wheel running phenotype is a complex trait. Its components include motivation and ability, both likely controlled by multi-allelic systems. This dissertation focuses on crosses of mice from lines selectively bred for high voluntary wheel running (HR lines). Cross 1 examined how a gene of major effect (GOME) known as the "mini muscle" (MM) would operate in a genetic background different from HR lines. Cross 2 tested for heterosis in a cross of two HR lines. Cross three investigated dominance and parental effects in a cross between one HR line and one control line. Finally, cross 3 mice were bred to produce F2 and backcross generations to estimate the minimum number of genes that contribute to the HR phenotype.

When one HR line fixed for MM was crossed with inbred strain (C57BL/6J), effects were similar to those seen in the HR and control lines that possess the allele (e.g., homozygotes exhibit ~50% reduction in mass of the triceps surae muscle complex, but enlarged hearts). A 50:50 ratio of normal/MM was observed in the backcross generation, confirming Mendelian recessive inheritance. Cross 2 hybrid males ran more

revolutions/day than purebred males, but hybrid females ran intermediate distances compared with purebred females. This result demonstrates differential and sex-specific responses to selection in two HR lines, implying divergent genetic architectures. Cross 3 found dominance for the HR phenotype in both sexes. Positive maternal influences were observed for all wheel measures, wherein F_1 mice from HR dams ran more than those from C dams. Estimates of the minimum number of genes that account for the difference between an HR and control line were 10 for females and 11 for males, showing that high voluntary wheel running has a polygenic basis, as expected.

In conclusion, this research supports ideas that the HR trait has a complex genetic architecture, consisting of multiple genes, dominance, maternal effects, and at least one GOME on traits related to wheel running. It also shows that during the artificial selection protocol, changes to the genetic architecture have occurred and that high levels of running can be achieved through different genetic pathways.

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Introduction to the Dissertation

The genetic architecture of complex traits

Evolution is primarily a genetic process. Without genetic mutation that introduces new variation, all forms of life would remain equal under natural selection because evolution could not proceed. The product of natural selection is adaptation. Adaptation has allowed organisms to become better suited to their particular environmental conditions. Multiple adaptations may be involved in the evolution of complex traits.

Complex traits generally follow quantitative genetic principles, instead of simple Mendelian rules. This is because many genes throughout the genome usually influence complex traits. These genes interact with one another to form complex phenotypes. As multiple genes govern complex traits, they generally have continuous distributions that must be quantified to determine the phenotype. An excellent example of a complex trait is functionality of the human eye. Over extensive periods of time, many mutations experienced the process of natural selection to produce the organ we have today (Darwin, 1859). The utility of the eye is measured by the degree to which it can function. It cannot be examined with a single yes-or-no characterization.

To understand how a complex trait presents itself as a phenotype, one must begin to dissect its genetic architecture. Genetic architecture is comprised of the loci that affect the trait; the inter-allelic interaction at those loci; possible pleiotropic effects of contributing loci; the combined effects of all loci; and epistatic effects among influential loci (Hansen, 2006). It is important to fully comprehend how genes interact to form distinct genetic architectures resulting in complex phenotypes for a number of reasons, such as to discover new ways to treat and prevent genetic diseases and disorders.

Quantitative phenotypic differences generally depend on allelic variation at many different loci, most of which may have relatively small effects (Falconer and Mackay, 1996). Given that small-effect genes interact to create complex phenotypes, understanding how changes within small-effect genes can alter complex phenotypes is crucial to our understanding of the genetic basis of evolutionary change.

Model of Study

An excellent opportunity to study the quantitative genetics of a complex trait was found in the novel system developed by Dr. Theodore Garland, Jr. who used artificial selection to produce four replicate high runner (HR) lines while also maintaining four non-selected control lines. The selection experiment began in 1993 with the goal of developing a model vertebrate system with which to study the correlated evolution of behavior with physiology and morphology, and thus further elucidate of the evolution of complex phenotypes. This system allows for responses to selection to present themselves on multi-factorial levels. Since the inception of the selection experiment, we have seen behavioral, physiological, and morphological changes in the mice (Garland, 2003; Rhodes et al., 2005; Swallow et al., 2009). Thus, the genetic basis of wheel running encompasses all these levels of biological complexities, which are all cornerstone areas of the evolution of complex traits.

Mice from the outbred Harlan Sprague Dawley (Hsd): ICR strain were used for the base population of the experiment. This strain of mice was chosen because its genetic

variability was similar to that of wild populations of house mice. Mice were randomly mated for two generations, paired, and then randomly separated into eight lines: four of which were designated selection lines (lab designation HR lines 3, 6, 7, 8), while the other four were designated control lines (C lines 1, 2, 4, 5).

The selection protocol for HR is described briefly here; however, it is described in more detail elsewhere (Swallow et al., 1998a). At age 6-8 weeks, mice are given access to 1.12-meter circumference wheels for six days. A standard housing cage is attached to the wheel so the mouse may exit and enter the wheel whenever it desires. Daily wheel running activity is monitored with photocell counters linked to a computer-automated system that registers wheel revolutions in 1-minute intervals. Wheel running is recorded for ~23.5 hours for the duration of wheel access. Therefore, measurement of time spent running (the number of 1-minute intervals with at least one revolution) and average running speed (revolutions/minute, the two components of total wheel running, can also be examined. Selective breeding is conducted based on the total revolutions on days five and six of the six-day trial. This is done so the mice have an opportunity to become accustomed to the wheel, which reduces the chance that selection would be acting primarily against neophobia of the wheels.

Once wheel running on days five and six has been analyzed and adjusted for variations in age, wheel freeness, and sex, breeders are chosen. In the four C lines, one male and one female are randomly chosen from each family. In the HR lines, the top-running males from each of 10 families from each line are bred randomly to the top-running females from each family within the same line, excluding sibling mating

(Swallow et al., 1998a). This within-family selection is performed to increase the effective population size ($N_{e)}$. This also reduces effects of maternal variance and genotype-environment interactions (Henderson, 1989).

The response to this selection has been dramatic. By generation 16, HR lines increased their total revolutions/day by ~170%. The primary cause of this increase was HR mice running faster rather than for running longer periods. However, there are differences between the sexes when further analysis is conducted (Swallow et al., 1998a; Koteja et al., 1999a, b; Rhodes et al., 2000; Girard et al., 2001). In particular, males in the HR lines have also shown a statistically significant increase in the amount of time spent running. After generation 16, there has been a minimal response to continued selection, indicating that a selection plateau may have been reached.

The continued selection applied to the HR lines has caused numerous trait differences between them and the non-selected C lines. These changes encompass many areas of biology including morphological, physiological, and behavioral. Morphological and physiological changes include, but are not limited to, the HR lines having reduced body fat (Swallow et al., 2001), more symmetrical hind limb bones with thicker femurs and tibia-fibulas (Garland and Freeman 2005; Kelly et al., 2006), elevated maximal oxygen consumption (VO_2 max) (Rezende et al. 2006b; Kolb et al., 2010), and elevated circulating corticosterone levels (Malisch et al, 2007)

Behavioral differences include nest-building behavior (Carter et al., 2000), results from open-field trials (Bronikowski et al., 2001), predatory aggression (Gammie et al.,

2003), and aspects of dopamine signaling in the brain, apparently giving higher motivation for running in selected lines (Rhodes et al., 2005; Belke and Garland, 2007).

On a final note, during the selection experiment a mutation was discovered via serendipity (Davisson, 2005) that halves hindlimb muscle mass (Garland et al., 2002; Houle-Leroy et al., 2003). Operating as a simple Mendelian recessive, this mutation was observed in three of the 8 lines of the selection experiment. One control line (lab designation line 5) apparently lost the allele, presumably by genetic drift, by generation 15. The other two lines affected were selected lines where the mutation has increased in frequency, going to fixation in one line (lab designation line 3) while remaining polymorphic in the other (lab designation line 6). This phenotype has a multitude of pleiotropic effects, including differences in organ mass, muscle fiber types, muscle enzymatic activity, glycogen levels, skeletal muscle contractile properties, HSP72 expression, and skeletal phenotype (Garland et al., 2002; Houle-Leroy et al., 2003; Belter et al., 2004; Swallow et al., 2005; Syme et al., 2005; Guderley et al., 2006, 2008; Kelly et al., 2006; Gomes et al., 2009).

Dissertation Research Summary

The highly complex phenotype of HR can be broken down into less complex phenotypes at many lower levels of biological organization. The main objective of this dissertation is to better understand the genetic architecture underlying the HR phenotype.

Chapter #1 examines pleiotropic effects of the Gene of Major Effect (GOME) known as the "Mighty Mini Muscle" (Garland et al., 2002; Houle-Leroy et al., 2003) by seeing if it breeds true into a novel genetic background of the C57BL/6J inbreed strain of mouse. This project had the added effect of creating a population suitable for mapping the genomic location of the MM allele (Hartmann et al., 2008).

Chapter #2 compares two selected lines and examines how multiple solutions to a given type of selection are possible (see also Garland et al., 2010). It also determines if the genetic architecture of the two selected lines are significantly different from one another by analysis of heterosis (hybrid vigor). The finding of significant heterosis suggests that renewed selection on a population derived by crossing two or more of the HR lines could allow breaking through the apparent selection limit that has been observed since approximately generation 16 (e.g., see Bult and Lynch, 2000)).

Chapter #3 tests for dominance and parental effects in the F1 of a reciprocal cross between a line of mice bred for high voluntary wheel-running behavior (HR) line and a non-selected control (C) line. When a trait is under sustained directional selection, alleles with dominance effects in the favored direction are expected to increase in frequency. In addition, parental effects (indirect genetic effects) may be expected to coadapt with the trait under direct selection under certain circumstances (xxgive a ref or two here).

Finally, Chapter #4 investigates the number of genes that contribute to the HR phenotype. To fully understand the genetic structure of the HR phenotype, an estimate of the number of genes that contribute to the HR phenotype is essential. Accurate estimates of genes that contribute to a phenotype will begin to answer several evolutionary

questions. The Castle\Wright method of gene estimation is the most widely used method to estimate the number if genes. Using phenotypic measurements of 2 populations differing in the trait in question, F1, F2, and backcross generations are produced. The mean and variance estimates are then used to calculate the number of independently segregating loci influencing the trait.

In summary, the results of these studies begin to identify the many intricacies of the genetic architecture of the high voluntary running trait in the HR lines of mice. The parallels between voluntary wheel running in mice and voluntary exercise in humans has been noted in the scientific literature (Eikelboom 1999; Garland et al., 2011).

Understanding the genetic components that surround voluntary exercise not only provides information on how a complex trait can evolve, but will also inform us regarding how genetics influences daily activity levels. It is a fact that when an organism's daily activity decreases, weight gain and other health issued generally follow (Booth et al, 2002). With the growing concern over obesity in the United States, information about genetic influences that contribute to activity levels are of relevance to public health and biomedicine. There are also many physiological implications surrounding physical activity. Human studies have suggested that increased physical activity has a direct link to positive mental health (Raglin, 1990; Gavin and Spence, 1996). There is also the proposed issue of addiction to exercise. Some people who maintain a regular exercise routine cannot stop, even if medically necessary (Pierce, 1994). Some forms of anorexia have exercise addiction as a symptom. A broader

information base of genes that possibly contribute to these issues is preliminary to treatments and cures for these disorders.

We know little about what causes the variability in motivation for physical activity. The experiments in this dissertation examine components that putatively contribute to this variability. They will also provide insight as to what influences the desire for voluntary activity. These are the beginning steps to help understand human health problems.

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Chapter 1

Phenotypic Effects of the "Mini-Muscle" Allele in a Large HR x C57BL/6J Mouse Backcross

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Summary

From outbred Hsd:ICR mice, we selectively bred four replicate lines for high running (HR lines) on wheels, while maintaining four non-selected lines as controls (C lines). An apparent Mendelian recessive, the "mini-muscle" (MM) allele, whose main phenotypic effect is to reduce hindlimb muscle mass by 50%, was discovered in two HR lines and one C line. This gene of major effect has gone to fixation in one selected line, remains polymorphic in another, and is now undetectable in the one control line. Homozygotes exhibit various pleiotropic effects, including a doubling of mass-specific muscle aerobic capacity, and larger hearts, livers, and spleens. To create a population suitable for mapping the genomic location of the MM allele and to better characterize its pleiotropic effects, we crossed females fixed for the MM allele with male C57BL/6J. F1 males were then backcrossed to the MM parent females. Backcross mice (N = 404) were dissected, and a 50:50 ratio of normal to MM phenotype was observed with no overlap in relative muscle mass. In the backcross, analysis of covariance revealed that MM individuals ran significantly more on days 5 and 6 of a 6-day exposure to running wheels (as in the routine selective-breeding protocol), were smaller in body mass, and had larger ventricles and spleens.

Introduction

A basic assumption of quantitative genetics is that most aspects of the phenotype are affected by alleles segregating at multiple loci, each having a small effect on the trait (Fisher, 1930). However, recent studies suggest that genes of major effect (GOMEs) are important during the adaptive evolution of many traits and that these genes are almost always associated with a large number of pleiotropic effects (Agrawal et al., 2001; Bradshaw, 1998; Orr, 1998; Orr and Coyne, 1992). GOMEs have been identified in both wild and captive populations. Some important examples in domestic livestock are the muscle-doubling gene in cattle (Rollins et al., 1972), dwarfing gene in poultry (Merat and Ricard, 1974), DGAT1 gene in dairy cattle (Grisart et al., 2002), and IGF2 gene in pigs (Van Laere et al., 2003). Notable examples in natural populations are the PITX1 gene, which alters the pelvis in stickleback fishes (Shapiro et al., 2004), and the Mc1r gene, which affects coat color in beach mice (Hoekstra et al., 2006). GOMEs also play a critical role in genetic research surrounding several major human health problems. For example, gene expression studies of GOMEs have provided valuable insight concerning treatments for both leukemia (Mullighan et al., 2007) and autism (Muhle et al., 2004).

We identified a gene of major effect, termed the "mini-muscle" allele (MM; Garland et al., 2002), within some of the replicate lines of a long-term selection experiment for high locomotor activity in house mice. Selection began in 1993 from a base population of outbred Hsd:ICR mice (Garland, 2003; Swallow et al., 1998). After initial generations of random mating, the base population was divided randomly into 8 lines, 4 of which serve as controls, while the other 4 are selectively bred for high

voluntary wheel running (High Runner or HR lines). After 16 generations, mice from the HR lines ran an average 170% more revolutions per day than control-line mice. These mice have been the subjects of many behavioral and physiological studies, which are summarized elsewhere (e.g., Belke and Garland, 2007; Garland, 2003; Girard et al., 2007; Malisch et al., 2007; Rezende et al., 2006a, b; Rhodes et al., 2005; Swallow et al., 2005).

The putative mini-muscle allele was discovered via dissections of early generations that revealed a subset of individuals with an approximate 50% reduction in "triceps surae" (gastrocnemius, soleus, and plantaris) mass relative to wild-type mice (Garland et al., 2002). Further analyses indicated that the allele operated as a simple Mendelian recessive. The phenotype was only observed in three of the eight lines (one control, two HR). In the one control line (lab designated line 5), the phenotype was apparently lost sometime after generation 22. In one HR line (lab designated line 6), the phenotype remains polymorphic as of generation 50. In the other HR line (lab designated line 3), the mutation apparently had gone to fixation by generation 36 (Syme et al., 2005). Model fitting and statistical analysis indicated that the MM allele must have been favored by the selection protocol in the HR lines (Garland et al., 2002). Loss of the allele from two of the HR lines and from all four control lines can be explained by random genetic drift. Whether the MM allele occurs at an appreciable frequency in any wild population of house mice is presently unknown.

The most obvious phenotype associated with the mini-muscle allele in homozygotes is a 50% reduction in mass of the triceps surae muscle complex (Garland et

al., 2002), as well as in mass of mixed hindlimb muscle exclusive of the triceps surae (Houle-Leroy et al., 2003). However, the MM allele has many pleiotropic effects. For example, homozygous individuals exhibit a doubling of mass-specific aerobic capacity as compared with normal muscle (Houle-Leroy et al., 2003), along with altered mitochondrial density and myosin heavy chain composition (Guderley et al., 2006), altered fiber type composition in the gastrocnemius (Guderley et al., 2008), elevated HSP72 expression in triceps surae (Belter et al., 2004), altered muscle contractile performance (Syme et al., 2005), an increase in size of their ventricles, liver, and spleen (Garland et al., 2002, Swallow et al., 2005), and longer and thinner hind limb bones (Kelly et al., 2006). Many of these pleiotropic effects would appear conducive to the support of sustained aerobic exercise (Garland, 2003; Guderley et al., 2006; Rezende et al., 2006a).

The aim of the present study was to determine whether the Mendelian recessive nature of the mutation is unique to Hsd:ICR mice or robust against a different genetic background, and whether some of the pleiotropic effects segregate with the mutation and display a similar expression pattern within a different genetic background. Finally, if the mutation breeds true, then the resulting population would be ideal for marker analysis to map the genomic position of the mutation and identify the underlying gene.

Materials and Methods

Animals

Full details of the selection experiment have been provided elsewhere (see

Swallow et al., 1998), but we provide a brief overview here. The original progenitors of the selection experiment were 224 individuals of the outbred, genetically variable house mice (*Mus domesticus*; Hsd:ICR; Harlan-Sprague-Dawley, Indianapolis, IN, USA). Mice were randomly mated for 2 generations and randomly assigned to eight closed lines (10 mating pairs in each). In successive generations, mice from the each of the eight lines did not mix. In each line for each generation, offspring were weaned at 21 days of age, and at 6-8 weeks of age were individually housed with access to a Wahman-type running wheel (circumference = 1.12 m) for 6 days. Food (Harlan Teklad, Madison, WI, Rodent Diet [W] 8604) and water were available *ad libitum*. Daily wheel-running activity was monitored with a computer-automated system. Wheel running was quantified as the total number of revolutions run on days 5 and 6 of the 6-day test.

In the four HR lines, the highest-running males and females from each family (highest number of revolutions on days 5 and 6) were chosen to propagate the lines into the next generation (i.e., within family selection). In the four control lines (C), breeders were randomly chosen from each family. Within all lines, males and females were randomly paired, excluding sibling mating.

Females for the current experiment originated from generations 42 (n = 60) and 43 (n = 17) from the HR line (lab designated line 3) that is fixed for the MM allele (Syme et al., 2005). Mice were weaned at 21 days of age and then wheel-tested at 6-8 weeks of age following the routine selection protocol (as described above). Following wheel testing, a portion of the females were used as breeders to supply animals for the next

generation. Therefore, some of the females had given birth to one litter prior to being used in the current study.

Adult males (n = 24; C57BL/6J strain) were purchased from The Jackson Laboratory (Bar Harbor, Maine). C57BL/6J was the source of DNA for the first draft sequence of the mouse genome (Mouse Genome Sequencing Consortium, 2002), thus making it ideal for future genetic mapping studies.

Breeding Design

We harem-mated 20 (of 24 available) C57BL6/J males with three HR females. When a dam appeared to be pregnant, it was removed from the cage and housed separately. From the 60 mating pairs, 33 litters were produced, yielding a total of 316 F1 individuals at weaning (21 days of age). Ninety males from these F1s were randomly chosen and represented all 33 sibships (1-3 males were chosen from each sibship).

At 8 weeks of age, F1 males were backcrossed to the original 60 HR line 3 parent females to produce the backcross (BC) generation. To ensure enough backcross animals for the eventual mapping of the mini-muscle allele, seventeen additional HR line 3 females from generation 43 were also mated to F1 males (13 of 90 F1 males were kept as backups). All breeding pairs were randomly assigned with mother-son and aunt-nephew mating disallowed. When the females appeared pregnant, the F1 males were removed. Of 77 mating pairs, 53 litters were produced, yielding a total of 553 BC individuals at weaning. The total number of BC individuals was reduced from 553 to 404 by randomly choosing a maximum of 4 males and 4 females from each litter, when available. The 404

individuals represented all 53 families that produced a successful litter.

Wheel Access and Dissection

When BC individuals reached 43 ± 3 (± *SD*) days of age, 384 (due to space constraints) were singly housed and given access to a Wahman-type running wheel (circumference = 1.12 m) for 6 days as in the routine selection regime (see above and Swallow et al.,[1998] for details). Food (Harlan Teklad, Madison, WI, Rodent Diet [W] 8604) and water were always available *ad libitum*. Rooms were controlled for temperature (~22°C) and photoperiod 12:12 light/dark cycle (lights on 0700). Wheels were checked daily to ensure freedom of rotation. Wheel running was monitored with a computer-automated system and revolutions were recorded in one-minute bins (intervals). Wheel running was quantified as means for days 5 and 6 of the 6-day test. We analyzed means for total revolutions per day, the number of intervals per day with at least one revolution, the mean speed when running (revolutions/intervals), and the highest single one-minute interval per day.

Following wheel testing, mice were sacrificed by CO_2 inhalation in batches to allow for the harvesting of organs and muscle tissue. Mean age at sacrifice was 234 ± 41 (± *SD*) for MM parent females (generations 42 and 43), 94 for C57BL6/J males, 74 ± 9 for F1 animals, and 57 ± 8 for BC individuals. Following sacrifice, mice were weighed and dissected. The heart was detached and ventricles were removed from the atria and connecting blood vessels. Ventricles were blotted to remove any excess blood prior to

weighing. The spleen was excised followed by the right and left triceps surae muscles (which include the lateral and medial heads of the gastrocnemius, soleus, and the plantaris, as described in Carter et al., 1999). Wet masses of all tissues were recorded to the nearest 0.001 g on an electronic balance (Denver Instruments, Denver CO, USA, model M-220).

Statistical Analysis

The MIXED procedure in SAS (version 9.1; SAS Institute, Cary, NC, USA) was used to apply analysis of covariance models (ANCOVA). A two-way ANCOVA was used to test for the effects of mini-muscle (normal phenotype vs. mini-muscle phenotype) and sex (male vs. female) on wheel running, ventricle mass, and spleen mass in the backcross generation of mice. Effects of the mini-muscle X sex interaction were also examined. Age, body mass, and wheel freeness were used as additional covariates when applicable. Family was a random effect in all analyses. Body mass and organ masses were $\log_{10}$ transformed because this was expected to achieve linearity of allometric relations. Wheel-running traits were transformed as necessary to achieve normality of residuals.

Results

Phenotype Observation and Characterization

The relation of triceps surae muscle mass to body mass in mice from the parent, F1, and BC generations is shown in Figure 1. Muscles from mini-muscle (MM) individuals could be identified visually in such graphs regardless of their body mass, sex,

or lineage. As expected, all Line 3 HR females expressed the mini-muscle phenotype, whereas none of the C57 or F1 individuals did. For the 74 HR females (3 excluded before dissection), average age at dissection was 234 ± 41 (± *SD*) days, range = 146-261. Mean triceps surae mass was 0.0788 ± 0.0102 g (± *SD*), range = 0.0544-0.1020 g, whereas mean body mass was 35.64 ± 5.080 g (± *SD*), range = 27.54-53.65 g.

For 21 C57BL6/J males (20 of which were part of the actual breeding design), the mean age at dissection was 94 days, range = 91-97. Mean triceps surae mass was 0.1416 ± 0.0073 g (± *SD*), range = 0.1269 - 0.1507 g, with a mean body mass of 25.34 ± 1.235 g (± *SD*), range = 22.94 - 28.01 g.

Mean age at dissection for F1 individuals (*n*=89, one died before dissection) was 74 ± 9 (± *SD*) days, range = 61-106. Mean triceps surae mass was 0.1663 ± 0.0116 g (± *SD*), range = 0.1435-0.1934 g, with a mean body mass of 30.44 ± 2.313 (± *SD*), range = 25.32-39.34 g.

At the time of dissection, the 404 BC individuals shown in Figure 1B were an average age of 57 ± 8 (± *SD)* days, range = 50-70. Two hundred and one individuals expressed the mini-muscle phenotype and 203 did not. This obviously does not differ from the 1:1 expectation given inheritance as a simple Mendelian recessive (χ^2=0.0099, *P* = 0.95). Mean triceps surae mass of the BC individuals expressing the mini-muscle phenotype was 0.0643 ± 0.0095 g (± *SD*), range = 0.0442-0.0887 g, with a mean body mass of 23.50 ± 3.079 g (± *SD*), range = 17.55-33.26 g. Mean triceps surae mass of individuals not expressing the phenotype (i.e., normal muscles) was 0.1327 ± 0.0224 g (±

SD), range = 0.0907-0.1932 g, with a mean body mass of 25.79 ± 3.730 g (± SD), range = 18.11g-39.19 g.

Analysis of Backcross Generation

Adjusting for variation in age, ANCOVA (Table 1) indicated that MM individuals were significantly lighter than normal mice ($P < 0.0001$) and that female mice were lighter than males ($P < 0.0001$), with no statistically significant MM X sex interaction ($P = 0.2964$).

After adjusting for variation in age and body mass, MM individuals had significantly smaller triceps surae muscles ($P < 0.0001$), males had significantly larger muscles than females ($P < 0.0001$), and the MM X sex interaction was significant ($P = 0.0066$: see footnote to Table 1 for adjusted means). MM individuals had larger ventricles relative to normal individuals ($P < 0.0001$), with no statistical effect of sex or a MM X sex interaction (Table 1). MM individuals had significantly larger spleens, and spleens were larger in females than in males ($P < 0.0001$) with no MM X sex interaction.

Analysis of wheel running data revealed that MM individuals ran significantly more revolutions because they ran at higher average and maximum speeds, with no statistical difference in the amount of time spent running per day (Table 1). Females ran significantly more than males by all four measures. The MM X sex interaction was not statistically significant for any measure of wheel running (Table 1).

Discussion

In the backcross (BC) generation, the numbers of animals exhibiting the mini-muscle (MM) phenotype did not significantly differ from the expected 1:1 ratio under Mendelian laws. No affected mice were found in the F1 generation, providing strong additional evidence that this phenotype is the result of a gene that segregates as a simple Mendelian recessive within this mouse lineage (see also Garland et al., 2002).

Body Size and Organ Measurements

The reduced body mass of backcross individuals expressing the MM phenotype follows the same pattern as has been reported for MM individuals within the selection experiment (Garland et al., 2002; see also Syme et al., 2005). This reduction can be accounted for in part by the reduction in triceps surae mass, as well as thigh muscle mass (Houle-Leroy et al., 2003). All else being equal, a reduction in body mass would reduce the absolute amount of energy needed during exercise, and it is conceivable that this is advantageous for high wheel running (see also Rezende et al., 2006b).

The 51.9% reduction in mass of the triceps surae muscle complex in affected BC mice is similar to that shown by MM individuals within the selection experiment (Belter, et al. 2004; Garland et al., 2002; Kelly et al., 2006; Rezende and Gomes et al., 2006; Swallow et al., 2005). BC males had larger triceps surae than females regardless of their mini status, but males showed a relatively greater reduction in mass of the triceps surae when expressing the mini phenotype (see Table 1). Many factors could account for a sex difference in the effects of the mini-muscle allele, one being testosterone, which affects muscle differentiation and growth in mammals.

BC individuals expressing the MM phenotype had significantly larger ventricles and spleens then their non-MM counterparts. Female BC mice had significantly larger spleens than male mice regardless of mini status, as was also reported within the selection experiment (Garland et al., 2002; but see Swallow et al., 2005). Both of these effects may be advantageous to HR mice. Increased ventricle size would be expected to increase stroke volume and hence cardiac output (Rezende et al., 2006a), whereas increased spleen size might indicate enhanced red blood cell production (or immune function).

The foregoing pleiotropic effects of the mini muscle mutation can be explained in three possible ways. First, the mutation itself may affect the sizes of other organs. Second, the presence of the smaller muscle may cause indirect pleiotropic effects on masses of other organs. For example, if smaller muscles cause increased peripheral resistance in the cardiovascular system, then blood pressure would increase and could lead to cardiac hypertrophy. Third, genes that affect organ mass could be tightly linked to the locus of the mini-muscle mutation. If other genes that affect ventricle or spleen mass were relatively close to the MM gene on the chromosome, then deviation from the observed segregation pattern would be rare (Falconer and Mackay, 1996). Given that organ mass is a complex trait (Deschepper et al., 2002), a quantitative genetic approach could be helpful in determining which of these hypotheses is correct.

Wheel Running

BC individuals expressing the MM phenotype showed a significant increase in wheel revolutions/day compared to non-MM individuals. However, within the selection

experiment, individuals with the MM mutation exhibit revolutions/day similar to other HR individuals (Garland et al., 2002; Houle-Leroy et al., 2003; Kelly et al., 2006; Swallow et al., 2005; but see Syme et al., 2005 who only studied lines 3 and 6). Thus, the mini-muscle allele may have a stronger positive effect on wheel revolutions in the BC population than in the context of the selection experiment. Alternatively, statistical power may be higher in the BC population, with a large sample size and a 1:1 ratio of normal to affected individuals. Aside from total daily running distance, the increased average running speed of mini-muscle individuals (Table 1) has been reported previously in the context of the selection experiment, at least for some samples (Kelly et al., 2006; see also Syme et al., 2005).

Females ran significantly more revolutions per day, at higher speeds, and for more minutes/day than males regardless of MM phenotype. These results are consistent with previous reports on the selection experiment (Garland et al., 2002; Koteja and Garland, 2001; Swallow et al., 1998; Swallow et al., 2005).

Future Directions

The putative mini-muscle allele exhibits "classic" properties of a gene of major effect, including its dramatic effect on muscle mass, its Mendelian recessive nature, and its many other pleiotropic effects. Non-deleterious genes that have all the above properties are rare, and so this mutation provides an excellent model in which to study the quantitative genetics of major-effect genes (Orr, 1998).

Typically, phenotypes affected by genes of major effect (GOMEs) are also

affected by so-called "modifier" genes that have smaller phenotypic effects (Futuyma, 1998). These modifier genes may either magnify or mask some of the properties of the major gene (Lynch and Walsh, 1998). Consistent with the presence of modifier genes, there are minor differences between the phenotypes of mice within the line where the mutation is fixed (line 3, used for the present crosses) and the line in which it is still polymorphic (6). For example, in line 3 there is a >50% reduction of myosin heavy chain (IIB) fibers in the gastrocnemius, whereas in line 6 the reduction is only 30%. Also, mitochondrial volume density in the plantaris muscle was significantly higher in MM individuals of line 3, but not so for line 6 (Guderley et al., 2006). Breeding experiments between lines could discern the direct effects of the mutation, and variance measures of muscle and organ mass could identify the presence of background genes of small effect that contribute additive genetic variance to the phenotype.

Future studies will include mapping the MM gene. The backcross population produced for the present study is highly suitable for mapping of the chromosomal position of the MM allele, and eventually detection of the underlying gene and the nature of variation causing MM.

Other proposed research into this mutation includes examining effects on other muscles. Within the triceps surae, it has already been shown that the reduction in mass is greater for the gastrocnemius than for the plantaris, and that the soleus is actually enlarged in mini-muscle individuals (Guderley et al., 2006; Syme et al., 2005). If the effects of the mutation are not limited to the triceps surae, then this GOME could prove useful in several biological areas of study that deal with muscle mutation/dysfunction.

With the growing base of information linking GOMES with common health disorders, this mutation could provide important insight into muscle degenerative diseases. For example, blockade of myostatin has been proposed as a treatment for muscle-wasting disorders (e.g., see Amthor et al., 2007). Importantly, MM individuals actually show enhancement of some muscle functional properties, i.e., increased fatigue resistance (Syme et al., 2005).

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Table 1.1. Analysis of covariance for effects of the mini-muscle phenotype in the backcross generation.

Trait	*n*	Mini	Sex	Mini x Sex	Age	$\log_{10}$ Body Mass
log Body Mass (g)	403[a]	$F(1,397) = 129.6$ $P <$ **0.0001** [-]	$F(1,397) = 587.0$ $P <$ **0.0001** [+]	$F(1,397) = 1.1$ $P = 0.2964$	$F(1,397) = 60.4$ $P <$ **0.0001** [+]	
log Triceps Surae Mass (g)	404	$F(1,397) = 6686.6$ $P <$ **0.0001** [-]	$F(1,397) = 69.6$ $P <$ **0.0001** [+]	$F(1,397) = 7.5$[b] $P =$ **0.0066**	$F(1,397) = 19.0$ $P <$ **0.0001** [+]	$F(1,397) = 373.8$ $P <$ **0.0001**
log Ventricle Mass (g)	402[a]	$F(1,395) = 157.5$ $P <$ **0.0001** [+]	$F(1,395) = 2.9$ $P = 0.0890$ [+]	$F(1,395) = 0.1$ $P = 0.7544$	$F(1,395) = 17.9$ $P <$ **0.0001** [-]	$F(1,395) = 479.4$ $P <$ **0.0001**
log Spleen Mass (g)	403[a]	$F(1,396) = 85.6$ $P <$ **0.0001** [+]	$F(1,396) = 44.2$ $P <$ **0.0001** [-]	$F(1,396) = 1.3$ $P = 0.2646$	$F(1,396) = 0.5$ $P = 0.4716$ [-]	$F(1,396) = 192.7$ $P <$ **0.0001**
(Revolutions/Day)^0.5	384	$F(1,328) = 25.4$ $P <$ **0.0001** [+]	$F(1,328) = 14.6$ $P =$ **0.0002** [-]	$F(1,328) = 2.2$ $P = 0.1395$	$F(1,328) = 3.4$ $P = 0.0648$ [-]	$F(1,328) = 2.3$[$] $P = 0.1332$ [+]
Intervals/Day	384	$F(1,328) = 0.5$ $P = 0.4634$ [+]	$F(1,328) = 15.1$ $P =$ **0.0001** [-]	$F(1,328) = 0.9$ $P = 0.3481$	$F(1,328) = 4.5$ $P =$ **0.0357** [-]	$F(1,328) = 2.5$[$] $P = 0.1128$ [+]
Mean Speed (RPM)	384	$F(1,328) = 35.0$ $P <$ **0.0001** [+]	$F(1,328) = 5.5$ $P =$ **0.0200** [-]	$F(1,328) = 1.7$ $P = 0.1898$	$F(1,328) = 1.0$ $P = 0.3315$ [-]	$F(1,328) = 0.9$[$] $P = 0.3531$ [+]
Maximum Speed (RPM)	384	$F(1,328) = 41.6$ $P <$ **0.0001** [+]	$F(1,328) = 12.7$ $P =$ **0.0004** [-]	$F(1,328) = 0.9$ $P = 0.3449$	$F(1,328) = 2.0$ $P = 0.1613$ [-]	$F(1,328) = 3.9$[$] $P =$ **0.0490** [+]

For *P* values, bold indicates $P < 0.05$, two-tailed, unadjusted for multiple comparisons. Signs following *P* values indicate direction of effect: + mini-muscle mice > normal, + male > female.

[a] One or two individual removed as a statistical outliers.

[b] Least squares means $\pm$ standard errors are: Normal Females 2.0785 $\pm$ 0.003156, Normal Males 2.1263 $\pm$ 0.004087, Mini Females 1.8021 $\pm$ 0.004115, Mini Males 1.8332 $\pm$ 0.003043. Back-transformed mean values (mg) are, respectively, 119.8, 133.8, 63.4, and 68.1.

[$] [c] Square root of wheel freeness rather than body mass was used as a covariate for the wheel-running trait

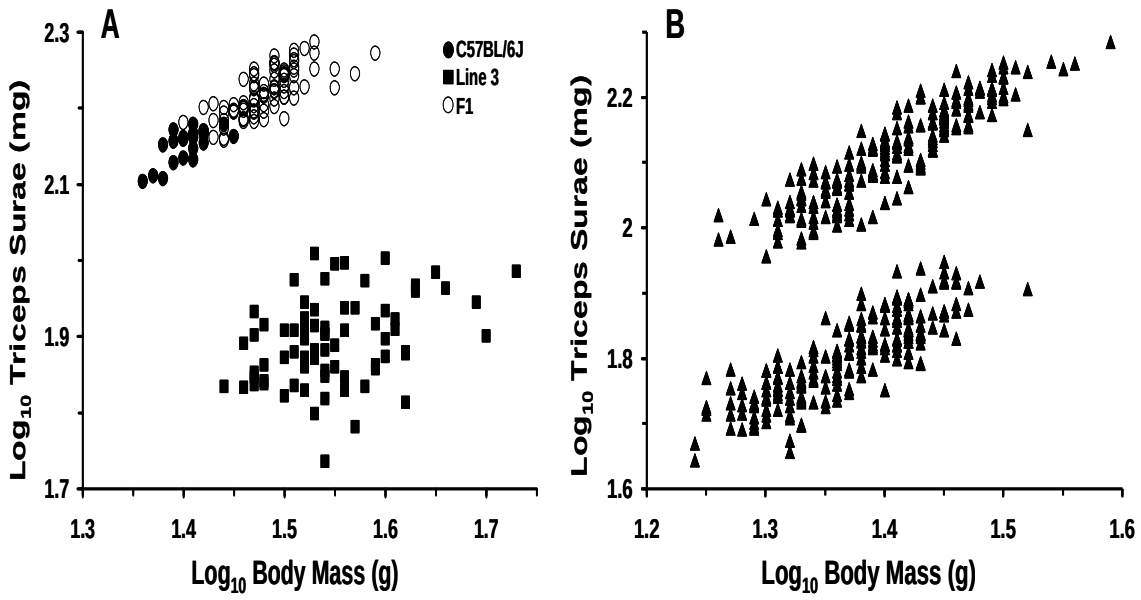

Figure 1.1. Relation between $\log_{10}$ triceps surae mass and $\log_{10}$ body mass for line 3 High-Runner females, C57BL6/J males, their F1 offspring, and backcross individuals. As shown in panel A, the mini-muscle phenotype was observed in all line 3 parent females, no C57BL6/J parent males, and no F1 individuals, thus indicating its recessive nature. As shown in panel B, the backcross generation of mice exhibited a 1 to 1 ratio of normal (N = 203) to mini-muscle (N = 201) phenotypes, with no intermediates. See text for statistical analyses.

Chapter 2

Sex-Specific Heterosis in Line Crosses of Mice Selectively Bred for High Locomotor Activity

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Keywords: Artificial selection; Body size; Complementation; Experimental evolution; Heterosis; Hyperactivity; Voluntary exercise; Wheel running

Summary

When populations with similar histories of directional selection are crossed, their offspring may differ in mean phenotype as compared with the average for the parental populations, often exhibiting enhancement of the mean phenotype (termed heterosis or hybrid vigor). We tested for heterosis in a cross of two replicate lines of mice selectively bred for high voluntary wheel running for 53 generations. Mice were paired to produce four sets of F1 offspring, two purebred High Runner (HR) lines and the hybrid reciprocal crosses. The purebred HR showed statistically significant, sex-dependent differences in body mass, wheel revolutions, running duration, mean running speed, and (controlling for body mass) organ masses (heart ventricles, liver, spleen, triceps surae muscle). Hybrid males ran significantly more revolutions than the purebred males, mainly via increased running speeds, but hybrid females ran intermediate distances, durations, and speeds, as compared with the purebred females. In both sexes, ventricles were relatively smaller in hybrids as compared with purebred HR. Overall, our results demonstrate differential and sex-specific responses to selection in the two HR lines tested, implying divergent genetic architectures underlying high voluntary exercise.

Introduction

Breeders of crops and livestock have known for centuries that matings between distantly related individuals often produce better offspring than those between closely related individuals (Darwin, 1868). This phenomenon is commonly known as heterosis (since Shull, 1914), or hybrid vigor, denoting the superiority of offspring. When inbred populations are crossed, the offspring will often exhibit mean values higher than those of the mid-parent level for any traits that have exhibited inbreeding depression, including aspects of Darwinian fitness (reproductive success, e.g., Falconer and Mackay, 1996; Birchler et al., 2006). This is not always the case, however, as outbreeding depression can also occur in distantly related populations due to breakup of coadapted gene complexes that contribute to a phenotype affected by a high degree of epistasis (Lynch, 1991; Lynch, 1994; Burke and Arnold, 2001; Birchler et al., 2006).

As noted by Mayr (1961), independent lines (populations) experiencing apparently identical directional selection will often respond at different paces and with different correlated traits. Although directional selection works to increase the frequency of favorable alleles while reducing the frequency of unfavorable alleles, the simultaneous effects of random genetic drift are indifferent to any particular allele's selective relevance. Therefore, drift potentially fixes alleles whose effects are neutral or even counter to what selection favors. As drift and mutation are stochastic processes, their effects will, on average, cause populations to diverge genetically, and the generation-to-generation response to directional selection will be contingent on existing genetic variation. For these reasons (and others), identical selection may often lead to "multiple solutions" in

different populations (Garland and Rose, 2009; Garland et al., 2010) and when these populations are mixed, as during an intentional cross, heterosis for many traits will often occur (e.g., Ehiobu and Goddard, 1990; Bult and Lynch, 1996; reviews in Lynch and Walsh, 1998; Lippman and Zamir, 2007).

Heterosis has been documented for many traits, within many different species, such as high-temperature growth rate in yeast (Steinmetz et al., 2002), post-weaning success in pigs (Young et al., 1976), and mannose-binding lectin in humans (Hellemann et al., 2007). In house mice, heterosis has been observed for traits including food competition (Manosevits, 1972), motor behavior (Guttman, 1980), growth rates (Bhuvanakumar et al., 1985), body size (Lynch et al., 1986), litter size (Peripato et al., 2004), activity rhythms (Beau, 1991), and nest-building behavior (Bult and Lynch, 1996).

The primary purpose of the present study was to test for heterosis using two (of four) replicate lines of mice that have been bred for high voluntary wheel-running behavior (Swallow et al., 1998, 2005, 2009). Wheel running is a behavior that generally will involve aspects of both motivation and ability (Waters et al., 2008; Meek et al., 2009; Garland et al., 2011). For example, an individual rodent that is highly motivated to run (e.g., because it is highly rewarding in a neurobiological sense) but lacks the inherent endurance capacity to do so simply will not be able to run as much as another individual with both high motivation and high ability. Rodent wheel running has been the subject of numerous studies, with goals ranging widely across behavior, physiology, and genetics (e.g., Slonaker, 1912; de Kock and Rohn, 1971; Holloszy and Smith, 1987; Belke and Garland, 2007). Despite a century of study, precisely what wheel running in laboratory

rodents represents remains controversial (Mather, 1981; Sherwin, 1998; Garland et al., 2011). Heterosis has been observed for wheel-running behavior (and other aspects of locomotor activity, e.g., exploratory behavior) when inbred strains of mice were crossed (Bruell, 1964a, b).

The crosses necessary to study heterosis also allowed us to test for line differences. On average, the four replicate High Runner (HR) lines run 2.5-3.0-fold more revolutions/day as compared with four non-selected control (C) lines, a differential that has been maintained from approximately generation 16 to the time of the present study at generation 53 (Middleton et al., 2008; Swallow et al., 2009). The nature of this selection limit is as yet unknown, but does not appear to be simply an exhaustion of additive genetic variance for wheel running (unpublished results). Phenotypically, the selection limit may be related to availability of lipids to fuel the many hours of running that occur during each 24-hour period (Gomes et al., 2009; Kolb et al., 2010a; Meek et al., 2010). Whatever the precise phenotypic characteristics that underlie the selection limit, if a cross between two HR lines results in hybrid vigor, then selection applied to a population derived from such a line cross would have the potential to break through the prevailing selection limit (e.g., Bult and Lynch, 2000). In addition to measures of wheel running, we report data for masses of four organs, at least three of which (heart ventricles, calf muscles, liver) may have important roles during endurance running (e.g., see Dumke et al., 2001; Garland et al., 2002; Swallow et al., 2005; Rezende et al., 2006c; Meek et al., 2009; and references therein).

Methods

Animals

Mice used in this study were from an ongoing selection experiment for high voluntary wheel running. Full details of the selection experiment are found in Swallow et al. (1998), and only a brief synopsis is presented here. The original progenitors were 224 mice of the outbred, genetically variable (e.g., see Carter et al. 1999) Hsd:ICR strain of house mice (*Mus domesticus*). This population was randomly mated for two generations and then divided into eight closed lines, four of which were deemed high runner (lab designations HR 3,6,7,8) and four control (C 1,2,4,5). A minimum of ten pairs from each line were used to propagate the subsequent generations. Pregnant dams are given a breeder diet (Harlan Teklad, Madison, WI, Mouse Breeder Diet [S-2335] 7004). At other times, food (Harlan Teklad, Madison, WI, Rodent Diet [W] 8604) and water are available *ad libitum*. Pups are weaned at 21 days of age. Each generation, at 6-8 weeks of age, mice are individually housed with access to a Wahman-type running wheel (circumference = 1.12 m) for 6 days, during which daily wheel running is monitored by a computer-automated system. The selection criterion is the total number of revolutions run on days 5 and 6 of the 6-day test. In the four HR lines, the highest-running male and female from each family are chosen as breeders (i.e., within-family selection). In addition, second-highest running males and females are chosen to provide backup pairings. In the four control lines (C), two males and two females are randomly chosen from each family without regard to wheel running. Within all lines, breeders are randomly paired, excluding sibling mating.

Selected lines 7 and 8 were chosen for this study due to the absence of the mini-muscle allele, which affects numerous traits, including wheel running and organ masses (see Garland et al. 2002, Swallow et al., 2005; Rezende et al. 2006a,c; Hannon et al., 2008; Hartmann et al., 2008; Middleton et al., 2008; Gomes et al., 2009). All line 7 and line 8 breeders (see previous paragraph) from generation 53 were repaired to produce mice for the present study (i.e., second litters). Sires were housed individually from time of removal from first pairing to time of second pairing. Dams were housed 3-4 to a cage from time of weaning of first litter to time of pairing for this experiment.

Due to the within-family selection method used to choose breeders for the selection experiment, the breeders for the present experiment usually had three siblings (one of the same sex, two of the opposite sex) also included in the experiment. Therefore, mice were repaired using the following guidelines. Sibling mating was disallowed and all females were mated with a novel male. Considering two siblings of the same sex, one sibling was randomly chosen to be mated with a mouse from the same line, while the other sibling was mated to the other line. For families represented by other than four (3 or 5) siblings, the odd mouse was randomly assigned as a breeder.

This protocol produced a total of 43 breeding pairs: 11 pairs were purebred line 7 X line 7; 10 were purebred line 8 X line 8; 11 were male line 7 X female line 8 hybrids; 11 were male line 8 X female line 7 hybrids. Purebred offspring of the replicate selected lines (7 X 7 and 8 X 8) were used because direct comparison to parental individuals could be compromised due to possible generational effects, which can be substantial (e.g., see figures in Swallow et al., 1998, 2009; Middleton et al., 2008). Reciprocal crosses for the

hybrids were conducted to test for parental effects. Eighteen days after pairing, the male was removed if the female was visibly pregnant; otherwise, he remained with the female until she appeared pregnant. Mice were weaned at 21 days of age and housed 4 per cage by sex and cross type. Total sample sizes were 171 females and 166 males for wheel-running traits, with the breakdown by cross type was as follows: 47 female and 38 male for line 7 X line 7; 42 female and 48 male for line 8 X line 8; 38 female and 37 male for male line 7 X female line 8 hybrids; 45 female and 43 male for male line 8 X female line 7 hybrids. For organ masses, total sample sizes were 177 females (176 for ventricle mass) and 166 males (165 for ventricle mass and triceps surae mass).

Measurement of Wheel Running and Organ Masses

F1s were wheel-tested in the same manner as in the regular selection experiment (described above). Rooms were controlled for temperature (~22°C) and photoperiod 12:12 light/dark cycle (lights on 0700). Wheels were checked daily to ensure freedom of rotation. Wheel running was monitored with a computer-automated system and revolutions were recorded in one-minute bins (intervals). Wheel running was quantified as means for days 5 and 6 of the 6-day test (Swallow et al., 1998). Following previous studies, we analyzed means for total revolutions per day, the number of 1-minute intervals per day with at least one revolution (minutes/day), the mean speed when running (revolutions/minutes), and the highest single 1-minute interval per day (e.g., Swallow et al., 1998; Hannon et al., 2008; Kelly et al., 2010a,b). We also analyzed body mass at the start of the wheel trial.

Following wheel testing, mice were returned to standard cages without wheels, housed 4 per cage. Approximately 7 days following wheel testing, mice were sacrificed by CO_2 inhalation in batches to allow for harvesting of organs and muscle tissue. Mean age at sacrifice was 69 ± 3 (± *SD*) days. Following sacrifice, mice were weighed and dissected to determine masses of organs that have potential relevance for exercise physiology. The heart was detached and ventricles were removed from the atria and connecting blood vessels. Ventricles were blotted to remove any excess blood prior to weighing. The liver was excised followed by the spleen, then the right and left triceps surae muscles [(which include the lateral and medial heads of the gastrocnemius, soleus, and the plantaris, as described in Carter et al. (1999)]. Wet masses of all tissues were recorded to the nearest 0.001g on an electronic balance (Denver Instruments, Denver CO, USA, model M-220).

Statistical Analyses

To test for differences in wheel running, body mass, and organ masses, a two-way analysis of covariance model (ANCOVA) was applied using the MIXED procedure in SAS (version 9.1; SAS Institute, Cary, NC, USA). All analyses used age as a covariate. Analyses of organ masses used body mass as an additional covariate. Analyses of wheel-running traits did not include body mass as a covariate, but did use a measure of wheel freeness. To measure wheel freeness, each wheel was accelerated to a constant velocity, then the number of revolutions spun until stopping was recorded. For analyses, wheel freeness was transformed by raising measured values to the 0.4 power to obtain a more

homogeneous spread of values. Deviations from linearity were not apparent in plots of the wheel-running traits versus transformed wheel freeness, and preliminary analyses indicated that the interaction between group and transformed wheel freeness were not statistically significant (all $P > 0.08$). Therefore, this interaction term was not included in final statistical models. Family was a random effect, nested within cross type. Preliminary analyses combined the sexes and tested for effects of cross type, sex, and the cross type * sex interaction. Because we found significant interactions (e.g., for revolutions/day, $P = 0.0012$; see Results), subsequent analyses were done separately by sex.

The hybrid groups were expected to exhibit greater variance than the parental types. Therefore, we considered a range of models that allowed for different variances among families within types and/or among individuals within families (i.e., the residual variance). Specifically, we considered models with (1) a single estimate for residual variance, (2) a single estimate for residual variance and a single estimate for variance among families (nested random effect), (3) a single estimate for residual variance and separate estimates of family variance for each of the four cross types, (4) a different residual variance for each cross type and no variance among families, (5) a different residual variance for each cross type and a single estimate of variance among families, (6) a different residual variance for each cross type and separate estimates of family variance for each cross type. We used a priori contrasts to compare the two parental types (i.e., test for line differences), the two reciprocal hybrid crosses (test for parental effects), and the two parental groups with the two hybrid groups (test for heterosis). In

general, significance levels for these contrasts were similar across the six models listed above. For simplicity and consistency, we report results only for the most parameter-rich model, i.e., number (6) above. In some cases, traits were transformed to improve normality of residuals.

Because we performed a number of tests on closely related data, our Type I error rate for the entire experiment may exceed the nominal 5% alpha level. Therefore, we performed a positive false discovery rate (pFDR) analysis using the QVALUE package (Version
1.1; Storey 2002) for R (Version 2.8.0; R Core Development Team 2008), allowing for 5% false significant results (pFDR = 0.05). Based on analysis of the 60 *P* values presented in Table 1, those < 0.016 can be considered significant, and we emphasize those results.

Results

In preliminary analyses, we found significant sex * cross type interactions for revolutions/day ($P = 0.0012$), minutes/day ($P = 0.0140$), and maximum speed in any 1-minute interval ($P = 0.0255$), but not for mean speed ($P = 0.0850$) or body mass ($P = 0.7866$). Therefore, subsequent analyses were done separately by sex.

Females

After adjusting for multiple comparisons, purebred females from line 7 ran significantly more revolutions per day ($P = 0.0032$), at higher mean ($P = 0.0005$) and

maximum speeds ($P = 0.0008$), but not for more minutes per day ($P = 0.6163$), as compared with line 8 females (Tables 1, 2 and Figure 1). Line 7 females were significantly smaller than those from line 8 (Tables 1, 2). Controlling for variation in body mass, lines 7 and 8 differed significantly for ventricle, spleen, and triceps sure mass, but not liver mass (Tables 1, 2 and Figure 2).

Female hybrids were intermediate between the purebred lines for body mass at the start of wheel access and for all running traits (Figure 1, Tables 1, 2). Female hybrids had significantly smaller ventricles ($P = 0.0034$) than purebreds after adjusting for body mass. Hybrids from the two reciprocal cross populations were not significantly different for any trait (Tables 1, 2; Figures 1, 2).

Males

Purebred males from HR lines 7 and 8 differed significantly for minutes/day of wheel running, but not for revolutions/day, mean speed or max speed (Tables 1, 2; Figure 1). Purebred males from line 8 were significantly larger than those from line 7, and they also had significantly larger livers, spleens, and triceps surae muscles (Figure 2, Tables 1, 2).

Unlike female hybrids, as compared with the mean for purebred lines, male hybrids showed a significant increase in revolutions per day ($P = 0.0016$), mean speed ($P = 0.0037$), and maximum speed ($P = 0.0101$), but did not differ in body mass at the start of wheel access (Tables 1, 2). Consistent with females, male hybrids from the reciprocal

crosses (7 X 8 vs. 8 X 7) were not significantly different for any trait (Tables 1, 2; Figures 1, 2).

Discussion

Results of our crosses between two replicate lines bred for high voluntary wheel running, intended primarily to examine heterosis, also show that the two lines differ for a number of traits, often in a sex-specific fashion. For example, revolutions run per day -- the target of selective breeding -- were higher in purebred HR line 7 than 8 for females (14,607 vs. 10,878, respectively, 2-tailed $P = 0.0032$), but not for males (9,123 vs. 11,257, $P = 0.1759$) (Figure 1, Tables 1, 2). Moreover, the patterns of heterosis that we identified differ between males and females. Therefore, we separate much of the subsequent discussion by sex. It is important to note that the higher wheel running of females than males in line 7 is not peculiar to this generation (e.g., see Garland et al., 2010 for results from generation 43).

Males

For males, examination of the two components of wheel revolutions/day indicates that the two HR lines have responded differently to artificial selection (Figure 1, Tables 1, 2). Line 8 males ran substantially more minutes/day as compared with line 7 (542 vs. 441 minutes/day), but the direction of this differential was reversed for mean running speed (18.17 vs. 20.02 revolutions/min). The end result was no statistical difference in revolutions/day (10,086 vs. 9,123), thus demonstrating approximate functional

equivalence achieved by "multiple solutions" in response to selective breeding (e.g., Endler et al., 2001; Spitschak et al., 2007; see also Swallow et al., 2009; Garland et al., 2010). Line 7 males were smaller than those of line 8, and also had significantly smaller body-mass adjusted spleens, livers, and triceps surae muscles (Tables 1, 2; Figure 2), but whether this is causally related to the differences in running behavior is unclear (see also Garland et al., 2002).

Consistent with the partial evolutionary independence of average running speed and duration found in the present study, within an advanced intercross mapping population of HR line #8 and inbred C57BL/6J, two statistically significant QTL were detected for average running speed on days 5 plus 6, and a different QTL was detected for time spent running on days 5 and 6 (Kelly et al., 2010b), although a formal test for epistasis was not performed. Similarly, a QTL analysis of an F2 population from a cross between relatively high-running C57L/J and low-running C3H/HeJ inbred strains found two QTL for wheel-running speed, one of which did not colocalize with the single QTL identified for duration (Lightfoot et al., 2008), although a subsequent paper detected considerable epistasis by use of a full genome epistasis scan for all possible interactions of QTL between each pair of 20 chromosomes (Leamy et al., 2008).

Hybrid males showed a significant increase in revolutions/day over purebred males (hybrid vigor), caused mainly by higher running speed, with a trend also for more time spent running (Figure 1). This result demonstrates that the underlying genetic architecture of high wheel running in males differs between these two HR lines (e.g., Bult and Lynch, 1996). In contrast to the results for wheel running, hybrids were intermediate

to the parental groups for relative liver, spleen, and triceps surae muscle masses. It is interesting that these lower-level traits do not follow the same pattern of heterosis as the target of selection, which could be explained by their not being functionally necessary to support the higher levels of wheel running and/or by a change in their genetic correlation with wheel running in the cross populations (e.g., see Eisen, 1975). In previous publications that reported masses for these organs, no consistent, statistically significant differences were found in comparisons of the four High Runner and four control lines (Dumke et al., 2001; Garland et al., 2002; Swallow et al., 2005; Rezende et al., 2006c; Meek et al., 2009).

Females

Unlike males, purebred line 7 females ran significantly more revolutions/day than line 8 females, almost entirely because the former ran faster, with no statistical difference in duration of running (Figure 1, Tables 1, 2). Also unlike males, hybrid females were intermediate between the two parental phenotypes for both revolutions/day and speed. In spite of the differences from males, overall these comparisons again indicate different genetic responses to selection.

As with males, females of line 7 were smaller than line 8 and had smaller size-adjusted spleens and triceps surae. In contrast to males, line 7 females had relatively larger hearts than their line-8 counterparts (Tables 1, 2), which could contribute to their higher running speeds via positive effects on endurance (Meek et al., 2009) or maximal aerobic capacity (Rezende et al., 2009). Arguing against this, however, hybrid females

had relatively smaller heart ventricles ($P = 0.0034$) than either purebred line, but exhibited intermediate levels of wheel running (Figure 1, Tables 1, 2).

Parental Effects

In a reciprocal cross between HR line 8 and a control line, we found parent-of-origin effects in the F1 for both body mass and wheel running (Chapter 3). Similarly, in a cross between HR line 8 and inbred C57BL/6J, we found parent-of-origin effects on body composition and wheel-running traits in a fourth-generation intercross population (Kelly et al., 2010a). In the present cross, however, we found no such effects that were statistically significant. The lack of such effects in the present cross may reflect the fact that the two replicate HR lines studied here are more similar, both phenotypically and genetically, than for a control line or C57BL/6J vs. HR line 8.

Summary and Future Directions

The line crosses presented here demonstrate different responses to selection for high voluntary wheel running in two (of four total) replicate HR lines, as well as sex-by-line interactions in the response to selection. In addition, the two HR lines not studied here have shown an increase in the frequency of a Mendelian recessive allele that causes a 50% reduction in hindlimb muscle mass and increased wheel-running speed, among many other identified pleiotropic effects (Garland et al., 2002; Swallow et al., 2005; Rezende et al., 2006a; Hannon et al., 2008; Middleton et al., 2008; Gomes et al., 2009). The "mini-muscle" phenotype was never detected in the two lines studied here, again

demonstrating different genetic responses to selection. Thus, overall, results of the long-term selection experiment reinforce the concept that directional selection favoring a particular phenotype, and hence altering the frequencies of alleles that affect the phenotype, will occur simultaneously with other evolutionary processes, especially random genetic drift in the relatively small populations used for rodent selection experiments (e.g., Eisen, 1975; Swallow et al., 2009).

Hormonal differences may contribute to the line (or sex: Lightfoot, 2008) differences we observed. For example, it has been shown previously that HR lines have higher circulating corticosterone (CORT) concentrations than C, and that differences among replicate lines are also statistically significant (Malisch et al., 2007, 2009). As suggested elsewhere (Malisch et al., 2008), organisms with elevated corticosterone levels could have higher available energy and/or motivation to perform during exercise such as wheel running (Dallman et al., 1993; Pecoraro et al., 2006). However, whether HR lines 7 and 8 show consistent differences in baseline CORT or in levels during wheel running is not yet known (see Malisch et al., 2007, 2009).

Our results show some clear examples of sex-specific heterosis, as has occasionally been reported in the literature. White et al. (1970) report heterosis involving body mass in mice, with both sexes experiencing heterosis, but one sex showing it to a greater degree. Line crosses involving body mass in beef cattle and poultry (Stonaker, 1963), fecundity in *Drosophila* (Brown and Bell, 1960), and survival in swine (Cox, 1960) also showed one sex to exhibit a greater degree of heterosis. However, the pattern of sex-specific heterosis reported in this study seems to be rare. Unlike the examples

cited, we show cases (Fig. 1) in which the F1 of one sex exhibits clear heterosis, whereas the F1 of the other is intermediate between the phenotypic means of the parental populations.

The mechanisms underlying the cases of sex-specific heterosis that we observed are not yet apparent. Using a backcross between a different HR line (#3) and inbred C57BL/6J, Nehrenberg et al. (2010) reported several sex-specific QTL, including for aspects of wheel running. That study probably underestimates the magnitude of such effects, because the cross design used did not allow examination of markers on the sex chromosomes. Kelly et al. (2010b) included markers on the X chromosome in a QTL study that used a large advanced intercross line (G4) population originated from a reciprocal cross between HR line #8 (one of the two used here) and C57BL/6J, but did not any detect any QTL on the X chromosome nor any sex-specific QTL. As noted in the Introduction, Leamy et al. (2008) detected a large amount of epistasis using a full genome scan of SNP markers in an F2 population of mice derived from a cross of two inbred strains, and some of the epistatic interactions involved markers on the X chromosome. To date, no study of mouse wheel-running QTL has included markers on the Y chromosome. Molecular imprinting is widespread in the mouse genome (Searle and Beechy ,1978; Cattanach and Kirk, 1985; Cattanach, 1986), and sex-specific molecular imprinting (Hager et al., 2008) could potentially account for the differential heterosis we see between the sexes in the F1 hybrids.

Experimental evidence has shown that both dominance and over-dominance play a role in heterosis, with some involvement of epistasis, although the relative contribution

of each of these mechanisms is still unclear (Birchler et al., 2006; Lippman and Zamir, 2007) and is likely to vary among organisms, strains, and traits. Additionally, epistatic interactions among loci can also play a significant role in heterosis. For example, in an F2 population of mice derived from a cross of two strains exhibiting large differences in wheel running (C57L/J, high active; C3H/HeJ, low active), a full-genome epistasis scan for all possible interactions of QTL between each pair of 20 chromosomes indicated that epistatic interactions contributed an average of 26% of the total genetic variation for the three measures of daily wheel running (total distance, duration, and average speed) (Leamy et al., 2008). As with most other studies of heterosis in rodent behavior (e.g., Bruell ,1964a, b; Lynch et al., 1986; Bult and Lynch, 1996, 2000), the present study provides no evidence as to which mechanism(s) account(s) for the observed instances of heterosis. Nonetheless, our results do indicate that crossing of replicate selected lines can yield offspring that exceed what was an apparent selection limit, as in Bult and Lynch (1996).

Given that heterosis for wheel running was only observed in male hybrids, it raises the interesting possibility that female mice might be closer to a true selection limit as compared with males. This suggests that further selection on a population descended from the hybrids (Bult and Lynch, 2000) might be able to break the limit for males but not females.

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Table 2.1. Statistical comparisons of body mass, wheel running, and organ masses (with body mass as a covariate) separated by sex

		Female				Male	
	7X7 vs. 8X8	Hybrid vs. Purebred	7X8 vs. 8X7		7X7 vs. 8X8	Hybrid vs. Purebred	7X8 vs. 8X7
Body Mass at start of wheel access	F(1,27)=22.79 **P<0.0001(-)**	F(1,27)=3.16 P=0.0869(-)	F(1,27)=0.18 P=0.6754(-)		F(1,28)=8.27 **P=0.0076(-)**	F(1,28)=2.53 P=0.1229(-)	F(1,28)=4.34 P=0.0464(-)
Revolutions/Day	F(1,27)=10.48 **P=0.0032$^{\$}$(+)**	F(1,27)=0.03 P=0.8618$^{\$}$(-)	F(1,27)=0.31 P=0.5853$^{\$}$(+)		F(1,27)=1.93 P=0.1759(-)	F(1,27)=12.23 **P=0.0016(+)**	F(1,27)=1.41 P=0.2457(+)
Minutes/Day	F(1,27)=0.26 P=0.6163(+)	F(1,27)=0.00 P=0.9879(-)	F(1,27)=0.53 P=0.4735(-)		F(1,27)=14.98 **P=0.0006(-)**	F(1,27)=3.27 P=0.0819(+)	F(1,27)=1.58 P=0.2195(+)
Mean Speed	F(1,27)=15.92 **P=0.0005(+)**	F(1,27)=0.03 P=0.8666(-)	F(1,27)=2.30 P=0.1412(+)		F(1,27)=4.54 P=0.0423(+)	F(1,27)=10.09 **P=0.0037(+)**	F(1,27)=0.65 P=0.4263(+)
Max Speed	F(1,27)=14.09 **P=0.0008(+)**	F(1,27)=0.00 P=0.9935(-)	F(1,27)=1.10 P=0.3037(+)		F(1,27)=0.77 P=0.3877(+)	F(1,27)=7.65 **P=0.0101(+)**	F(1,27)=0.22 P=0.6427(+)
Body Mass at dissection	F(1,29)=23.66 **P<.0001(-)**	F(1,29)=5.18 P=0.0305(-)	F(1,29)=2.87 P=0.1012(-)		F(1,28)=15.33 **P=0.0005(-)**	F(1,28)=6.66 **P=0.0154(-)**	F(1,28)=0.82 P=0.3739(-)

Table 2.1. Statistical comparisons of body mass, wheel running, and organ masses (with body mass as a covariate) separated by sex (cont.)

		Female				Male	
	7X7 vs. 8X8	Hybrid vs. Purebred	7X8 vs. 8X7		7X7 vs. 8X8	Hybrid vs. Purebred	7X8 vs. 8X7
Ventricle Mass	F(1,29)=6.59 **P=0.0157(+)**	F(1,29)=10.18 **P=0.0034(-)**	F(1,29)=0.00 P=0.9906(+)		F(1,28)=0.61 P=0.4402(+)	F(1,28)=4.64 P=0.0399(-)	F(1,28)=0.01 P=0.9274(-)
Liver Mass	F(1,29)=3.13 P=0.0875(-)	F(1,29)=3.32 P=0.0789(-)	F(1,29)=0.65 P=0.4263(-)		F(1,28)=11.18 **P=0.0024(-)**	F(1,28)=1.64 P=0.2106(-)	F(1,28)=0.04 P=0.8471(+)
Spleen Mass	F(1,29)=10.62 **P=0.0028(-)**	F(1,29)=3.19 P=0.0845(+)	F(1,29)=1.52 P=0.2274(-)		F(1,28)=25.31 **P<.0001(-)**	F(1,28)=0.00 P=0.9527(+)	F(1,28)=1.13 P=0.2966(+)
Triceps Surae Mass	F(1,29)=21.53 **P<.0001(-)**	F(1,29)=0.35 P=0.5598(-)	F(1,29)=0.88 P=0.3555(-)		F(1,28)=13.76 **P=0.0009(-)**	F(1,28)=0.01 P=0.9327(-)	F(1,28)=0.21 P=0.6521(-)

[$] Full model (#6 as described in Methods) did not converge for female revolutions/day, so results are for a reduced model (#5 in Methods).

All analyses used age as a covariate. Analyses of wheel-running traits also used a measure of wheel freeness (see Methods).

P values significant after controlling for multiple comparisons (see Methods) are in **bold**.
Signs after *P* values indicate direction of effect: for purebreds, minus indicates 7 < 8, plus indicates 7 > 8; for reciprocal hybrids, minus indicates 7x8 < 8x7, plus indicates 7x8 > 8x7; for hybrids vs. purebreds, minus indicates hybrid > purebred, plus indicates purebred > hybrid.

Table 2.2. Least squares means and (standard errors) for body mass, wheel running, and organ masses (corresponding to statistical analyses in Table 1)

	Female					Male			
	7X7	7♂X8♀	8♂X7♀	8X8		7X7	7♂X8♀	8♂X7♀	8X8
Body Mass at start of wheel access (g)	22.40 (0.35)	22.68 (0.56)	22.95 (0.32)	24.75 (0.36)		28.23 (0.48)	28.20 (0.82)	29.20 (0.49)	30.17 (0.52)
Revolutions/ Day	14,607 (994)	12,893 (787)	12,294 (762)	10,878 (619)		9,123 (504)	12,288 (494)	11,257 (700)	10,086 (537)
Minutes/Day	545 (39)	519 (22)	549 (36)	524 (16)		441 (20)	553 (15)	513 (28)	542 (20)
Mean Speed (RPM)	26.71 (1.13)	24.95 (1.51)	22.20 (1.03)	20.87 (0.98)		20.22 (0.79)	22.07 (0.61)	21.25 (0.80)	18.17 (0.62)
Max. Speed (RPM)	43.50 (1.16)	40.84 (1.82)	38.64 (1.06)	36.01 (1.66)		34.82 (0.95)	37.48 (1.13)	36.85 (0.99)	33.57 (1.13)
Body Mass at dissection (g)	22.15 (0.29)	21.68 (0.63)	22.90 (0.34)	24.36 (0.35)		28.80 (0.45)	28.26 (0.84)	29.09 (0.36)	31.65 (0.58)

Table 2.2. Least squares means and (standard errors) for body mass, wheel running, and organ masses (corresponding to statistical analyses in Table 1) (cont.)

	Female					Male			
	7X7	7♂X8♀	8♂X7♀	8X8		7X7	7♂X8♀	8♂X7♀	8X8
Ventricle (g)	0.1262 (.0018)	0.1158 (.0032)	0.1158 (.0014)	0.1192 (.0019)		0.1505 (.0024)	0.1417 (.0041)	0.1421 (.0020)	0.1471 (.0036)
Liver (g)	1.1743 (.0107)	1.1429 (.0318)	1.1738 (.0209)	1.2340 (.0325)		1.5703 (.0258)	1.6153 (.0458)	1.6038 (.0369)	1.7560 (.0493)
Spleen (g)	0.0606 (.0022)	0.0685 (.0025)	0.0729 (.0025)	0.0720 (.0026)		0.0689 (.0025)	0.0805 (.0028)	0.0764 (.0026)	0.0877 (.0027)
Triceps Surae (g)	0.1013 (.0012)	0.1040 (.0028)	0.1069 (.0011)	0.1119 (.0019)		0.1404 (.0018)	0.1441 (.0039)	0.1462 (.0026)	0.1504 (.0020)

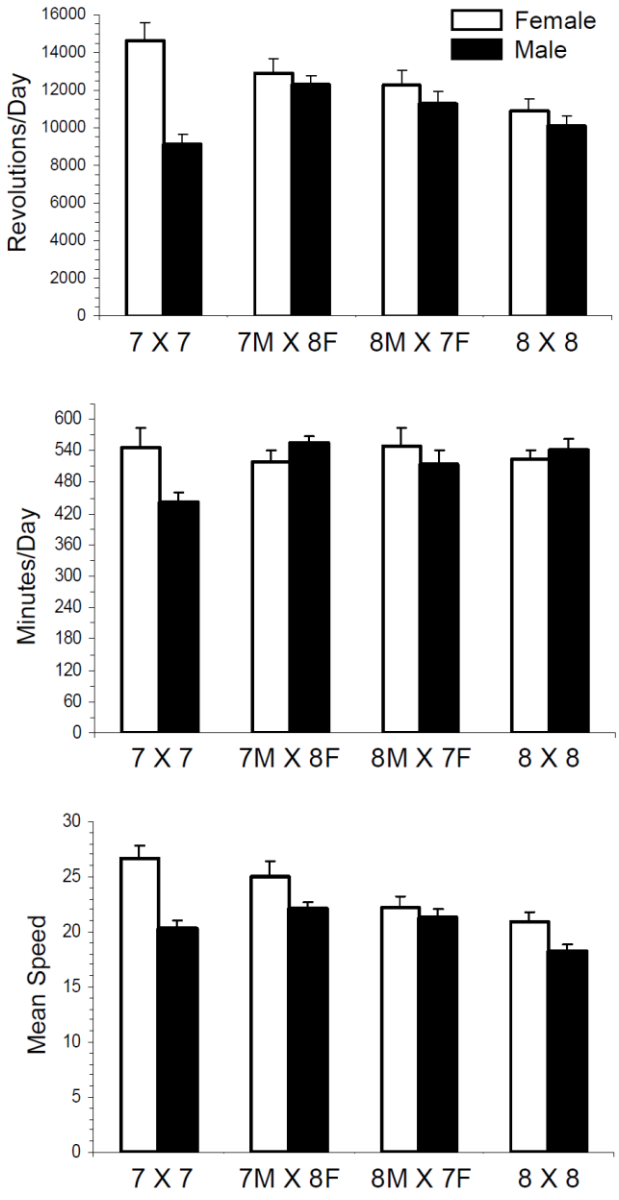

Figure 2.1. Wheel-running activity during days 5 and 6 of a 6-day exposure to wheels (1.12 m circumference) attached to standard housing cages. Values are least squares means + SEs from analysis of covariance models in SAS Procedure Mixed (see text and Table 1 for statistical results). 7 X 7 and 8 X 8 denote purebred mice from two different HR lines bred for high voluntary wheel running (Swallow et al. 1998). Values in between these are for reciprocal crosses. See Table 2 for numerical values.

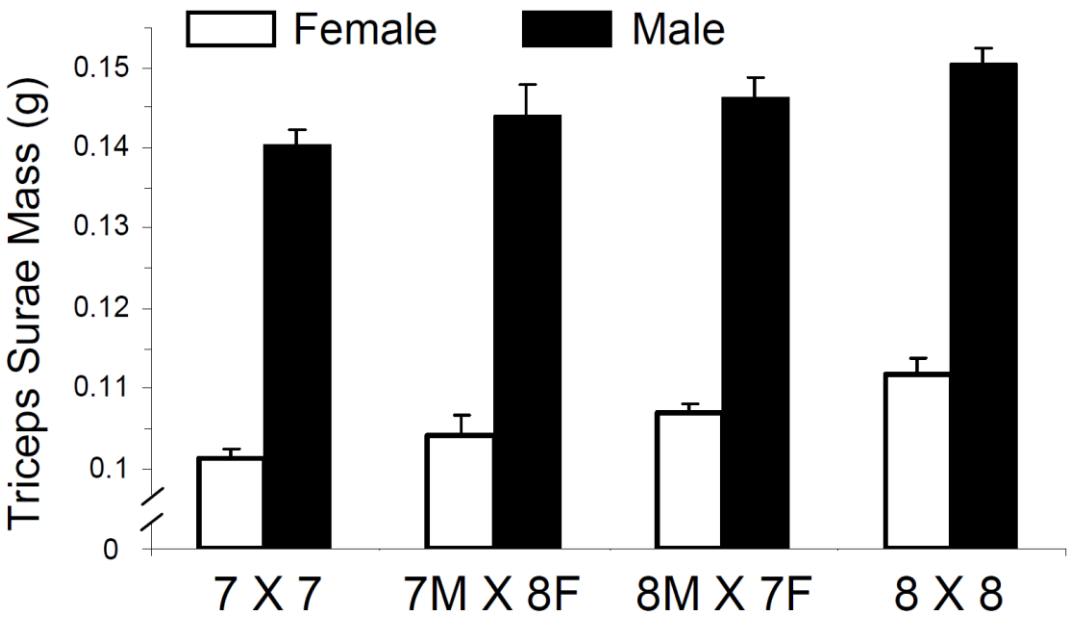

Figure 2.2 Triceps surae muscle mass, adjusted for body mass. Values are least squares means + SEs from analysis of covariance models in SAS Procedure Mixed (see Table 1 for statistical results and Table 2 for numerical values). Note broken Y-axis to emphasize differences among groups. 7 X 7 and 8 X 8 denote purebred mice from two different HR lines bred for high voluntary wheel running (Swallow et al. 1998). Values in between these are for reciprocal crosses.

Chapter 3

Genetic dominance and maternal influences on body mass and wheel running in mice selectively bred for high voluntary wheel running

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Summary

When a trait is under sustained directional selection, alleles with dominance effects in the favored direction are expected to increase in frequency. In addition, parental influences (e.g., non-genetic maternal effects) may be expected to coadapt with the trait under direct selection, at least if the cross-generational genetic covariances are permissive. We tested for net genetic dominance and parental influences in the F_1 of a reciprocal cross between a line of mice bred for high voluntary wheel-running behavior (HR line) and a non-selected control (C) line. At the time of sampling (generation 42), mice from the HR line ran almost 3-fold more revolutions/day than C, and were significantly smaller in body mass. Consistent with previous studies of mice, we found largely additive inheritance of body mass, and a positive maternal influence for both sexes. However, we found strong directional dominance for high revolutions/day, minutes/day, average speed, and maximum speed, in both sexes. We also found positive maternal influence for all wheel measures, wherein F_1 mice from HR dams ran more than those from C dams. These influences were stronger for males than for females, and statistically significant only for males for revolutions/day and minutes/day. This is the first report of positive maternal influences on wheel running in an F_1 population, and could have important implications for understanding the basis of individual variation in levels of voluntary exercise.

Introduction

Quantitative traits, such as body size and most behaviors, generally have complex genetic architectures, with multiple genes contributing to the phenotypic variance within a population. From the perspective of natural or artificial selection, the magnitude of additive genetic effects (typically expressed as the narrow-sense heritability, i.e., additive genetic variance/total phenotypic variance) is of particular importance because it determines the rate of response to selection (Falconer and Mackay, 1996). However, quantitative traits are also influenced by non-additive genetic effects of alleles within and among loci, and by various environmental factors (e.g., Cheverud, 1984; Roff, 2007).

Dominance interactions are perhaps the most common deviation from additivity of allelic effects, and dominant alleles become especially important when considering populations under selection (Fisher, 1930; Crnokrak and Roff, 1995). For example, under directional phenotypic selection, alleles with dominance effects in the favored direction are expected to increase in frequency more rapidly than those with purely additive effects or that are recessive (e.g., Freeman and Herron, 2007, pp. 198-199). When the alleles affecting a trait act in an entirely additive fashion, its "genetic architecture" is relatively stable, but with dominance it can become unstable across generations, even in the absence of selection (de Brito et al., 2005).

When a population has been under directional selection for a particular trait, net directional dominance of contributing alleles should occur in the direction of selection (Broadhurst and Jinks, 1974; Crnokrak and Roff, 1995). Several previous studies of wheel running in crosses of mouse lines have demonstrated net directional dominance for

higher levels of running (Bruell, 1964; Dohm et al., 1994; Lightfoot et al., 2008; Nehrenberg et al., 2009), and Bruell (1964) argued that the heterotic nature of wheel running in crosses of inbred strains suggests that the trait is important to the Darwinian fitness of mice.

The expression of such quantitative traits as locomotor activity is also subject to numerous parental influences. In principle, parental influences can be separated into at least two components, parental "effects" (parental environment) and parental "inheritance" (parental non-autosomal genetic/epigenetic factors) (Wolf and Wade, 2009). Similarities between parent and offspring can arise from either of the above or a combination of such factors (e.g., on mouse open-field behavior, see de Mooij-van Malsen et al., 2009). According to Badyaev and Uller (2009, p. 1169), parental influences "*enable* evolution by natural selection by reliably transferring developmental resources needed to reconstruct, maintain and modify genetically inherited components of the phenotype" (italics in original). Thus, directional selection may also be expected to alter parental influences. In laboratory rodents, maternal influences may be more common than paternal influences, in part because males are typically removed prior to birth of offspring. Thus, one might predict that directional selection would tend to lead to the evolution of positive maternal influences on the trait under selection, at least when the genetic effects in offspring have a positive covariance with the maternal environment and/or non autosomal inheritance (e.g., see Wolf et al., 1998). In any case, identification and study of parental influence is important in understanding the evolution of traits under selection (Badyaev, 2008).

The purpose of the present study was to test for net dominance and parental influences in the F_1 of a cross between a line of mice bred for high voluntary wheel running (HR) and a non-selected control (C) line. Since reaching an apparent selection limit at approximately generation 16, the HR lines have been running, on average, 2.5-3.0-fold more revolutions/day as compared with C lines (Garland, 2003). We hypothesized that we would find both directional dominance and maternal influences that favor high levels of wheel running. Documenting the existence of one or both phenomena could lead to insights regarding the fundamental neurobiological and physiological mechanisms that underlie individual variation in voluntary exercise, which has important implications for both physical and mental health and wellbeing (Nehrenberg et al., 2009; Kelly et al., 2001a,b; Garland et al., in revision). Total revolutions run per day can be broken into components of running duration and mean speed, which have shown different selection responses in males and females (Garland et al., 2010), and so they were analyzed separately by sex. We also examined body mass because it has generally shown a lack of net dominance in previous studies of mice (Chai, 1956, 1957) and because it has decreased as a correlated response to selection in the HR lines (Swallow et al., 1999; Girard and Garland, 2002; Garland et al., 2010).

Materials and Methods

Animals

Full details of the selection experiment can be found in Swallow et al. (1998), but a brief overview is provided here. The original progenitors were 224 outbred, genetically

variable house mice (*Mus domesticus*; Hsd:ICR; Harlan-Sprague-Dawley, Indianapolis, IN, USA). These mice were randomly mated for two generations, then randomly assigned to eight closed lines, each to be maintained with 10 mating pairs in each. Each generation, offspring are weaned at 21 days of age. At 6-8 weeks of age mice are individually housed with access to a Wahman-type running wheel (circumference = 1.12 m) for 6 days. Food (Harlan Teklad, Madison, WI, Rodent Diet [W] 8604) and water are always available *ad libitum*, and photoperiod is 12:12. Daily wheel-running activity is monitored by a computer-automated system at 1-minute increments for six days. For purposes of selection, wheel running is quantified as the mean number of revolutions run on days 5 and 6 of the 6-day test.

In the four HR lines, the highest-running male and female from each of 10 families are selected to propagate the lines to the next generation (i.e., within-family selection). In the four C lines, two males and two females are chosen randomly from each family, wheel testing is administered, and then a subset of these individuals is chosen randomly as breeders. Within all lines, sibling matings are disallowed. Effective population size (Ne) is approximately 35 per line (Swallow et al., 1998).

Mice used for the parents in the current experiment were from generation 42. Twenty-eight males and 28 females were randomly chosen from a single control line (lab-designated line 2) and from a single HR line (lab-designated line 8). Body mass was recorded immediately prior to wheel access (see above). Following wheel testing, a portion of the mice were used as breeders to supply animals for the next generation of the

ongoing selection experiment. Therefore, some of the mice had given birth to one litter prior to being used in the current study.

Breeding and Wheel Testing

Control line 2 females were paired with line 8 HR males, and the reciprocal crosses were also made. Mice were paired randomly, one male to one female, and housed together for 17 days. On the 18th day, males were removed if the female was pregnant based on visual inspection; otherwise, males were removed when pregnancy was evident. As in our routine breeding protocol, pregnant dams were then given a breeder diet (Harlan Teklad, Madison, WI, Mouse Breeder Diet [S-2335] 7004) until weaning of their pups at 21 days of age. A total of 519 F_1 pups were weaned, then culled to 392 by randomly choosing two males and two females from each litter, if available. F_1 mice were housed 4 per cage, in same-sex groups, and by cross type (C X HR or HR X C). At approximately 7 weeks of age, they were wheel-tested in two batches, using the routine 6-day protocol described above. Three hundred and seventy five F_1 mice completed wheel testing with acceptable data (e.g., no wheel malfunction). These data were analyzed in conjunction with the values for their parents, which had been obtained during testing of the previous generation. Following previous studies, we analyzed body mass at the start of wheel trials and four aspects of running on days 5 and 6: the mean number of revolutions run on days 5 and 6 (which is the selection criterion: Swallow *et al*., 1998), the mean number of 1-minute intervals with at least one revolution recorded (an index of the amount of time spent running), the average speed when running (total

revolutions divided by intervals run), and the mean maximum speed achieved (average of the two daily highest 1-minute intervals)(e.g., Girard and Garland, 2002; Garland et al., 2010; Kelly et al., 2010a, 2010b).

Statistical analysis

The MIXED procedure in SAS (version 9.1; SAS Institute, Cary, NC, USA) was used to apply analysis of covariance (ANCOVA) models with three specified *a priori* contrasts among groups (C parent vs. HR parent, Expected midpoint of C and HR parents vs. F_1, F_1 from C dam vs. F_1 from HR dam). In addition, for completeness, we present *a priori* contrasts of the C parent vs. F_1 and of the HR parent vs. F_1 in Online Supplemental Table 1. In preliminary analyses, the sex-by-group interaction was significant for some traits (see Results), so the sexes were analyzed separately. Age and a measure of wheel freeness to rotation were used as covariates for all wheel traits (wheel freeness is the number of revolutions after acceleration by a motor to a constant velocity [Girard and Garland, 2002; Garland et al., 2010]); only age was used for body mass. Family, nested within group, was included as a random effect in all analyses. To allow for different variance among groups (e.g., due to genetic segregation), we specified separate estimation of the among-family and residual variances components for each group, so each statistical model included up to eight covariance parameter estimates (in some cases, not all eight were estimable).

Some of the traits showed skewness of the residuals of ~0.5 or greater in magnitude, so they were also analyzed after a transformation that reduced skewness of

the residuals. In no case did the analysis of transformed data make a change in the significance of the P value versus the *a priori* criterion of P = 0.05 (see Table 1). Because we performed a number of tests on closely related data, our Type I error rate for the entire experiment may exceed the nominal 5% alpha level. Therefore, we performed a false discovery rate (FDR) analysis using the QVALUE package (Version 1.1; Storey 2002) for R (Version 2.8.0; R Core Development Team 2008), allowing for 5% false significant results (FDR = 0.05). For these analyses, we used the P values from the non-transformed data for all traits. Based on analysis of the 30 P values presented in Table 1, those < 0.04 can be considered significant, and we emphasize those results. We also present least squares means and associated standard errors from SAS Procedure Mixed.

Results

The sex-by-group interaction was significant for body mass ($F = 8.96$, d.f. = 3,69, $P < 0.0001$) and intervals/day ($F = 8.77$, d.f. = 3,69, $P < 0.0001$), but not for revolutions/day ($F = 0.47$, d.f. = 3,69, $P = 0.7067$), mean speed ($F = 0.68$, d.f. = 3,69, $P = 0.5658$) or maximum speed ($F = 0.44$, d.f. = 3,69, $P = 0.7252$). Hence, as noted in the Methods, subsequent analyses were split by sex.

As expected from previous studies of all four C and all four HR lines (e.g., Swallow et al., 1999, Girard et al., 2002), HR mice were smaller than C, although this was only statistically significant for males (Tables 1, 2). Body mass of F_1 mice was not statistically different from the midpoint of HR and C for either sex (Table 1). For both

sexes in the F_1, mice from C dams were significantly larger than those from HR dams (Table 1, 2).

For both sexes, wheel-running revolutions/day of F_1 individuals was similar to that of the HR-line parent and significantly greater than the HR-C midpoint (Tables 1, 2). In the F_1, mice from an HR dam ran more than those from a C dam (Figure 1), although this difference was statistically significant only for males (Table 1).

The increased daily running distances of HR mice were a function of significantly increased (Table 1) average running speeds for both sexes (+110% in females, +87% in males; Table 2), and also amount of time spent running, especially for males (for males +75%, for females +22%). For both sexes, the F_1 resembled the HR line for both speed and duration of wheel running (Table 2).

For F_1 males, those from HR dams ran significantly more revolutions/day because they ran more minutes per day, but not faster, as compared with those from C dams (Tables 1, 2). For F_1 females, those from HR dams showed a non-significant trend for higher revolutions/day, based on higher mean (and maximum) running speeds.

Discussion

Many previous studies of mice have found largely additive inheritance of body mass (e.g., Chai, 1956, 1957; Baker, 1976; Eisen and Prasetyo, 1988; Dohm et al., 1994). Consistent with these results, we found that the F_1 were generally intermediate in body mass (Tables 1, 2). Previous studies of mice have also reported positive maternal influences on body mass (e.g., Chai, 1956, 1957; DeFries et al., 1967; White et al., 1968;

Cowley et al., 1989; Rhees et al., 1999). Again in agreement with these previous studies, we found that mice from Control-line dams were significantly heavier than those from HR dams, for both sexes (Tables 1, 2).

For total daily wheel running, we observed net dominance in the direction of the HR parent. Dominance for high wheel running has also been observed in crosses of inbred strains (Bruell, 1964; Lightfoot et al., 2008), in a cross of wild house mice with Hsd:ICR mice that formed the base population for the present selection experiment (Dohm et al., 1994), and in a cross of the HR line used here (lab designation #8) with the inbred strain C57BL/6J (Nehrenberg et al., 2009; Kelly et al., 2010b). Thus, the net dominance of genes that favor high wheel running seems to be a rather general feature of the genetic architecture of house mice. Considering the components of wheel running separately, F_1 mice ran 1.43-fold more minutes/day and at an average speed 1.87-fold faster than their Control-line parents (Table 2). In both cases, net dominance was again in the direction that favors high values of wheel running. Overall, our results support the concept that net directional dominance is an indication that a trait has been under selection in that direction (e.g., Bruell, 1964; Crnokrak and Roff, 1995; Crusio and Schmitt, 1997; Garland and Kelly, 2006; references therein).

Inspection of Table 2 indicates that F_1 mice from HR dams always ran more total revolutions (Figure 1), more minutes/day, and at higher mean and maximum speeds, as compared with mice from Control-line dams. However, the differences were only statistically significant for males for revolutions/day and minutes/day (Table 1). This seems to be the first report of a positive maternal influence on voluntary wheel running

within an F_1 generation of mice (see Kelly et al., 2010a for a positive maternal influence in the F4 generation). In a previous study of crosses between the HR line studied here (lab designation #8) and another HR line (lab designation #7), we found sex-dependent line differences for all aspects of wheel running (see also Garland et al., 2010), but in no case did we find evidence for a parental influence on these traits in the F_1 offspring (Hannon et al., in revision). Few other studies have tested for parental influences on aspects of locomotor activity in rodents. In a diallel cross of six lines from a replicated selection experiment, Halcomb et al. (1975) found little evidence of parental influences on open-field behavior. In an earlier study using ovarian transplantation with inbred lines, DeFries et al. (1967) also found little evidence for parental influences on open-field behavior, but relatively large influences on body mass.

The present study provides no direct information as to whether the apparent maternal influences are a result of genetic parental inheritance, environmental effects, or both. It is important to differentiate between these possibilities because they can have different evolutionary consequences. Although environmental maternal effects would increase the phenotypic variation within the next generation, this influence would only continue through many generations if there were no change in environment. However, genetic maternal inheritance would likely maintain influence in changing environments and have a direct influence on the response to selection (Wolf and Wade, 2009). If the influence of genetic maternal variance (and covariance between direct genetic inheritance and maternal genetic inheritance) is not included in heritability studies, then the result could change the estimate of the potential for the trait to respond to selection (Wilson et

al., 2005). It is also important to note that there can be an interaction between epigenetic parental inheritance and the maternal environment (Hager et al., 2009). In any case, genetic parental inheritance can have a large influence on a population under selection. If a trait under selection exhibits a genetic parental inheritance component, then the rate of response to selection can change across generations (Kirkpatrick and Lande, 1989).

Genetic maternal inheritance can be caused by several factors, the most common being the maternal mitochondrial genome. Phenotypic changes caused by mitochondrial genes are commonly viewed as maternal effects because generally only the maternal mtDNA is present in zygotes (Birky, 1976). Many genes within the mitochondrial genome can significantly impact the phenotype of an individual, and one can hypothesize that this would be particularly true for wheel running, which can entail high aerobic metabolic rates, especially in the HR mice (e.g., see Rezende et al., 2005, 2009). Additionally, mitochondrial and nuclear genes can have epistatic interactions that cause variation in gene expression (Gusdon et al., 2007; Yang et al., 2008).

A prime candidate for the sex-differential portion of the maternal influences observed in the present study, with F_1 males showing stronger effects (see Tables 1, 2, Figure 1), would be genes located on the X chromosome. As males receive their only X chromosome from their female parent, those from HR dams would have an HR X chromosome contributing to their phenotype, whereas females would receive X chromosomes from both parents. Following random inactivation in females, where one X chromosome is randomly inactivated during embryonic development (Starmer and Magnuson, 2009), half would have an active HR X chromosome, while the other half

would have an active C X chromosome. Thus, at the level of the population mean, F_1 males should show a stronger maternal X influence as compared with F_1 females. Two studies have examined genetic markers on the X chromosome for associations with wheel running in mice, and neither identified statistically significant markers on the X (Lightfoot et al., 2008; Kelly et al., 2010b), although Leamy et al. (2008) detected a large amount of epistasis using a full genome scan of SNP markers in the F_2 population from Lightfoot et al. (2008), with some of the epistatic interactions involving markers on the X chromosome.

Beyond these possibilities, parental influences can result from such epigenetic processes as paramutation (Chandler, 2007) and genetic imprinting (da Rocha et al., 2008). All of these phenomena could be involved in the positive maternal influence that we observed. Additionally, there is evidence that these genetic parental effects can be sex dependent (Hager et al., 2008).

Environmental maternal effects in mammals can be caused by various traits expressed in the mother, including body size, nesting behavior, milk quality, and locomotor activity, often times having a developmental impact on offspring, thus causing physiological and behavioral changes to the offspring's phenotype (Koenig et al., 1988; Meek et al., 2001; Parsons et al., 2005). Because we removed sires before birth of pups, one might imagine that paternal influences should be negligible. However, it is possible that being housed for 19 days with a "hyper" male could have an influence on the dam that would end up affecting her offspring.

Future studies to elucidate the causes of the apparent maternal influences on

wheel running, including determining the genetic vs. environmental components, could follow various paths. By removing non-genetic variation that is known to be caused by environment, one can gain a better understanding of parental genetic influences (see Wilson et al., 2005). Analysis of environmental components could include post-natal cross-fostering, analysis of milk quality, and measurement of home-cage activity by dams both pre- and post-partum. Additionally, more investigation would be helpful in understanding the differences between the sexes, particularly when total wheel running is broken down into its respective components (speed and duration).

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Table 3.1. Analysis of covariance with a priori contrasts for differences in means between Control, High Runner, and F_1 mice. P values in **bold** are significant after use of the False Discovery Rate procedure (using values for non-transformed data) to control the table-wide Type I error rate at 5% (P<0.04).

Trait	Sex	N	Trans-form	Resid-ual Skew	Control vs. High Runner Line	Midpoint of Control and HR vs. F_1	F_1 from Control Dam vs. F_1 from High Runner Dam
Body Mass	Female	240	none	-0.258	$F(1,69)=1.84$ $P=0.1789$(+)	$F(1,69)=0.31$ $P=0.5795$	$F(1,69)=5.63$ $P=$**0.0204**(+)
	Male	243	none	0.078	$F(1,71)=20.60$ $P<$**0.0001**(+)	$F(1,71)=0.42$ $P=0.5176$	$F(1,71)=28.26$ $P<$**0.0001**(+)
Revolutions/Day	Female	240	none	0.464	$F(1,69)=43.18$ $P<$**0.0001**(-)	$F(1,69)=22.26$ $P<$**0.0001**	$F(1,69)=3.03$ $P=0.0861$(-)
	Female	240	^0.5	0.016	$F(1,69)=48.26$ $P<$**0.0001**(-)	$F(1,69)=30.31$ $P<$**0.0001**	$F(1,69)=2.86$ $P=0.0952$(-)
	Male	243	none	-0.028	$F(1,71)=81.77$ $P<$**0.0001**(-)	$F(1,71)=32.36$ $P<$**0.0001**	$F(1,71)=7.45$ $P=$**0.0080**(-)
1-minute Intervals/Day	Female	240	none	0.282	$F(1,69)=4.68$ $P=$**0.0340**(-)	$F(1,69)=7.11$ $P=$**0.0096**	$F(1,69)=1.76$ $P=0.1895$(-)
	Male	243	none	-0.553	$F(1,71)=54.86$ $P<$**0.0001**(-)	$F(1,71)=26.26$ $P<$**0.0001**	$F(1,71)=8.02$ $P=$**0.0060**(-)

Table 3.1. Analysis of covariance with a priori contrasts for differences in means between Control, High Runner, and F_1 mice. (cont.)

Trait	Sex	N	Transform	Residual Skew	Control vs. High Runner Line	Midpoint of Control and HR vs. F_1	F_1 from Control Dam vs. F_1 from High Runner Dam
	Male	243	^1.8	0.061	$F(1,71)=49.94$ $P<$**0.0001**(-)	$F(1,71)=17.23$ $P<$**0.0001**	$F(1,71)=9.47$ $P=$**0.0030**(-)
Mean Speed	Female	240	none	0.728	$F(1,69)=77.27$ $P<$**0.0001**(-)	$F(1,69)=23.08$ $P<$**0.0001**	$F(1,69)=3.09$ $P=0.0833$(-)
	Female	240	^0.1	0.022	$F(1,69)=89.89$ $P<$**0.0001**(-)	$F(1,69)=45.38$ $P<$**0.0001**	$F(1,69)=1.74$ $P=0.1921$(-)
	Male	243	none	-0.125	$F(1,71)=66.30$ $P<$**0.0001**(-)	$F(1,71)=33.58$ $P<$**0.0001**	$F(1,71)=0.29$ $P=0.5903$(-)
Max Speed	Female	240	none	0.911	$F(1,69)=86.94$ $P<$**0.0001**(-)	$F(1,69)=20.24$ $P<$**0.0001**	$F(1,69)=4.29$ $P=0.0420$(-)
	Female	240	^0.1	0.467	$F(1,69)=109.37$ $P<$**0.0001**(-)	$F(1,69)=41.19$ $P<$**0.0001**	$F(1,69)=2.56$ $P=0.1138$(-)
	Male	243	none	0.112	$F(1,71)=69.01$ $P<$**0.0001**(-)	$F(1,71)=29.14$ $P<$**0.0001**	$F(1,71)=3.49$ $P=0.0660$(-)

Note: + after P values indicates first group > second group, - indicates converse. Age was a covariate for analysis of body mass; age and a measure of wheel freeness were covariates for wheel-running traits.

Table 3.2. Least square means and associated (standard errors) corresponding to analyses shown in Table 1 for untransformed data.

Trait	Sex	Control	F_1 from Control Dam	F_1 from High Runner Dam	High Runner
	N	27 Female, 27 Male	93 Female, 94 Male	94 Female, 94 Male	26 Female, 28 Male
Body Mass (g)	Female	24.46 (0.69)	24.10 (0.41)	22.97 (0.22)	23.20 (0.63)
	Male	31.51 (0.46)	30.93 (0.36)	28.61 (0.24)	28.54 (0.48)
Revolutions/Day	Female	4,137 (363)	8,922 (309)	9,637 (260)	9,828 (793)
	Male	3,017 (324)	8,168 (191)	9,126 (283)	9,646 (660)
1-minute Intervals/Day	Female	494.7 (29.9)	578.6 (25.2)	583.0 (11.4)	604.3 (10.6)
	Male	332.3 (24.1)	530.8 (9.5)	571.1 (9.8)	581.5 (23.8)
	Male	8.67 (0.65)	15.41 (0.36)	15.71 (0.40)	16.19 (0.66)
Max Speed (revs/min)	Female	16.69 (0.68)	27.03 (0.57)	28.70 (0.55)	31.09 (1.40)
	Male	17.47 (0.99)	27.46 (0.53)	28.98 (0.59)	29.80 (1.12)

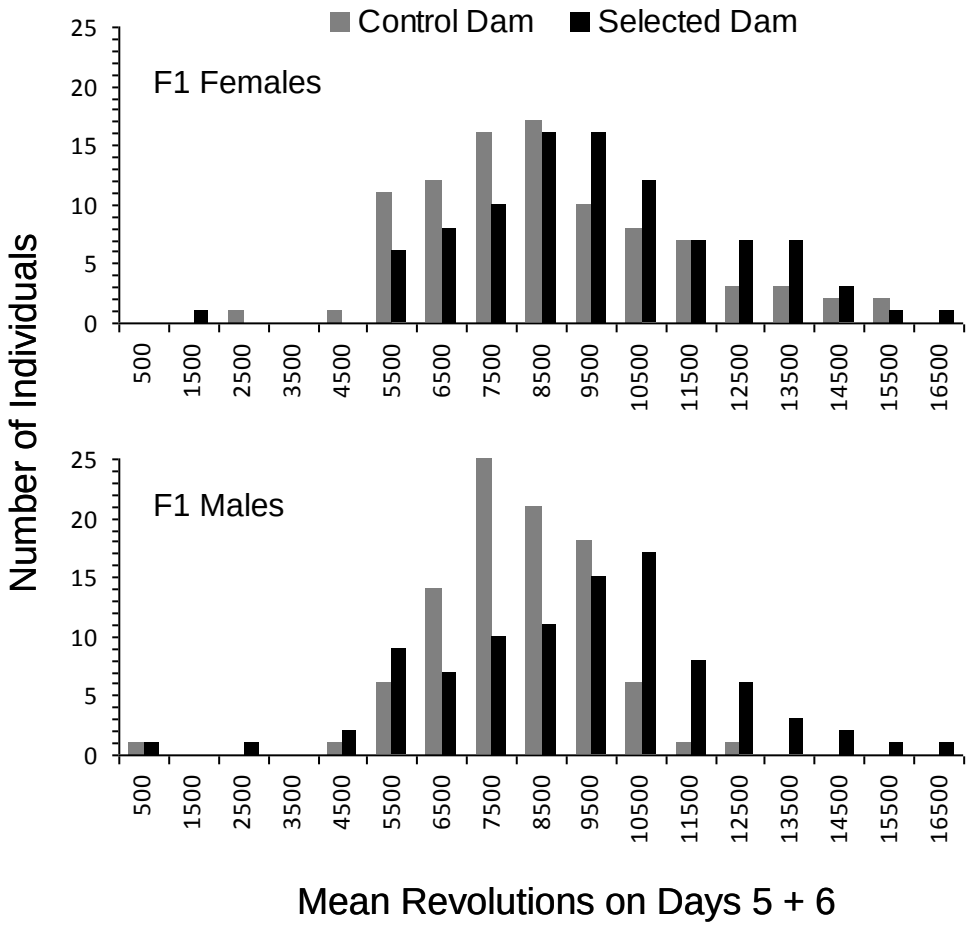

Figure 3.1. Average revolutions run (raw data, non-transformed) by females (top panel) and males (bottom panel) of the F_1 generation. For males, F_1 individuals from crosses of an HR dam with a Control sire ran significantly more (P = 0.0080) than those from the reciprocal cross; for females, the trend was similar, but not statistically significant (P = 0.0861; see Table 1 for statistical comparisons and Table 2 for least square means and sample sizes).

Chapter 4

Estimating the number of genes contributing to the high voluntary running trait in selectively bred house mice

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Summary

Estimating the number of genes that contribute to the expression of a quantitative trait is important for the study of mechanisms of heredity, evolution, and how these traits express themselves within populations. Voluntary physical activity is a highly complex behavioral phenotype, comprised of many lower-level traits that are influenced by alleles segregating at multiple loci.

The purpose of the present study was to estimate the number of genetic loci contributing to the high voluntary wheel running (HR) phenotype in populations created by crossing one HR line with one non-selected Control line. Using wheel-running measurements from parental, F1, F2, and backcross generations, the equations in Fenster and Ritland (1994) were used to produce estimates of the minimum number of independently segregating genetic factors for both body mass and wheel running (including its components, i.e., mean speed of running and duration of running). Based on analysis of the F2generation, our data for total wheel running (revolutions/day) yield an estimate of 11 genes for males and 10 genes for females. These results support the hypothesis that voluntary wheel running trait is polygenic. Estimating the number of genes that contribute to the HR phenotype is one of the first steps to understanding the complex genetic architecture of voluntary exercise.

Introduction

Estimating the number of genes that contribute to quantitative phenotypic expression is important for the study of mechanisms of heredity, evolution, and how these traits express themselves within populations. The rate and ultimate amount of genetic (evolutionary) adaptation in response to selection depend, in part, on the number and mode of action of loci and alleles that affect traits under selection (Maynard Smith, 1983; Barton and Charlesworth, 1984; Coyne, 1992). For example, the number of genes involved in the inheritance of quantitative characters influences the limits to recurrent directional selection (Park, 1977). The simplest and least expensive methods for estimating this number involve statistical analysis of the means and variances of populations that differ in mean phenotype as well as offspring from crosses of these divergent populations (Zeng et al., 1990). These methods were first proposed in 1921 (Castle, 1921a, b) and allow estimation of the minimum number of independently segregating genetic factors that contribute to the phenotype difference between the parental populations.

The original estimating equations make several assumptions (Castle, 1921a, b). First, the two parental populations must be divergently selected so that all genetic loci contributing to the trait are fixed for the alleles that have opposite effects on the phenotype. Second, there can be no genetic linkage. Third, all genetic factors that contribute to the phenotype must have effects of equal magnitude. In addition, there can be no dominance effects occurring among alleles at any loci influencing the trait. Lastly, as was pointed out later (Wright, 1968), no epistasis among loci can occur.

Given that very few populations will ever meet all assumptions, as time progressed; there have been many adjustments to the original equation of Castle (1921b). Sewall Wright added two backcross generations to the experimental procedure to help quantify the positioning of the parental classes and relax the assumption that they needed to be divergently selected to opposite extremes (Wright, 1968). Lande (1981) then adjusted the equation so that it could be applied to populations that have not reached fixation of all alleles contributing to a phenotype. Further alterations are presented in Zeng (1992).

Castle (1921a) presented examples involving seed weight in corn and body weight in rabbits, and since then the Castle/Wright method has been used to estimate the number of genes for various phenotypes in various species. Examples in plants include tomato weight (Powers, 1942) and percent oil in a kernel of maize (Sprague and Brimhall, 1949). Animal examples include fish eye diameter (Wilkens, 1971), *Drosophila* head shape (Val, 1977; Templeton, 1977), and human skin color (Harrison and Owen, 1964). For other examples, see Zeng (1992) and Roff (1997).

One other adjustment to the equation was made by Fenster and Ritland (1994). Their equation was chosen for the present experiment because not only does it incorporate the Wright and Lande adjustments, but it also corrects for dominance effects among alleles at loci contributing to the trait. In previous studies of mice, including a study of the F1 generation of the mice used for this experiment, wheel-running activity has been shown to have a high net dominance toward high activity (Bruell, 1962; Lightfoot et al., 2008; Dohm et al.,1994; Nehrenberg et al., 2009; Kelly et al., 2010b,

Chapter 3). Therefore, relaxing the assumption of no dominance removed a major violation that is present in the populations of mice used for this study.

The purpose of the present study was to estimate the number of genetic loci contributing to the high voluntary wheel running (HR) phenotype. Knowledge of the number of independently segregating loci that contribute to this trait could lead to insights regarding the fundamental neurobiological and physiological mechanisms that underlie individual variation in voluntary exercise, which has important implications for both physical and mental health and wellbeing (Nehrenberg et al., 2009; Kelly et al., 2001a, b; Garland et al., 2011). Total revolutions run per day can be broken into components of running duration and mean speed, which have shown different selection responses in males and females (Garland et al., 2010; Chapter 2), and so they were analyzed separately by sex. We also examined body mass because it has decreased as a correlated response to selection in the HR lines (Swallow et al., 1999; Girard and Garland, 2002; Garland et al., 2010).

Materials and Methods

Selection experiment

Full details of the selection experiment are provided elsewhere (Swallow et al., 1998), and only a brief overview is provided here. The original progenitors were 224 outbred, genetically variable house mice (*Mus domesticus*) from the Hsd:ICR strain (Harlan-Sprague-Dawley, Indianapolis, IN, USA). These mice were randomly mated for

two generations, and then randomly assigned to eight closed lines (10 mating pairs in each). In each generation, offspring are weaned at 21 days of age. At 6-8 weeks of age, mice are individually housed with access to a Wahman-type running wheel (circumference = 1.12 m) for 6 days. Food (Harlan Teklad, Madison, WI, Rodent Diet [W] 8604) and water are always available *ad libitum*, and photoperiod is 12:12. Daily wheel-running activity is monitored by a computer-automated system at 1-minute increments for six days. For purposes of selection, wheel running is quantified as the total number of revolutions run on days 5 and 6 of the 6-day test.

In the four HR lines, the highest-running male and female from each family are selected to propagate the lines to the next generation (i.e., within-family selection). In the four C lines, breeders are randomly chosen from each family. Within all lines, breeders are randomly paired, excluding sibling mating.

The parental cohort in the current experiment was from generation 43. From a total of 14 families from a single control line (lab-designated line 2), we chose 28 males and 28 females to use as breeders. Similarly, from a total of 14 families from a single HR line (lab-designated line 8), we chose 28 males and 28 females. Body mass was recorded both when mice were allowed wheel access and when they were removed from wheel access, six days later. Following wheel testing, a portion of the mice were used as breeders to supply animals for the next generation of the ongoing selection experiment. Therefore, some of the mice had given birth to one litter prior to being used as breeders in the current study.

Breeding

Control line 2 females were paired with HR line 8 males, and the reciprocal crosses were also made. Mice were paired randomly, one male to one female, and housed together for 17 days. On the 18th day, males were removed if the female was pregnant based on visual inspection; otherwise, males were removed when pregnancy was evident. As in our routine breeding protocol, pregnant dams were then given a breeder diet after removal of the male (Harlan Teklad, Madison, WI, Mouse Breeder Diet [S-2335] 7004) until weaning of their pups at 21 days of age. A total of 519 F_1 pups were weaned, and then culled to 392 by randomly choosing two males and two females from each litter, if available. F_1 mice were housed 4 per cage, in same-sex groups, and by cross type (HR male X C female or C male X HR female). At approximately 7 weeks of age, they were wheel-tested in two batches, using the routine 6-day protocol described above. Three hundred and seventy five F_1 mice completed wheel testing with acceptable data (e.g., no wheel malfunction)

Seven days after wheel testing, F1 individuals were paired in one of 3 ways. One hundred thirty two pairs of F1 mice produced 479 F2 offspring. Fifty three pairs of F1 X C mice produced 266 BC1 offspring. Fifty one pairs of F1 X HR mice produced 201 BC2 offspring. Offspring number represents offspring that survived wheel testing with acceptable data (e.g., no wheel malfunction).

Pairing was done randomly within lineage, one couple per cage. On day 18, sires were removed from the cage if the dam appeared pregnant; otherwise they remained with the dam until pregnancy was evident. Upon removal of the sire, pregnant dams were

given breeder chow. When pups were 21 days old, they were weaned from their dam and placed 4 per cage, in same-sex groups, and by cross type. When approximately seven weeks of age, all BC and F2 mice were wheel tested in 5 batches. Mice were assigned to a batch based on age (oldest mice were tested first) to minimize age variation.

Statistical Analysis

As mentioned in the Introduction, the equations given in Fenster and Ritland (1994) were used to calculate the minimum number of independently segregating loci between these two lines of mice. Following previous studies, we did this for body mass at the start of wheel trials and for the following wheel traits based on days 5 and 6: the mean number of revolutions run (which is the selection criterion: Swallow et al., 1998), the mean number of 1-minute intervals with at least one revolution recorded (an index of the amount of time spent running), the average speed when running (revolutions divided by intervals run), and the mean maximum speed achieved (average of the two daily highest 1-minute intervals)(e.g., Girard and Garland, 2002; Garland et al., 2010; Kelly et al., 2010a, b). Due to the known sex-specific responses to selection that have occurred (Garland et al., 2010) and a significant sex-by-group interaction in preliminary analyses, all analyses were split by sex. Age was used as a covariate for analyses of body mass. Age and a measure of wheel freeness were used as covariates in analyses of wheel traits.

When using the F2 generation for estimation, the equation below was used, which is equation #2 in Fenster and Ritland (1994):

$$Ne= ((2+ (D/A)^2) (U_{p1}-U_{p2})^2-C)/ (16S)$$

U_{p1} and U_{p2} are the parental means. The dominance ratio (D/A) is the ratio of dominance to additive effects, estimated as $(2(U_{f2}- E_{f2}))/ (U_{p1}-U_{p2})$. E_{f2} is calculated as $(U_{p1}+U_{p2})/2$. This is an estimate of the average of the parental means in the absence of dominance. C is a correction factor equal to the statistical variance and is calculated via $(2+ (D/A)) (U_{p1}-U_{p2})$. Finally, S is segregational variance. This is normally found by subtracting the F1 variance, which is assumed to be entirely environmental, from the variance of the generation in question. For our studies, we chose to calculate this by including data from both parents and the F1 to average the biases due to the dependence of environmental variance on heterozygosity (Wright, 1968). Therefore, our S was equal to $V_{f2}-(1/4\ V_{p1} +1/2\ V_{f1} +1/4\ V_{p2})$.

When the backcrosses were examined, the following equation was then used, which is equation #4 in Fenster and Ritland (1994):

$$Ne= ((1+ (D/A)^2) (U_{p1}-U_{p2})^2-C)/ (16S)$$

Where the dominance ratio (D/A) is now: $(4(U_{b1}-E_{b1}))/ (U_{p1}-U_{p2})$ when data from mice produced from the backcross to the control parent group were used in the analysis, and $(4(U_{b2}-E_{b2}))/ (U_{p2}-U_{p1})$ when data from mice produced from the backcross to the HR parent group were used. E_{b1} was calculated as $(3/4)\ U_{p1}+ (1/4)\ U_{p2}$, while E_{b2} was $(3/4)\ U_{p2}+ (1/4)\ U_{p1}$.

Preliminary analyses indicated a significant correlation between the mean wheel running measurement and the variance within groups. Therefore, data were transformed to eliminate this correlation. Transformations were done by raising the trait to a power

ranging from 0.025 to 3.0 in increments of 0.005. The power that altered the correlation to a value closest to 0 was chosen for continued analysis (see Table 1).

Results

Parental, F1, F2, and backcross means for the raw measurements of each trait are given in Table 1, while the raw variances are given in Table 2 (see also Figures 1, 2). As noted in the Materials and Methods section, data transforms were required to minimize any mean-variance correlation. In some cases an appropriate transform could not be found, so the calculation was not conducted. The transform used, the new mean-variance correlation, and the resultant means for each trait are given for Parental, F1, F2, and Backcross generations in Table 3, while the transformed variances are given in Table 4 (see also Figures 3, 4). Means of the F1, F2, and both backcrosses for body mass were intermediate between the two parental lines. F1, F2, and both backcrosses showed higher means than the Control parent for all running components, while the backcross to HR females showed higher revolutions per day than the HR parent (Figures 1, 2, 3, 4). F1, F2, and Backcross to HR female mice all showed higher intervals per day than the HR parent. Male Backcross to HR mice exceeded Parental HR mean speed and maximum speed. All running traits were statistically more variable in Parental HR mice than control mice, except for time spent running. F1, F2, and both backcross generations had intermediate variances between the two parental lines, except for a higher variance for male Backcross to HR mice in terms of mean speed. Male F1, F2, and Backcross to HR mice all had lower variance in time spent running than either parental group.

Estimates of the dominance ratio (see Materials and Methods for formula) for untransformed values using the equations above are presented in Table 5, with values for transformed data presented in Table 6. In a previous study, a high degree of net dominance was observed in the F1 generation of this experiment (Chapter 3). Dominance ratio estimates show net dominance for all wheel running traits in F1, F2, and both backcross generations.

Estimates of the total number of independently segregation loci, using both non-transformed and transformed data, for body mass and all wheel running components are presented in Table 7. Estimations that resulted in a negative number are listed as undefined. For revolutions per day, we obtained an estimate of 5 loci for both males and females when using untransformed data. After transformation, we see a slight sex difference, with males estimated to have 11 genes and females 10.

Discussion

Many of the gene number estimates derived from this experiment were negative (Table 7). In general, these impossible estimates occur because the pattern in the data (see Figures 1-4) does not closely resemble the expected (assumed) "triangle" (e.g., see Figure 1 for theoretical expectations and Figure 2 for tomato fruit weight Lande, 1981). Theoretically, the variances of the two parental groups and of the F1 should be equal, and the mean of the F1 should be exactly intermediate between the means of the two parental means (i.e., no dominance exists). The mean of the F2 should be similar to that of the F1, but the variance should be much greater. The backcross means should be intermediate to

the parentals and F1, while the backcross variances should be intermediate to the parentals (and F1) and the F2.

However, we were able to obtain positive numbers for the minimum number of segregating loci that contribute to the difference between Control line 2 and HR line 8 for total wheel running, our main trait of interest. The number of segregating loci differs slightly between the sexes (11 male, 10 female) for transformed data, but it is doubtful these estimate would be statistically different if estimates of the associated standard errors were computed (see Fenster and Ritland, 1994). In any case, we have strong evidence that wheel running is polygenic, as would be expected from first principles of complex traits and as indicated by recent QTL mapping studies (Lightfoot et al., 2008; Nehrenberg et al., 2010; Kelly et al., 2010b).

Due to violation of several of the assumptions (see Introduction) concerning the equations used (see Materials and Methods), our estimates are likely to underestimate the true number of independently segregating loci contributing to the HR - C differences in the wheel-running traits. For instance, it is known that genetic linkage is abundant in the mouse genome (Mouse genome sequencing consortium, 2002). Likewise, in a mouse F2 population derived from an original intercross of two strains that exhibited large differences in wheel running, the results from a full genome epistasis scan showed that epistatic interaction contributed, on average, 26% of the genetic variation in daily voluntary wheel-running measurements, including daily distance, duration, and speed (Leamy et al., 2008). In addition, it is unlikely that all loci have equal effects on wheel running in our mice. At least one Gene of Major Effect (GOME) for hindlimb muscle

mass has been detected segregating within three of the eight lines of the selection experiment (Garland et al., 2002; Hannon et al., 2008; Nehrenberg et al., 2010). Even though this GOME was not detected in the lines used for this experiment, it is possible that others not yet identified are within the two specific lines used. Overall, the violations of all the assumptions listed above would lead to an underestimate of the number of genes contributing to the HR phenotype.

In a previous study, reciprocal crossing of the parental groups showed a significant positive maternal effect on wheel running in the F1 generation (Chapter 3). Individuals from HR dams ran more than those from Control dams. Any parental effect (e.g., non-genetic maternal effect) in the F1 generation would increase the variance of the F1 group, which is assumed to show only environmental variance, resulting in a decrease in the estimated number of genes (Fenster and Ritland, 1994, p. 428). However, analysis of the F2 and backcrosses in our study showed no statistically significant parental effects (results not shown).

One thing that is rarely mentioned in terms of this sort of gene number estimation is the inclusion of the mitochondrial genome, which can cause maternal effects. Due to the tight linkage of genes, if the mitochondrial genome contributes to the phenotype, then one would expect the mitochondrial genes to add only 1 gene to the number obtained. However, due to the nature of inheritance of the mitochondrial genome, combined with our breeding design (reciprocal crosses to produce F1, F2, and backcrosses), any difference in the mitochondrial genome would cause increased variance within the F1,

F2, and backcross groups. Again, this should lead to underestimation of the number of genes contributing to the HR phenotype.

Means and dominance factors of the F2 and both backcross generations all show a continued high degree of net dominance in the direction of high wheel running (see Figure 5, Tables 5, 6). As in Chapter 3, this result was not unexpected, given that similar dominance relationships have been shown in previous studies in both F1 and F2 generations of mouse strains that differ in wheel running (Bruell, 1962; Lightfoot et al., 2008; Dohm et al., 1994; Nehrenberg et al., 2009; Kelly et al., 2010b).

One important consideration with regard to our conclusions is that the cross used represents only one of four control lines crossed with one of four selected lines. Although the selection criterion is the same for all four HR lines, identical selection may often lead to "multiple solutions" (Garland and Rose, 2009; Garland et al., 2010). Therefore, there may be additional alleles that could contribute to the HR phenotype, but were lost in the line used for this study due to founder effects when the initial base population was split into four lines or, more likely, subsequent genetic drift (e.g., Eisen, 1975; Swallow et al., 2009). Therefore, our results might not be an all-inclusive estimate of the loci that could possibly influence wheel running.

Identifying the number of genes that contribute to the HR phenotype is one of the first steps to understanding the complex genetic architecture of this trait. By exploring this genetic architecture, we can eventually ascertain how these genetic influences interact with environmental components to produce a phenotype. Understanding the intricacies of high wheel running in mice allows us to better understand the genetic

underpinnings of voluntary activity in general (Garland et al., 2011). This knowledge can be useful in developing new drug therapies to counteract activity disorders in human populations.

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Table 4.1. Means for untransformed data.

Trait	Sex	Mean-Variance Correlation	Control Parent Mean	HR Parent Mean	F1 Mean	F2 Mean	BC to Control Mean	BC to HR Mean
Body Mass (grams)	F	-0.739	24.42	23.11	23.47	23.49	23.78	23.48
	M	-0.723	31.34	28.20	29.72	29.58	30.32	28.94
Revolutions /Day	F	0.947	4169.9	9941.5	9337.2	8736.6	6670.9	10081.5
	M	0.818	3017.6	9678.4	8686.1	8507.2	5430.0	9495.4
Minutes/Day	F	-0.851	491.2	571.9	593.9	572.5	554.2	585.3
	M	-0.673	332.8	584.3	552.1	548.7	472.5	540.6
Mean Speed (RPM)	F	0.866	8.27	17.26	15.76	15.11	11.89	17.08
	M	0.852	8.68	16.30	15.60	15.29	11.40	17.41
Maximum Speed (RRP)	F	0.800	16.86	31.32	28.08	27.32	22.50	30.77
	M	0.801	17.53	29.98	28.30	27.80	22.34	31.71

Table 4.2. Variances for untransformed data.

Trait	Sex	Control Parent Variance	HR Parent Variance	F1 variance	F2 Variance	BC to Control Variance	BC to HR Variance
Body Mass	F	4.06	2.09	2.50	2.03	1.55	2.17
	M	1.43	2.50	2.46	2.34	2.52	2.37
Revolutions /Day	F	2003343	8876266	5737595	6607011	4067459	8098653
	M	2121738	9787289	4729829	6663425	3589989	5653091
Minutes/Day	F	13007.5	7745.2	8315.0	7974.4	8383.7	9503.6
	M	12187.8	10013.8	5900.8	8378.7	10138.8	5679.7
Mean Speed	F	2.47	14.98	10.25	11.58	7.75	8.80
	M	6.13	11.21	9.44	13.53	6.78	11.82
Maximum Speed	F	5.81	29.52	21.04	18.98	19.96	17.22
	M	13.26	31.52	19.52	32.91	14.92	27.70

Table 4.3. Means for transformed data.

Trait	Sex	Trans-form Used	Mean-Variance Correlation	Control Parent Mean	HR Parent Mean	F1 Mean	F2 Mean	BC to Control Mean	BC to HR Mean
Body Mass	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Revolutions /Day	F	0.350	-0.0180	18.17	24.74	24.31	23.65	21.54	24.93
	M	0.500	-0.0261	52.96	96.85	92.23	90.80	72.59	96.51
Minutes/Day	F	1.850	-0.0160	100201.0	129053.6	13419.1	129329.3	122125.8	135228.6
	M	1.400	0.0008	3531.1	7553.5	6948.1	6916.3	5643.1	6749.1
Mean Speed	F	0.050	-0.0297	1.11	1.15	1.15	1.14	1.13	1.15
	M	0.475	-0.5000	2.76	3.74	3.67	3.62	3.16	3.86
Maximum Speed	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	M	.300	-0.0311	2.35	2.76	2.72	2.70	2.53	2.81

Table 4.4. Variances for transformed data. Transforms used are identical to previous table, but are presented here for clarity.

Trait	Sex	Transform Used	Mean-Variance Corr	Control Parent	HR Parent	F1	F2	BC to Control	BC to HR
Body Mass	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	M	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Revolutions /Day	F	0.350	-0.0180	6.269	6.980	5.005	6.595	5.784	5.982
	M	0.500	-0.0261	215.12	259.45	164.01	228.87	187.00	165.09
Minutes/Day	F	1.850	-0.0158	1623041116	154588883	1593703660	1255754798	1237827411	1661002667
	M	1.400	0.0008	2396932	3166472	1669392	2372642	2512578	1618527
Mean Speed	F	0.050	-.0297	0.000143	0.000187	0.000133	0.000171	0.000164	0.000105
	M	0.475	-0.0500	0.1572	0.1444	0.1300	0.1734	0.1171	0.1366
Maximum Speed	F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	M	0.300	-0.031	0.023	0.024	0.017	0.024	0.016	0.019

Table 4.5. Estimates of dominance ratio in F1, F2, and backcross generations for untransformed data.

Trait	Sex	F1	F2	BC to Control	BC to HR
Body Mass	F	-0.440	0.413	0.969	-0.124
	M	0.030	0.120	0.120	0.060
Revolutions /Day	F	0.791	0.582	0.733	1.09
	M	0.702	0.648	0.449	0.890
Minutes/Day	F	1.544	1.014	2.121	1.663
	M	0.744	0.717	1.220	0.305
Mean Speed	F	0.668	0.522	0.611	0.923
	M	0.816	0.734	0.429	1.581
Maximum Speed	F	0.553	0.447	0.560	0.849
	M	0.729	0.649	0.546	1.557

Table 4.6. Estimates of dominance ratio in F1, F2, and backcross generations using transformed values. Transforms used are identical to those in previous tables, but are repeated here for clarity.

Trait	Sex	Transform used	F1	F2	BC to Control	BC to HR
Body Mass	F	N/A	N/A	N/A	N/A	N/A
	M	N/A	N/A	N/A	N/A	N/A
Revolutions /Day	F	0.350	0.869	0.667	1.051	1.114
	M	0.500	0.789	0.724	0.790	0.969
Minutes/Day	F	1.850	1.649	1.019	2.040	1.856
	M	1.400	0.699	0.683	1.100	0.200
Mean Speed	F	0.050	0.781	0.634	0.965	1.042
	M	0.475	0.849	0.757	0.627	1.504
Maximum Speed	F	N/A	N/A	N/A	N/A	N/A
	M	0.300	0.785	0.694	0.789	1.489

Table 4.7. Estimates of the number of independently segregating genetic factors influencing the high voluntary wheel running trait in a selectively bred High Runner line of house mice. Calculations that yielded negative numbers are listed as undefined.

Trait	Sex	F2	BC to Control	BC2	Transform	F2	BC to Control	BC to HR
Body Mass	F	Undefined	0.03	0.07	N/A	N/A	N/A	N/A
	M	6.78	0.70	1.33	N/A	N/A	N/A	N/A
Revolutions /Day	F	4.78	Undefined	1.83	0.350	9.85	Undefined	36.07
	M	5.08	Undefined	15.99	0.500	11.04	Undefined	Undefined
Minutes/Day	F	Undefined	Undefined	9.82	1.850	Undefined	Undefined	3.22
	M	Undefined	6.05	Undefined	1.400	16.96	7.79	Undefined
Mean Speed	F	6.14	Undefined	Undefined	0.050	316.32	498.35	Undefined
	M	2.35	Undefined	5.21	0.475	9.75	Undefined	Undefined
Maximum Speed	F	Undefined	32.15	Undefined	N/A	N/A	N/A	N/A
	M	2.13	Undefined	5.33	0.300	22.80	Undefined	Undefined

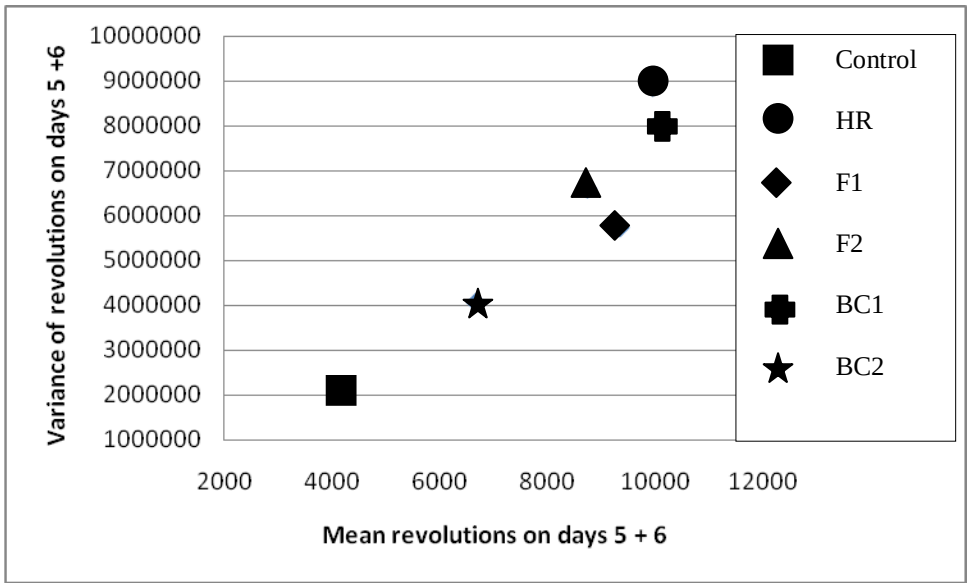

Figure 4.1. Means and variances for untransformed revolutions run by female mice of all groups on days 5 and 6 of a 6-day trial. See Table 1 for means of untransformed values. See Table 2 for variances of untransformed values.

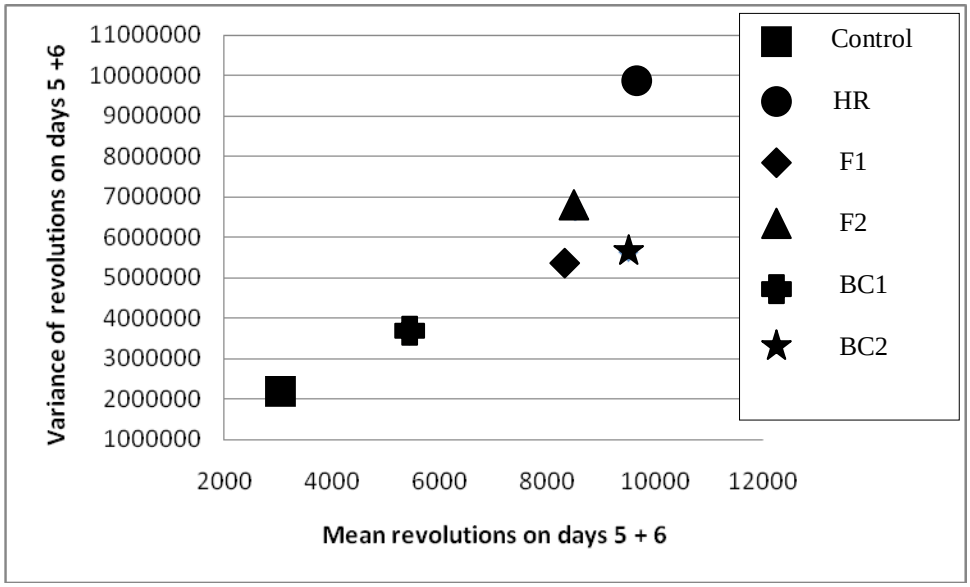

Figure 4.2. Means and variances for untransformed revolutions run by male mice of all groups on days 5 and 6 of a 6-day trial. See Table 1 for means of untransformed values. See Table 2 for variances of untransformed values.

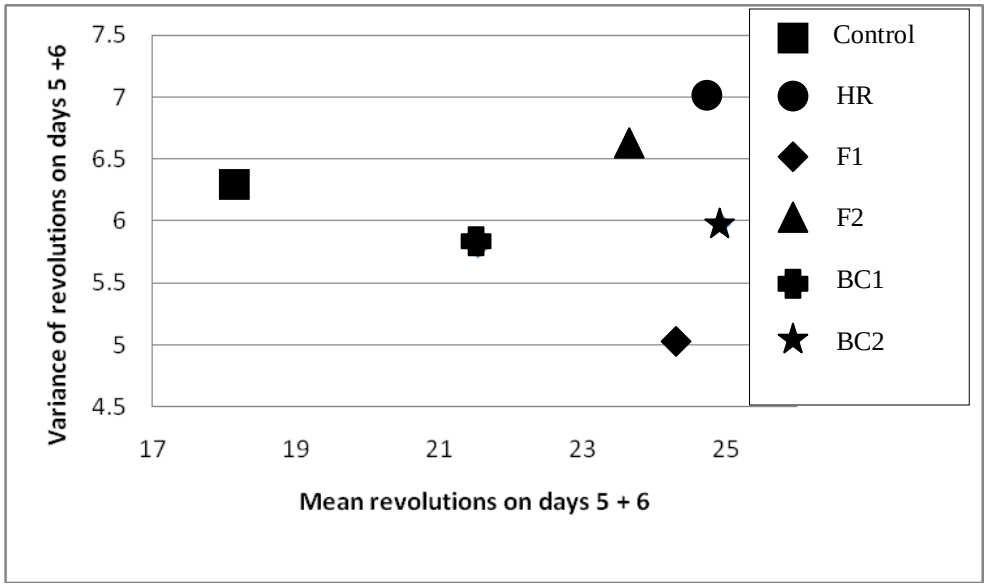

Figure 4.3. Means and variances for revolutions run by female mice raised to the 0.35 power. See Table 3 for means of transformed values. See Table 4 for variances of transformed values.

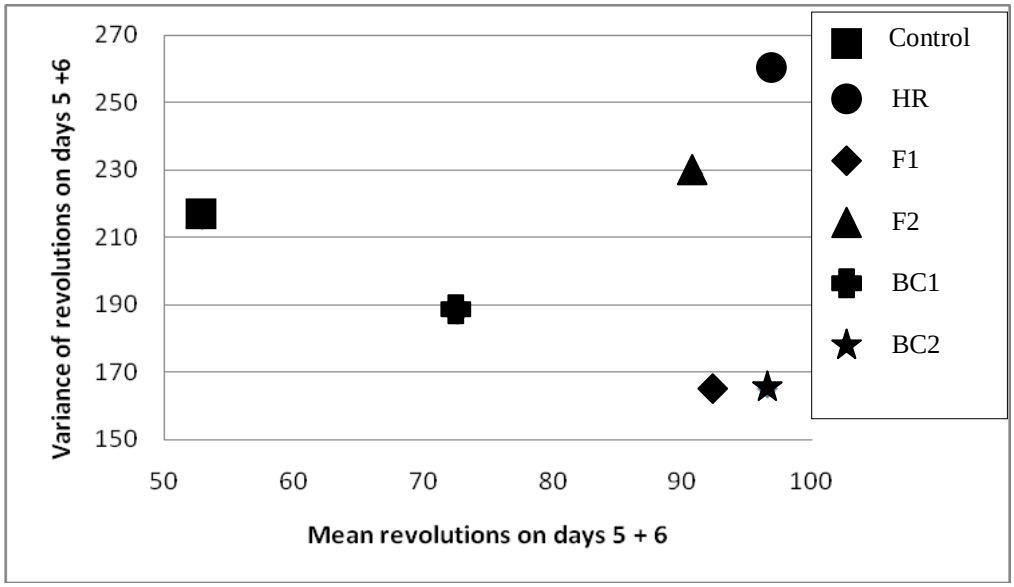

Figure 4.4. Means and variances for revolutions run by male mice raised to the 0.5 power. See Table 3 for means of transformed values. See Table 4 for variances of transformed values.

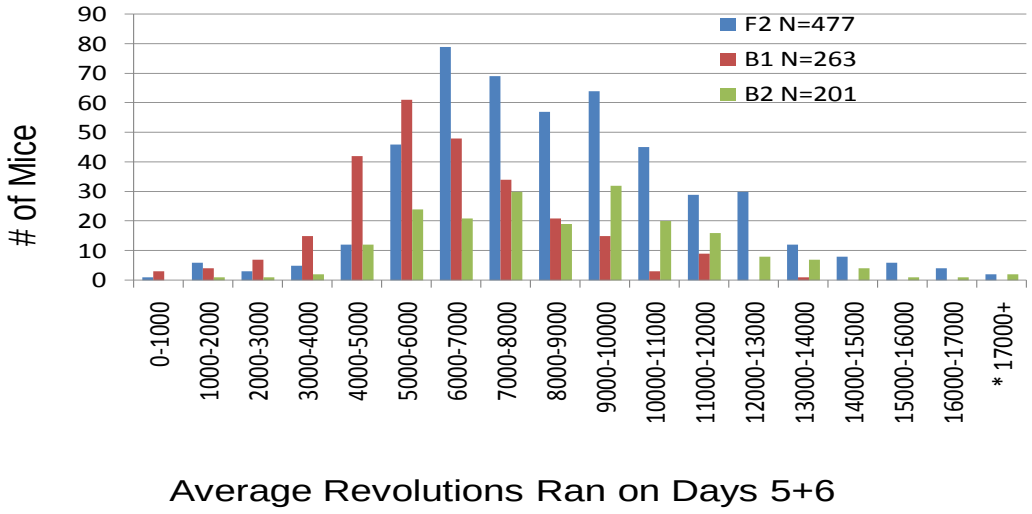

Figure 4.5. Average revolutions run by F2 and backcrosses to parental groups on days 5 and 6 of a 6-day trial. See Table 1 for means of untransformed values. Backcross to HR parent mean (B2) was not significantly different from HR parental group. F2 generation shows slight regression toward control group. Backcross to control group (B1) shows even further regression toward controls. These phenotypic measurements further support the hypothesis that genes affecting high wheel running act mainly in a dominant fashion.

Concluding Remarks

Knowledge about genetic architecture, including identification of genes and epigenetic mechanisms that affect particular traits, is of interest for two main reasons; first, it may have agricultural or biomedical relevance; second, it may lead to development of inferences about the evolutionary processes that maintain genetic variation and those that cause divergence among populations (Laurie et al., 2004; Hansen, 2006). The data presented in this dissertation contribute to our knowledge about the genetic architecture of voluntary wheel running in laboratory house mice, which is seen as a model for human voluntary exercise (Kelly et al., 2010b; Garland et al., 2011).

The goals of this dissertation were to: 1) examine the effects of the gene that causes the "mini muscle" phenotype in a genetic background other than HR (high runner); 2) consider the possibility of heterosis and the ideas of "multiple solutions" when considering two lines of HR mice; 3) investigate the direction of dominance of the HR phenotype and determine if it is influenced by any parental effects; 4) estimate the number of independently segregating genetic factors that contribute to the difference in voluntary wheel running between control and HR mice.

Chapter one investigated the effects of the "mini muscle" allele in a partially novel genetic background. The "mini muscle" allele appears to operate as a simple Mendelian recessive, causing the hind limb muscle mass and the triceps surae complex to be approximately 50% of normal size (adjusted for body mass). Pleiotropic effects of the allele on relative heart and spleen mass were also observed. All phenotypic and pleiotropic effects occurring in HR also were present in the same magnitude in resultant

backcross mice from a C57Bl/6J X HR intercross (see table 1.1 in chapter 1 or table 1 in Hannon et al., 2008), showing that this gene of major effect does not need the HR genetic background to manifest itself.

Chapter two provides evidence that HR lines have exhibited "multiple solutions" (Garland et al., 2010) to a uniform selection (Hannon et al., 2011). It also suggests that the two sexes have responded differently to selection. Sex-specific hybrid vigor is rare, and it is currently unknown why this is shown in our mice. Regardless, it does show that higher levels of wheel running are attainable (cf. Bult and Lynch, 2000)

Chapter three shows that the HR wheel-running phenotype exhibits net dominance over the control (C) phenotype. F1 mice from an HR X C reciprocal cross ran at similar levels as their HR parents. A maternal effect was also detected when the F1s were broken down by reciprocal cross type. F1 individuals from HR dams ran more revolutions per day than those from C dams. This apparent maternal effect was stronger for males than for females. This is the first report of positive maternal influences on wheel running in an F_1 population of mice (see also Kelly et al., 2010a).

Chapter four gives support to the hypothesis that the HR phenotype is a result of multiple independently segregating genetic factors (see also Kelly et al., 2010b). At least ten genes in females and 11 genes in males are estimated to influence the HR phenotype (wheel revolutions run per day), based on the cross between one HR and one C line.

This dissertation adds to our understanding of the complex genetic architecture of the high voluntary wheel running phenotype of HR mice. Data presented show that

selection for high voluntary wheel running not only can result in differences in genetic architecture among the replicate selected lines (populations), but also between the sexes.

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