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Physiological and Genetic Causes of a Selection Limit for Voluntary Wheel-Running in Mice

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by

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ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
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Dr. Theodore Garland, Jr., Chairperson

Populations under directional selection may reach a selection limit after which they no longer respond to selection. This dissertation examined four possible genetic and/or physiological causes of selection limits reached in four replicate lines of mice bred for high voluntary wheel running (HR lines).

Chapter 1 used individual locus models to test the hypothesis that "phenotypic epistasis" (non-additive interactions among components of a trait) can allow maintenance of additive genetic variance (V_A) for a complex behavioral trait at a selection limit. Models with phenotypic epistasis but purely additive genetic effects on component traits involving motivation and ability for speed and duration of running did not maintain V_A, nor did genetic dominance or pleiotropy. However, models with genetic antagonistic pleiotropy did sometimes allow maintenance of V_A.

Chapter 2 attempted to break the selection limits in HR lines by use of a hybrid cross with continued selection on it and the parental HR lines. The hybrid line did not break the limit for daily running distance. The genetic correlation between running duration and speed evolved from positive in the starting population to negative in the

parental lines, and remained so in the hybrid line, which represents a type of genetic constraint.

Chapter 3 studied body composition (i.e., lean and fat mass) of mice before and after 6 days of wheel access. Despite increased exercise, HR lines lost less fat, indicating that preserving a baseline fat mass may be a limiting factor in HR locomotor activity.

Chapter 4 examined energetic perturbations imposed in early-life. Dams were given high-fat, high-sugar "Western" diet (WD) or standard chow from 2 weeks prior to pairing until pups were 14 days of age, when all mice were switched to standard chow. From weaning to adulthood, offspring received physiological and behavioral tests. Maternal WD increased juvenile home-cage activity for both HR and C mice (only males tested). Maternal WD also increased fat and lean masses of adult mice, but 6 days of wheel access reversed the effect on fat. Offspring of dams given WD did not increase wheel running, indicating that fat availability itself does not increase wheel running.

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Introduction

Breeders have used artificial selection for thousands of years to increase the frequency or expression of desired traits of crops and livestock (Price 1984; Vigne 2011; Larson and Burger 2013). More recently, artificial selection has been used in hypothesis-driven experiments to identify processes that cause evolutionary change in phenotypes and genotypes (Garland 2003; Garland and Rose 2009). Artificial selection allows biologists to know, or make better estimates of, the target of selection and intensity of selection, both of which are difficult to measure in observations of natural populations. Artificial selection can also help predict outcomes of natural populations under similar types of selection (but see "multiple solutions" section below).

Especially important are selection experiments that target behavioral traits, because natural and sexual selection generally act most strongly on traits at relatively high levels of biological organization (see Fig. 1 in Garland and Kelly 2006; Garland and Carter 1994; Careau and Garland 2012; Storz et al. 2015). For example, an artificial selection experiment for maximal locomotor performance (e.g., sprint speed) might be interesting as a study of physiology, but it does not tell how evolution would occur in nature, because organisms may not always behave at their maximal capabilities. Thus, artificial selection experiments that target voluntary behavioral traits, as opposed to forced performance traits, may be particularly relevant for understanding the evolution of wild populations under natural selection.

However, predictions made from artificial selection experiments may not be as reliable as expected. Even under the same laboratory environment and artificial selection

regime, similar populations (or lines) may undergo different evolutionary paths, i.e., have "multiple solutions" (Garland et al. 2011a). As Mayr noted, "Probably nothing in biology is less predictable than the future course of evolution. ... independent parallel lines exposed to the same selection pressures will respond at different rates and with different effects, none of them predictable." (1961, p. 1505). Employing replicate lines in artificial selection experiments is therefore crucial in ascertaining whether or not a particular result is consistently reached (Garland 2003).

Replication is especially important for selection experiments with behavioral traits because they are complex. Although the term "complex traits" has been used in various contexts (e.g., Fuller 2005), we will treat them here as traits that have many lower-level component traits, are polygenic (and hence appropriate for quantitative genetics, (Kelly et al. 2010)), and often exhibit emergent properties (Ghalambor et al. 2003; Sinervo and Calsbeek 2003; Swallow and Garland 2005; Rezende et al. 2006b). Component (or subordinate) traits are all the lower-level traits that affect a complex trait. Genotype and environment produce primary phenotypic traits (e.g., morphology), which determine organismal performance abilities, which limit behavior and hence impact life-history traits (see Fig. 1 in Garland and Carter 1994; Storz et al. 2015). Finally, emergent properties occur when lower-level traits interact in complicated paths, so that a simple change in one component trait can result in a disproportional or otherwise unpredictable change in the higher-level complex trait. Complex traits can be difficult to study because of emergent properties.

Limits to the evolution of complex traits can occur at any one of many component traits, or via interactions of those components. Replicated selection experiments provide a fairly direct way to study complex traits, and their component traits, at their evolutionary limits (Barton and Partridge 2000). Many artificial selection experiments do reach limits after tens of generations of selection: body mass in mice (Roberts 1966), shock-avoidance in rats (Brush et al. 1979), post-weaning mass gain in mice (Barria and Bradford 1981a,b), litter size in mice (Buis 1988), protein mass in mice (Bünger et al. 1998), and nest-building behavior in mice (Lynch 1994), although some have not even after 100 generations (Bünger et al. 2001). However, most of these artificial selection studies that hit limits do not study the cause.

The most common theories as to what causes limits to trait evolution include: diminished selection differentials, loss of additive genetic variance (V_A), counterpoising natural selection, and negative genetic correlations between the trait under selection and other fitness-related traits (e.g., caused by antagonistic pleiotropy, i.e., alleles that affect the trait under selection and a fitness-related trait in opposite directions: (e.g., see Rose 1985)) (Falconer and MacKay 1996). The selection differential (written as "s") is the difference in phenotypic means between a population before selection and the subset of individuals that breed to produce the next generation (Falconer and MacKay 1996). The "breeder's equation" ($r = h^2 s$, where r is the response to selection across one generation and h^2 is the narrow-sense heritability) plainly indicates why selection differentials of zero will coincide with a selection limit. Narrow-sense heritability is the proportion of phenotypic variance that can be passed on from parent to offspring, i.e., the additive

genetic variance (Falconer and MacKay 1996). Thus, the breeder's equation also clearly shows why a narrow-sense heritability of zero results in no response to selection (i.e., a selection limit), regardless of how strong selection might be.

Natural selection is said to counterpoise artificial selection when Darwinian fitness is negatively affected by artificial selection on a given trait (e.g., as the trait under selection increases, fertility and/or litter size decreases; (Hill and Mbaga 1998)). (In such situations, the "realized" selection differential [weighted by litter size, e.g., see Careau et al. 2013], will be lower than the selection differential.) As an example of natural selection counterpoising artificial selection, mice bred for rapid post-weaning mass gain reached a selection limit associated with reduced fertility and embryo survival (Barria and Bradford 1981a,b). Subsequent subpopulations with reverse selection or relaxed selection regained fertility, but had reduced growth rate, indicating that natural selection was indeed counterpoising artificial selection (Barria and Bradford 1981a,b).

Behavioral traits offer interesting opportunities to further consider potential causes of selection limits. Almost all voluntary behavioral traits can be considered composites of 1) traits that affect motivation to engage in the behavior and 2) traits that affect physical ability to perform the behavior (e.g., see Garland et al. 2011b specifically on voluntary exercise and wheel running). If a limit to either motivation or ability exists, then the higher-level behavioral trait will be limited as well. For example, a limit to voluntary wheel running may be caused by a limit on the motivation to run as opposed to a limit on the ability to run. That is, mice may be physically capable of running more revolutions per day, but simply are not motivated to do so. In this way, directional

selection on a voluntary behavior could reach a limit that is imposed when either motivation or ability, or both simultaneously, reach a limit.

Mouse model

In this dissertation, I studied mice from an ongoing artificial selection experiment for high voluntary wheel-running (Swallow et al. 1998). The founding population was 224 outbred laboratory house mice (*Mus domesticus*) of the Hsd:ICR strain (Harlan-Sprague-Dawley; Indianapolis, Indiana, USA). Following 2 generations of random breeding, mice were randomly assigned to one of eight closed lines. Four replicate lines were selected for high voluntary wheel running (HR; lab designated lines 3, 6, 7, and 8) and four lines were bred without regard to wheel running (C; lab designated lines 1, 2, 4, and 5).

In each generation, ~600 mice (HR and C of both sexes) were wheel tested at ~6-8 weeks of age for 6 days in standard housing cages attached by a tunnel to Wahman-type running wheels (1.12 meter circumference; Lafayette Instruments, Lafayette, IN). Mice were kept with *ad libitum* food (Harlan Teklad Laboratory Rodent Diet 8604) and water, in a 12:12 light-dark cycle with room temperature maintained at 22 – 24°C. Wheel running was recorded every minute for approximately 23 hours per day using photocell counters and uploaded to an automated computer system. Wheel running was quantified as the total number of wheel revolutions on days 5 and 6 of the 6-day test.

Within-family selection was used to increase the effective population size (N_e) while reducing maternal, environmental, and genotype-by-environment interaction

variances (Henderson 1989). In HR lines, we paired the highest runner of every family to another family's highest runner of the opposite sex. We excluded sibling mating and generally avoided mating between first cousins. Generations did not overlap and we used first litters.

Since approximately generation 16, all four HR lines run 2.5 to 3 times more revolutions per day than C lines. Female HR mice increased wheel running primarily by increasing the average speed of running, rather than increasing the minutes per day spent running, while both components were important for increased wheel running in male mice (Swallow et al. 1998; Koteja et al. 1999a,b; Rhodes et al. 2000; Girard et al. 2001). By generation 25, HR lines (except possibly HR line 8) had reached an apparent selection limit (Careau et al. 2013).

Based on mean values for the sexes, the four HR lines had statistically significant differences in initial response to selection (realized heritability h^2_w, range: 0.14–0.25), timing at which they reached a selection limit (range: generation 17 – 25), and height of the plateau (revolutions per day; range: 6,394 – 8,344) (Careau et al. 2013).

Correlated trait responses in the mouse model

Artificial selection for increased wheel running in the HR mice resulted in multiple correlated responses, ranging from components of exercise capacity to morphology and physiology, as well as behavior and neurobiology (reviews in Rhodes and Kawecki 2009; Swallow et al. 2009; Garland et al. 2011b). In many cases, these

changes can be considered as primarily related to either ability or motivation for sustained, endurance-type locomotion.

HR mice have increased endurance (Meek et al. 2009) and maximal oxygen consumption (VO_2max) during forced exercise on a motorized treadmill, both of which are important components of locomotor performance abilities (Swallow et al. 1998; Rezende et al. 2006b,a). As mentioned previously, the three-fold increase in running in the HR lines was achieved mostly by higher running speeds (Swallow et al. 1999; Garland et al. 2011a).

HR mice have numerous anatomical differences that might enhance sustained locomotor behavior, including smaller and leaner bodies, increased hindlimb symmetry, and larger femoral heads (Swallow et al. 1999, 2001, 2005; Garland and Freeman 2005; Rezende et al. 2006b). They also have increased heart ventricle mass, which could have implications for stroke volume and cardiac output (Rezende et al. 2006b; Kelly et al. 2017).

Administration of Ritalin (methylphenidate) in female mice decreased wheel running in the HR lines, almost to the level of C lines, but increased wheel running in the C lines (inverse rate-dependent effect) (Rhodes and Garland 2003). Similarly, administration of cocaine in female mice decreased wheel running in HR lines but not C lines (Rhodes et al. 2001). This suggests that (at least female) HR mice have reduced dopamine function, particularly in D1-like receptors (Rhodes et al. 2001; Rhodes and Garland 2003). It is interesting to note that no pharmacological manipulation increased wheel running in HR lines, suggesting that they may already be near their maximal

motivation for wheel running. However, feeding mice a high-fat, high-sugar "Western" diet increased wheel running in HR lines and not C lines (Meek et al. 2010; only males were studied). The Western diet may have increased wheel running in HR lines by either increasing ability (e.g., related to fuel usage) or motivation, given that the rewarding effects of both Western diet and wheel running occur through the same brain circuitry.

Many of the pharmacological and behavioral studies on the mice have shown them to be possible model systems for various human conditions. One hypothesis is that the HR mice exhibit higher running because they are addicted to physical exercise, which can be a self-rewarding behavior (Sherwin 1998; Novak et al. 2012). During wheel withdrawal (no wheel access after several days of wheel access), HR mice exhibited behavioral despair (Malisch et al. 2009; only males tested), altered brain activity (Rhodes and Garland 2003; only females tested), and altered cardiovascular response (Kolb et al. 2013). HR mice also had higher levels of home-cage activity than C when housed without wheels (Malisch et al. 2009), which suggests a need to be more active.

In summary, information on correlated responses to selection in the HR lines suggests that the observed limits to selection may be related to either motivation or ability. It is possible that limits to either aspect of voluntary wheel-running behavior may be limiting further increases in the evolution of the behavior itself. In this dissertation, I performed a set of related studies that together contribute to our understanding of the genetic and physiological causes of the selection limits.

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Chapter 1

Individual-locus genetic simulations to study the evolution of hierarchical complex traits and selection limits

Layla Hiramatsu, Matthew Wolak, and Theodore Garland, Jr.

Author Contributions: LH and TG designed the research. LH and MW designed the computer model. LH collected data. LH and TG designed and performed the analysis. LH wrote the initial draft. All authors reviewed and edited the final chapter.

Abstract

For many complex behavioral traits, the total amount of a behavior expressed is the product of intensity and duration, each of which is potentially limited by the lower of motivation and physical ability. Expression of such behaviors thus involves non-additive interactions among lower-level traits (i.e., phenotypic epistasis), even if the alleles at all loci affecting the four lowest-level traits (motivation and ability for intensity and duration) have purely additive effects. Genetic epistasis is commonly mentioned as a possible cause of limits to selection, e.g., in artificial selection experiments, and could potentially maintain additive genetic variance (V_A) at the limit, but the role of phenotypic epistasis has rarely been considered. We created a simple model with the genetic architecture described above and purely additive genetic effects at the lowest levels. Population sizes were chosen to mimic typical selection experiments with rodents, and the intensity of selection was set by selecting 40% of the individuals each generation (census and effective population size = 40 per generation). Each of the four lowest-level traits was affected by 10 loci, and each locus had two alleles (+1 and -1) which were randomly assigned at the start of the model. Values for allelic effects, environmental effects, and population means in the base population and when a limit might be reached were chosen to mimic a long-term selection experiment that targeted voluntary exercise on wheels in laboratory house mice. V_A was calculated by simulating a large number of mating pairs in each generation to allow calculation of variance in breeding values and by use of an "animal model" which partitioned variance components. Similar to results from the mouse wheel-running selection experiment, the model reached a selection limit after

about 20 generations, but the limit coincided with depleted V_A and hence narrow-sense heritability, unlike the selection experiment. We then used more complex models to better mimic the likely genetic architecture of complex behaviors, including using a leptokurtic distribution of initial allelic effects (instead of a biallelic model), employing "within-family" selection (as in many rodent experiments), and adding dominance or pleiotropic effects of genes. Despite increased complexity, these models followed the same patterns of depleted heritability at the selection limit. However, in a few replicate simulations of a model with loci that had antagonistic pleiotropic effects, heritability was maintained ($h^2 > 0.1$) at the selection limit. We discuss biological relevance of these models and suggest modifications and additions which would further improve the model.

1. Introduction

Many artificial selection experiments reach limits, beyond which continued directional selection causes little or no further change (Falconer 1981, 1992; Lynch and Walsh 1998; Barton and Partridge 2000). Commonly cited explanation for such limits include reduced selection differentials (i.e., little phenotypic variance remains for selection to be applied), depletion of additive genetic variance (V_A), counterpoising natural selection (e.g., via reduced fertility), and negative genetic correlations with other fitness-related traits (Falconer 1981; Barton and Partridge 2000). In few cases have the causes of selection limits been identified with certainty.

Selection limits are often observed in experiments with rodents. One example involves bidirectional selection for nest-building behavior in laboratory house mice (Lynch 1994; Bult and Lynch 2000). In the low-selected lines, the selection limit approached, but did not reach, a physical limit of zero grams of cotton used per day to build nests (Lynch 1994). However, why these low-selected lines did not reach the physical limit of zero, and why the high-selected lines reached limits of approximately 40 grams of cotton per day, was never clearly determined (C. B. Lynch, personal communication to T.G. 26 May 2011).

Many behaviors, including nest-building and measures of physical activity, are composites of two traits, duration and intensity (e.g., average speed) (Fig. 1.1). Furthermore, both of these lower-level traits involve components of motivation and physical ability to perform the trait (Fig. 1.1). Thus, the limiting factor to the amount of physical activity expressed (e.g., total distance moved on a daily basis) by an organism

may be any one of the following: its ability to run fast, its motivation to run fast, its ability to sustain running, or its motivation to sustain running (Fig. 1.1). In this example, the relationships of the component traits with the total amount of physical activity expressed are non-additive: the expressed behavior is not simply the sum of its component traits.

We term non-additive interactions of component phenotypes "phenotypic epistasis." Non-additive interactions between or among alleles at different loci is termed epistasis (although many different definitions exist, see Cordell 2002; Ehrenreich 2017), but we believe that the term phenotypic epistasis is useful for describing situations in which the underlying genetic effects for the component traits might not include any non-additive genetic effects, i.e., no genetic epistasis. Studies on the *Drosophila* genome suggest that genetic epistasis is an important component of the genetic architecture of numerous traits (Forneris et al. 2017; review in Mackay and Huang 2017), but the possibility of phenotypic epistasis is rarely considered.

Our thesis here is that phenotypic epistasis can allow the maintenance of V_A for a complex behavioral trait at a selection limit, even if its lowest-level component traits (Fig. 1.1) are affected only by alleles with purely additive effects. As a hypothetical example, the selection limit for running distance will occur when both speed and duration of running have reached limits. The limits to these two lower-level traits will occur when V_A for either ability or motivation has been depleted. If, however, V_A for ability for speed has been depleted, V_A for motivation for speed might still remain, and similarly for

duration of running. In such a scenario, our conjecture is that V_A could remain for running distance.

One example of a selection experiment in which a behavioral trait reached a limit despite maintained V_A is artificial selection on voluntary wheel running in mice (Careau et al. 2013). Four replicate High Runner (HR) lines were bred for high wheel running, which resulted in a 2.5-3-fold increase in revolutions run over days 5+6 of a 6-day trial, compared against 4 replicate, non-selected control lines (Swallow et al. 1998). All four lines reached a selection limit at generation 17-25 (depending on line and sex), but V_A was maintained at these limits for at least 3 of the 4 lines (Careau et al. 2013). Other explanations for the selection limit (e.g., counterpoising natural selection via reduced fertility, reduced selection differential) were not supported (Careau et al. 2013). As voluntary wheel running can be broken down into the component traits of ability and motivation (e.g., Lightfoot et al. in press; Kelly and Pomp 2013; Garland et al. 2016) for both intensity and duration of running (cf. Fig. 1.1), a plausible explanation for the maintenance of V_A at the selection limit could be the non-additive phenotypic architecture of the trait.

We created an individual locus model to test whether the non-additive interactions between ability and motivation for both intensity and duration of a behavior (i.e., phenotypic epistasis) could cause the maintenance of V_A for a trait at a selection limit. With purely additive genetic effects at the four lowest-level traits (Fig. 1.1) and individual (mass) selection, V_A for the top-level trait was not maintained. We then incorporated within-family selection (as used for the mouse wheel-running selection

experiment), a leptokurtic distribution of allelic (and mutational) effects, which also did not lead to maintenance of V_A. Finally, we allowed dominance, positive pleiotropy or antagonistic pleiotropy for the lowest-level traits. Results show that antagonistic pleiotropy can sometimes maintain V_A at a selection limit.

2. The model

The simulation model was adapted from the individual-based model described by Roff (2010, sections 4.6 and 4.9). The initial model described below in section 2.1.1 through 2.1.3 was the basic model with simple genetic assumptions. In subsequent models, we added more biologically relevant complexities regarding selection and genetic effects (see sections 2.2-2.6). We analyzed twenty replicate simulations of every model. For each replicate simulation, we saved pedigree information (individuals' dam and sire identities) for all 100 generations and individual phenotypic data for 7 traits (i.e., the phenotypes in Fig. 1.1). From the pedigree information, we created a relationship matrix to obtain inbreeding coefficients (F) (Butler et al. 2007).

2.1.1. Starting population

Each simulation was started with 100 diploid individuals who were assigned 10 loci each for four traits that affected wheel running (referred to as "lowest-level traits" in the text): motivation for running speed, physical ability for running speed, motivation for running duration, and physical ability for running duration (10 loci per trait x 4 traits x 2 alleles per locus = 80 alleles total per individual). Alleles at every locus were assigned as

either +1 or -1 (biallelic model) with a probability of 0.5. These alleles were unlinked and autosomal (sexes were not defined). Generations were non-overlapping.

2.1.2. Assigning genotypes and phenotypes

All alleles at the 4 lowest-level traits were additive within and among loci, so that an individual's genotype for a trait was the sum of all 20 alleles plus a population mean. Because these 80 alleles were assigned independently, no genetic correlations between any of the 4 lowest-level traits were specified. The population mean added for motivation and ability for running speed was 16, and the effect of every allele was kept at +1 or -1. For duration, the population mean added was 500, and the effect of every allele was multiplied by 20. The resulting genotype was calibrated so that no individual would have a genotype for speed of less than 1 (i.e., any individual that had genotype of speed motivation or ability less that 1 was re-assigned 1 for that trait) or more than 39 revolutions per minute. Similarly, genotype for duration was truncated to a lower limit of 10 and upper limit of 960 minutes per day.

To obtain the phenotype for speed traits, we multiplied the genotype by 0.7 and added an environmental effect. The environmental effect was pulled from a normal distribution with a mean of zero and standard deviation (SD) of 1 and multiplied by 3. Environmental effects were independently assigned for each of 4 traits within each individual, so the expected environmental covariances between traits were zero. For duration phenotypes, we multiplied genotypes by 0.7 and environmental deviations by 25. Genotypic and environmental effects on phenotype were scaled to obtain phenotypic

values that were in the range we have observed for the control lines of the mouse selection experiment (duration ~ 350 minutes per day and average speed ~ 11.2 revolutions per minute (Swallow et al. 1998; Garland et al. 2011)). The phenotype for speed was truncated again to a lower limit of 1 (i.e., no mouse runs at an average speed of less than 1 revolution per minute) and an upper limit of 39 revolutions per minute. Similarly, the phenotype for duration traits were truncated to a lower limit of 10 minutes per day and an upper limit of 960 minutes per day (i.e., 16 hours per day).

Average speed was calculated per individual as the lower phenotype between motivation and physical ability for speed. That is, an individual would only run as fast as they were motivated or able to do (limited by motivation or ability, whichever was lower). Duration was calculated the same—as the lower of motivation or physical ability for running duration. Finally, the trait under selection (i.e., total wheel running) was calculated as the product of speed and duration (Fig. 1.1).

2.1.3. Selecting parents

In models with directional selection, selection intensity was set to 40 breeding individuals (= 20 pairs) per generation to mimic the mouse selection experiment, which has a N_e~40. In non-selected, control models, 40 individuals were randomly chosen as breeders for the next generation. Breeders were only allowed to mate once and we did not separate sexes. We kept note of family identity, and sibling-mating was disallowed. Each of 20 pairs produced 5 offspring to create 100 individuals for the next generation. (Note, litter size did not vary among pairs.) Offspring alleles were assigned by selecting

one allele at random from each parent for each locus. Mutations were allowed to occur right after offspring alleles were determined, and caused a change in the sign of allelic effect (i.e., +1 to -1 or vice versa). The number of mutations was pulled from a Poisson distribution with lambda (λ) equal to the number of alleles multiplied by 10^{-4} (Roff 2010). After mutations, the new generation of individuals were assigned genotypes and phenotypes (back to section 2.1.2.), and this was repeated for 100 total generations of breeding.

2.1.4. Heritability estimates

We estimated the narrow-sense heritability (V_A divided by phenotypic variance) of total wheel running in two ways: variances of breeding values and use of the "animal model".

Additive genetic variance was calculated in each generation as the variance in breeding values among all individuals. To obtain breeding values, every individual (N = 100) was "mated" to every other individual to produce 5 offspring per pair. Then, an average phenotypic value for total wheel running was obtained across all offspring for each individual (5 offspring x 99 pairs = mean of 495 offspring total). An individual's breeding value was calculated as twice the difference between the grand mean of all offspring (100 individuals x 99 mates x 5 offspring = 49,500 offspring total) and the mean of the individual's offspring. The variance in breeding values (100 individuals = 100 breeding values per generation) is the additive genetic variance. To obtain narrow-

sense heritability, the variance in breeding values was divided by phenotypic variance for wheel running for each generation.

We also estimated narrow-sense heritability by use of linear mixed-effects models (commonly referred to as the "animal model", software ASReml-R (Butler et al. 2007)), which separated variance components into common family environment (i.e., identity of the mouse's family), additive genetic variance (i.e., the identity of the mouse linked with the pedigree), and residual variance. Narrow-sense heritability was calculated as the ratio of the additive genetic variance component divided by the sum of all variance components. Confidence intervals for the variance components were estimated using profile likelihoods with the R package nadiv (Wolak 2012).

Given a pedigree, the animal model makes inference of variance component estimates back at the starting population. Thus, in order to estimate heritability over 100 generations, we analyzed 10 generation-blocks separately (i.e., generations 1-10, 11-20, 21-30, etc.). This procedure effectively assumed that individuals in the first generation of each block (i.e., generation 1, 11, 21, etc.) were unrelated—which is, of course, untrue. Therefore, we specified the inbreeding coefficient F of all individuals in the first generation of each block to account for inbreeding. The violation of the assumption (that the individuals are unrelated) is necessary to estimate V_A at a given time point in a pedigree (otherwise, the animal model makes inference back to generation 1).

We compared heritability estimates obtained from breeding values and animal models by matching the breeding value at generations 1, 11, 21, etc. to the animal model estimates for generation blocks 1-10, 11-20, 21-30, etc. for each replicate simulation of

each model. We calculated both Pearson's and Spearman's coefficient of correlation with replicate simulations pooled.

2.2. Within-family selection

To account for more biologically relevant genetic models, we introduced complexities to the model in increments. First, we mimicked the HR selection experiment by adding the use of within-family selection in our models, in which the top runner in each family is chosen as a breeder and bred to the top runner of other families (thus, selection is only employed at the family level; (Swallow et al. 1998)). As in the HR experiment, we used 20 breeding individuals with 10 offspring per family, for a total of 100 individuals per generation. (Note, litter size did not vary among pairs.)

2.3. Leptokurtic alleles

As an alternative to using biallelic genetic effects (+1 or -1, as described in section 2.1.1), we also tested the effect of assigning initial alleles by sampling from a leptokurtic distribution of allelic effects. We used a leptokurtic distribution with mean = 0, standard deviation (SD) = 1, and kurtosis of k~10 (a normal distribution has k = 3). The distribution we used had 82.5% values within one SD from the mean, while a normal distribution has 68.2% within one SD of the mean. That is, in the leptokurtic models, most alleles sampled had effects near the mean but some rare alleles had values very distant from the mean.

Leptokurtic distributions of allelic effects are more realistic than normal distributions at mutation-stabilizing selection-drift balance (for example, in outbred ICR house mice used to start the HR selection experiment) (Barton and Turelli 1989; Reeve 2000; Reeve and Fairbairn 2001). Such a distribution of allelic effects occurs under "house-of-cards" mutation, e.g., mutation rate is low but mutational variance is high compared with Gaussian assumptions (Turelli 1984). An empirical example of a rare allele with large effects was found in the HR selection experiment in the "mini-muscle" allele, a Mendelian recessive allele which causes a ~50% reduced hindlimb muscle mass (Garland et al. 2002; Kelly et al. 2013).

2.4. Dominance effects

We introduced directional dominance effects at the genetic level for the first 5 out of 10 loci for each lowest-level trait. We had positive dominance in the direction of selection so that if a locus had two unequal alleles, the value for that locus was equal to twice the allele of higher value. If the locus had two equal alleles, the value was simply the sum of the two alleles. The other loci which did not have dominance effects were simply summed for both alleles contributing to each locus.

2.5. Pleiotropic effects

We also introduced loci with overlapping effects for two of the four lowest-level traits because some genes are likely to affect two or more of these closely-related component traits of wheel-running. We coded these overlapping effects as genetic

pleiotropy, although linkage disequilibrium (especially tight genetic linkage) could cause the same or similar effects. In the pleiotropic model, each of the 4 lowest-level traits was affected by 10 loci total (as before), but only 4 loci were unique to that trait and 6 loci were shared in common with another trait (e.g., physical ability for speed was affected by 4 unique loci and 6 pleiotropic loci; (following pleiotropic model in Roff [2010], section 4.1.3)). Of the 6 pleiotropic loci, 3 loci affected the same "second-level" trait (speed or duration) but for the other type (ability or motivation), and 3 more loci affected the same type but for the other second-level trait (Fig. 1.2). In total, we modeled 12 pleiotropic loci: 3 loci that affected both motivation and physical ability for speed, 3 loci that affected both motivation and physical ability for duration, 3 loci that affected both motivation for speed and motivation for duration, and 3 loci that affected ability for speed and ability for duration. The number of pleiotropic loci was chosen arbitrarily.

The pleiotropic correlation was set to +1, so that the loci shared in common had the exact same effects in the two traits they affected (Fig. 1.2, Positive pleiotropy). This is equivalent to assuming the alleles are pulled from a bivariate distribution of phenotypic effects with a correlation of 1. The result is that the total number of loci are reduced from 40 to 26. However, each lowest-level trait was still affected by 10 total loci.

2.6. Antagonistic pleiotropic effects

Additive genetic variance can be maintained at a selection limit in the case of simultaneous selection on two phenotypes if some genes that act in pleiotropy for the trait have opposite effects (Falconer 1981, pg. 300). We modeled antagonistic genetic

pleiotropy using the same model described above (section 2.5), but allowed 6 of the 12 pleiotropic loci to have opposite effects in different lowest-level traits (Fig. 1.2, Antagonistic pleiotropy). Specifically, the 3 pleiotropic loci that affected physical ability (grey loci in Fig. 1.2) affected speed normally (as the sum of all alleles at the 3 loci), but for duration the sign of effect was flipped. Similarly, the 3 pleiotropic loci that affected motivation (white loci in Fig. 1.2) affected duration normally, but for speed the sign of effect was flipped. The other pleiotropic loci (blue and red in Fig. 1.2) could have had antagonistic effects as well, but we did not explore these models.

3. Results

3.1. The simple model

Both non-selected and selected simulations of the simple model produced changes in the wheel-running trait over 100 generations comparable to the HR selection experiment's Control and HR lines (Fig. 1.3A and 1.3E, 1.4A and 1.4E) (Garland et al. 2011). Specifically, the 20 replicates of the non-selection model maintained a mean value of ~2500 revolutions with expected increase in variability among replicates over 100 generations due to genetic drift (Fig. 1.3A, in blue). Narrow-sense heritability for wheel running calculated from the variance in breeding values started at $h^2 \approx 0.45$ and decreased to $h^2 \approx 0.3$ by generation 100 (Fig. 1.3A, in black). Heritability estimated from the animal model started at similar values but increased to $h^2 \approx 0.5$ over 100 generations (Fig. 1.3E). These estimates of narrow-sense heritability are higher than estimates obtained for the HR selection experiment (Careau et al. 2013). However, we confirmed

the general pattern of finding a non-zero, positive estimate for heritability in the Control lines.

All twenty replicates of the simple selected model increased from ~2500 revolutions to ~14000 revolutions over 100 generations, with a selection limit reached around generation 30 (Fig. 1.4A, in red). Estimates of narrow-sense heritability by breeding values and animal models followed the same pattern of starting $h^2 \approx 0.4$ and decreasing to barely above zero (Fig. 1.4A, in black). These estimates did not indicate the same maintenance of heritability at the selection limit reported for selection lines in the HR selection experiment (Careau et al. 2013).

3.2. Within-family selection

Adding within-family selection to the models did not significantly affect non-selected models for mean revolutions or heritability estimates (except possibly a slight increase in variance in animal model h^2 estimates, Fig. 1.3F). For selected models, within-family selection slightly shifted the selection limit and corresponding heritability estimates to the right (generation 40 instead of 30) (Fig. 1.4F). However, the limit did not correspond with maintained heritability.

3.3. Leptokurtic effects

Non-selected models were unaffected by biallelic vs. leptokurtic distributions of allelic effects (Fig. 1.3A vs. 1.3C). For selected models, sampling from a leptokurtic distribution of allelic effects increased the trait value at the limit (~16000 revolutions

instead of ~14000 revolutions in biallelic models; Fig. 1.4C). Another interesting effect of the leptokurtic distributions was the increase in narrow-sense heritability in the first ~10 generations in these models. This effect is expected under leptokurtic distributions of allelic effects (Turelli 1984) and has been observed for other theoretical models (Reeve and Fairbairn 2001).

3.4. Dominance effects

Including dominance effects in half of the total number of loci (i.e., 20 loci with dominance effects, 5 for each of the 4 lowest-level traits) did not affect the selection limit or heritability estimates (Fig. 1.5A and 1.6D, compared with Fig. 1.4D and 1.4H).

3.5. Pleiotropic effects

Including loci with pleiotropic effects in the model did not affect the selection limit or heritability estimates of models when the loci had the same effects in all traits (Fig. 1.5B and 1.5E). However, when these loci had antagonistic pleiotropic effects (Fig. 1.5 Antagonistic pleiotropy), some replicate simulations had much lower responses to selection and/or reached a limit much later than generation ~20 (Fig. 1.5C and 1.5F). Along with phenotypic differences, the heritability estimates for models with antagonistic pleiotropy were much more variable and higher compared with simpler models (Fig. 1.5C and 1.5F). Because of these differences from other models, we analyzed the antagonistic pleiotropic model replicates further to test whether h^2 was maintained at the selection limits. We defined the phenotypic selection limit as the mean phenotypic value at

generation 91-100 (Table 1.1, "Limit"). Then, we calculated the generation at which the simulation first reached 95% and 99% of this limit (Table 1.1, "Generation"). Finally, we obtained the h^2 estimates from breeding values at that generation for that replicate population (Table 1.1, "h^2"). Some replicate simulations (i.e., replicate run 16, 17, and 20) had high h^2 estimates (i.e., $h^2 > 0.1$) even at the selection limit (Table 1.1; Phenotypes of replicate run 16 are shown in Fig. 1.8).

3.6. Breeding value vs. animal model

The estimate of narrow-sense heritability differed somewhat based on method of estimation (i.e., variance of breeding values vs. variance components in an animal model) (Fig. 1.6). Correlations were lower in non-selected models (range of Pearson's r = 0.24 – 0.52) compared with selected models (range of Pearson's r = 0.93 – 0.97), especially in later generations (although no statistical tests were run for separate generation blocks) (Fig. 1.6). Estimates from animal models tended to be higher than from breeding values (points are above the 1:1 line) (Fig. 1.6). Although some estimates seem to be at $h^2 = 0$, no estimate from either method was actually $h^2 = 0$ (or negative; results not shown). We were also particularly interested in differences between heritability estimates obtained from the two methods at later generations when heritability estimates were low (after the selection limit was reached in selected models), particularly if the animal model gave substantial h^2 while the breeding value did not. That is, if the animal model overestimated heritability when breeding values suggested h^2 was not significantly higher than zero, then we might question the previous report that heritability was maintained in

the HR selection experiment at selection limits (Careau et al. 2013). In general, we did not find animal model heritability estimates to be much higher than breeding value estimates.

3.7. Inbreeding coefficient (F)

Inbreeding coefficients increased in all models (Fig. 1.7). Models without within-family selection increased from F = 0 to F = 0.6, while models with within-family selection increased from F = 0 to F = 0.7, with no discernable difference within these groups as result of any other model parameters. In each model, the 20 replicate simulations were very similar (Fig. 1.7 depicts mean F for all 20 replicates).

4. Discussion

Purely additive genetic effects coupled with non-additive interactions ("phenotypic epistasis") among component traits of a complex trait (Fig. 1.1) under selection did not maintain V_A at the selection limit (Fig. 1.4A). Thus, the proposed model of phenotypic epistasis by itself is not a general explanation for selection experiments which reach limits despite maintenance of narrow-sense heritability (e.g., the selection limit of an artificial selection experiment for high voluntary wheel-running behavior in mice (Careau et al. 2013)).

When genetic effects other than purely additive were considered, our results generally confirmed predictions from quantitative genetics literature. Within-family selection delayed reaching the selection limit (Hill 1971) by approximately 5-10

generations. When allelic effects were drawn from a leptokurtic distribution (Turelli 1984; Reeve 2000; Reeve and Fairbairn 2001), initial increases in genetic variance occurred, but again V_A was not maintained at the selection limits. Positive pleiotropic effects yielded similar results. With antagonistic pleiotropy, however, simulations varied much more among replicates in mean trait value over 100 generations and in a few cases V_A appeared to be maintained ($h^2 > 0.1$) at the selection limit.

The magnitudes of effects were set arbitrarily in several of the complexities we added. For example, pleiotropic correlation was set at +1, so that the loci shared in common had the exact same effects in the two traits they affected (Fig. 1.2, Positive pleiotropy). This correlation could have been set at any number between 0 and +1 (or 0 to -1 for antagonistic pleiotropy), and an intermediate correlation might have been more biologically relevant, although this would reduce the effect of pleiotropy. Also, in models with pleiotropy (positive or antagonistic), the total number of loci were reduced from 40 to 26. We could increase the number of loci which act in pleiotropy to maintain 40 total loci, although this would necessarily increase the number of loci that affect each of the 4 lowest-level traits. Thus, it is impossible in models with pleiotropy to maintain both the number of total loci at 40 and the number of loci which affect each of the four lowest-level traits at 10.

Also, we chose only to have antagonistic pleiotropy for two of the 4 groups of pleiotropic loci (black and white loci in Fig. 1.2), partially because antagonistic genetic correlations between speed and duration have evolved in lines of mice selected for high voluntary wheel running (Garland et al. 2011). Alternately or in addition, we could have

included antagonistic pleiotropy for loci which affect ability and motivation for speed (blue loci in Fig. 1.2) and/or for loci which affect ability and motivation for duration (red loci in Fig. 1.2). Correlations between ability and motivation for physical activity are relevant in rodent and human studies (Lightfoot et al. 2008).

When adding dominance effects to the model, we coded dominance in the high direction only—that is, alleles which had more positive effects were dominant over alleles which had less positive or more negative effects. Not all dominance effects should necessarily be for the high direction and we could have introduced models with differing numbers of loci with dominance in the high and low directions. However, some rodent studies suggest that alleles which were selected for over long evolutionary history would have net dominance, such as high levels of physical activity in house mice (Bruell 1964; Dohm et al. 1994; Nehrenberg et al. 2009).

In the future, we would like to add more biologically relevant complexities which might explain the selection limit with maintained genetic variance. For example, coding for two sexes is of interest because the mice from HR lines are sexually dimorphic in total wheel running, speed, duration, and correlated traits like body size (Garland et al. 2011). The selection limit could be related to sex-specific effects, and selection could be fluctuating between these sex-specific effects (especially if they are negatively correlated). Seasonal variation could also be included, as these mice show an increase in wheel running in Winter months and decrease in Summer months (Supplementary Fig. S4 in Careau et al. 2013). That is, some loci could be coded to have opposite effects when individuals are tested during Winter vs. Summer (we would code a change in

season every generation because each generation in the HR experiment takes ~3-4 months). This would also cause fluctuation in selection pressure.

In conclusion, the phenotypic architectural model analyzed in the present study is a simplified but powerful tool for elucidating the complex interactions within and among loci, lower-level phenotypes, and individuals that occur during the evolution of a population. We will continue to refine the model to allow simulations of a wide range of biologically relevant complexities. In addition, ongoing genetic analyses of the High Runner and control lines of mice that motivated the present simulation models should help in parameterization (e.g., Xu and Garland 2017).

The simulation code used in this chapter is available in the Appendix of this dissertation.

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Tables and Figures

Table 1.1. Heritability estimates at the selection limit from the 20 replicate simulations of the antagonistic pleiotropy model.
The selection limit was defined as the mean revolutions run per day averaged over generations 91-100. Then, we calculated the generation at which the simulation first reached 95% and 99% of this limit ("Generation") and obtained the h^2 estimates from breeding values at that generation for that replicate population. Note that when lines had reached 99% of the selection limit, three of 20 replicates maintained substantial narrow-sense heritability, with estimates ranging from 0.16 to 0.27. This pattern matches what was observed for three of four replicates in the long-term High Runner mouse selection experiment (Careau et al. 2013).

	Limit Revolutions per day, average of generations 91-100	**95% of Limit**		**99% of Limit**	
Replicate		Generation	h^2	Generation	h^2
1	16849	28	0.0590	37	0.0206
2	16868	26	0.0768	32	0.0158
3	16877	32	0.1289	51	0.0221
4	16871	18	0.1238	25	0.0309
5	14410	26	0.1254	53	0.0569
6	15976	29	0.1050	34	0.0469
7	16791	27	0.1320	37	0.0233
8	16769	21	0.2139	25	0.0857
9	16977	23	0.1359	32	0.0150
10	16807	22	0.0943	28	0.0983
11	16790	22	0.0943	24	0.0437
12	16704	32	0.0986	52	0.0290
13	16810	27	0.1179	35	0.0208
14	16838	20	0.1434	27	0.0271
15	16731	22	0.1876	26	0.0491
16	14429	23	0.2349	27	0.1622
17	14278	63	0.2079	91	0.2453
18	16782	35	0.1178	55	0.0280
19	16836	26	0.1536	33	0.0402
20	14731	26	0.2696	26	0.2696

Figure Legends

Fig. 1.1. Complex interactions governing the expression of a behavioral trait, using daily running distance as an example. Total running distance is the product of average running speed and duration. Speed and duration are each affected by an individual's physical ability and motivation to perform the trait. The lower of the two, physical ability or motivation, will limit the expression of the composite traits of speed and duration.

Fig. 1.2. Models of positive and antagonistic pleiotropy, depicting loci (each pair of 2 circles) that affect the 4 lowest-level traits (ability and motivation for speed and duration). Each trait is affected by 10 loci total (= 20 alleles), but only 4 are unique to the trait (in black). The other 6 loci are pleiotropic loci and they are color-coded: 3 blue loci affect both ability and motivation for speed, 3 red loci affect both ability and motivation for duration, 3 grey loci affect ability for both speed and duration, and 3 white loci affect motivation for both speed and duration. Thus, even though each trait is affected by 10 loci, in total only 28 loci are coded (4 unique x 4 traits + 3 common x 4 groups). In the **positive pleiotropy** model, all pleiotropic loci have the same effect in the two traits they affect (all arrows are +). In the **antagonistic pleiotropy** model, the blue and red loci have the same effects in the two traits they affect (all arrows are +), but the grey ("ability") and white ("duration") pleiotropic loci have opposite effects for speed vs. duration.

Fig. 1.3. Non-selected "Control" simulations: 4 models of increasing complexity (columns from left to right), with 20 replicates within each model. The models tested were A) biallelic genetic model with no within-family selection, B) biallelic genetic model with within-family selection, C) leptokurtic distribution of allelic effects with no within-family selection, and D) leptokurtic model with within-family selection. Mean ± standard error bars (se) of the 20 replicates are in bold, with the 20 simulations shown in lighter colors. Panels A-D show mean wheel-running revolutions (in blue) and heritability estimates from variance in breeding values (in black) for 100 individuals per generation. Panels E-H show heritability estimates from animal model analyses (h^2 ± se, estimates inferred to the initial generation of each 10-generation block within each simulation).

Fig. 1.4. Selected "High-Runner" simulations: 4 models of increasing complexity (columns from left to right), with 20 replicates within each model. The models tested were A) biallelic genetic model with no within-family selection, B) biallelic genetic model with within-family selection, C) leptokurtic distribution of allelic effects with no within-family selection, and D) leptokurtic model with within-family selection. Mean ± standard error bars (se) of the 20 replicates are in bold, with the 20 simulations shown in lighter colors. Panels A-D show mean wheel-running revolutions (in red) and heritability estimates from variance in breeding values (in black) for 100 individuals per generation. Panels E-H show heritability estimates from animal model analyses (h^2 ± se, estimates inferred to the initial generation of each 10-generation block within each simulation).

Fig. 1.5. Selected "High-Runner" simulations: 3 additional models of increasing complexity (columns from left to right), with 20 replicates within each model. The models tested all used leptokurtic distributions of allelic effects and within-family selection, with A) dominance effects, B) positive pleiotropy, and C) antagonistic pleiotropy. Mean ± standard error bars (se) of the 20 replicates are in bold, with the 20 simulations shown in lighter colors. Panels A-C show mean wheel-running revolutions (in red) and heritability estimates from variance in breeding values (in black) for 100 individuals per generation. Panels D-F show heritability estimates from animal model analyses (h^2 ± se, estimates inferred to the initial generation of each 10-generation block within each simulation).

Fig. 1.6. Comparison of heritability estimates by breeding values and animal models for each model. Top panels are the same models as Fig. 1.3, middle row panels are the same as Fig. 1.4, and bottom panels are the same as Fig. 1.5. Each model has 20 replicate runs and the heritability for every 10-generation block is depicted in a monochromatic scale from black to light grey, corresponding to generation 1 to 91.

Fig. 1.7. Inbreeding coefficients (F) increasing over 100 generations as mean of 100 individuals per generation, and then averaged across 20 replicates. Coefficients were calculated from the pedigree of each replicate simulation. Top panels are the same models as Fig. 1.3, middle row panels are the same as Fig. 1.4, and bottom panels are the same as Fig. 1.5.

Fig. 1.8. Phenotypic values and heritability estimates for one replicate (replicate #16) of the selected antagonistic pleiotropy model (Fig. 1.5C). The top panels depict A) mean phenotype of 100 individuals in each generation for total wheel revolutions, B) mean speed, and C) mean duration. The bottom panels depict D) heritability for total revolutions (in grey for estimates from breeding values and in blue for estimates from the animal model), E) mean phenotype of 100 individuals in each generation for ability (open square) and motivation (open circle) for running speed, and F) ability (filled square) and motivation (filled circle) for running duration. This replicate had h^2 estimates > 0.1 when it reached 95% and 99% of the selection limit (calculated as the mean trait value for generations 91-100; see Table 1.1).

Fig. 1.1. Complex interactions governing the expression of a behavioral trait.

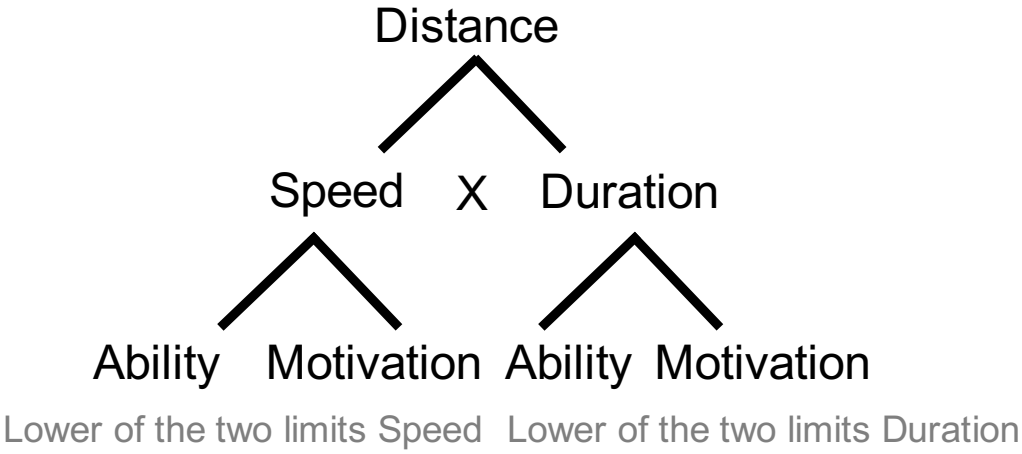


Fig. 1.2. Models of positive and antagonistic pleiotropy

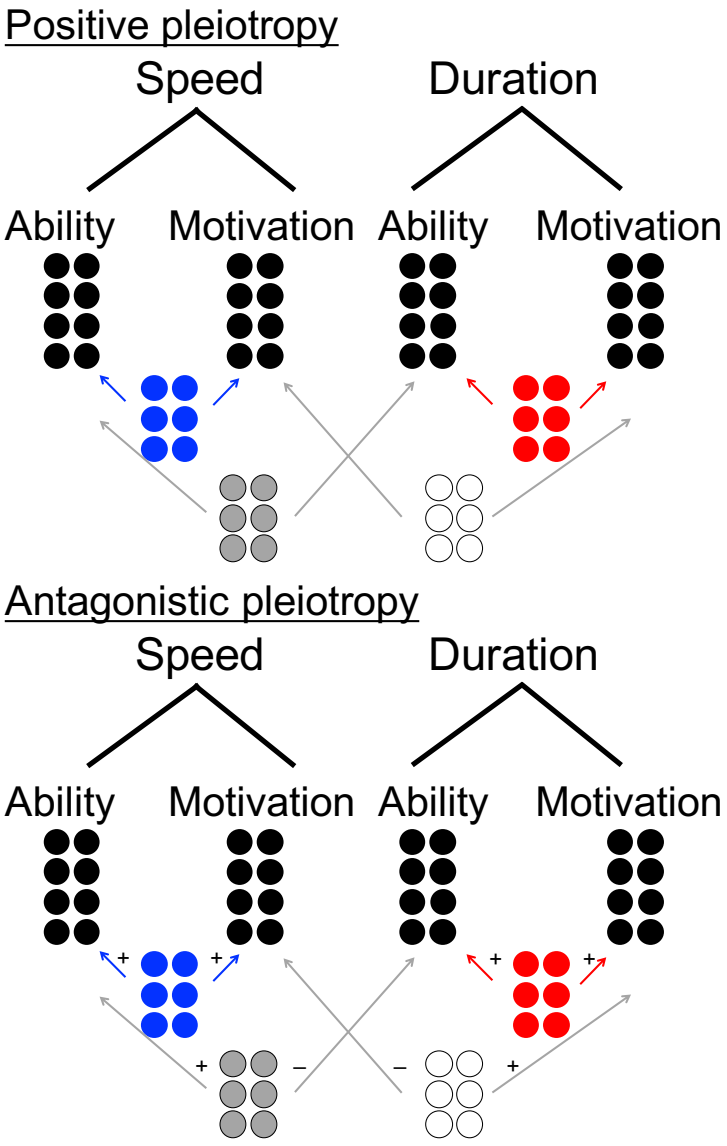


Fig. 1.3. Non-selected "Control" simulations: 4 models of increasing complexity.

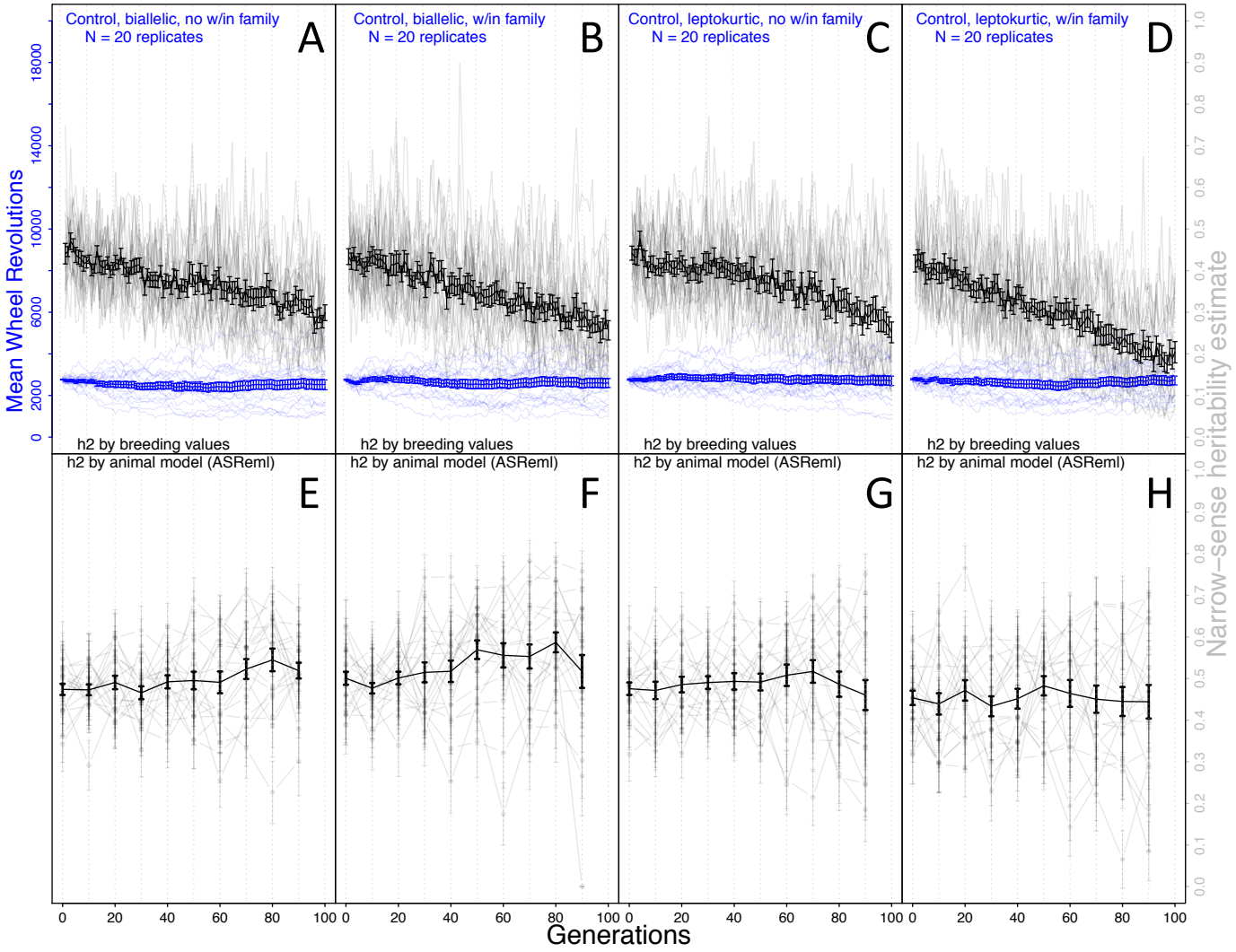


Fig. 1.4. Selected "High-Runner" simulations: 4 models of increasing complexity.

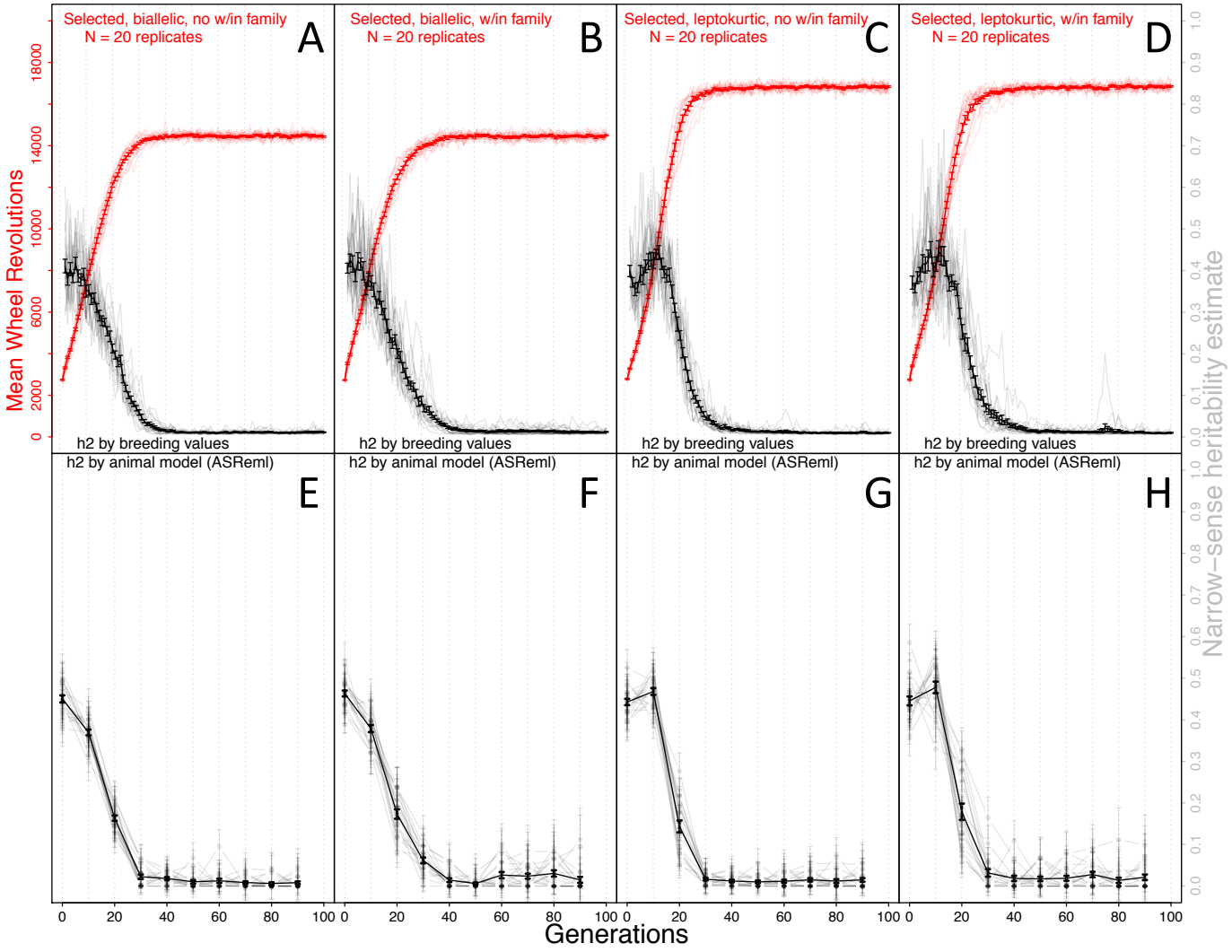


Fig. 1.5. Selected "High-Runner" simulations: 3 additional models of increasing complexity.

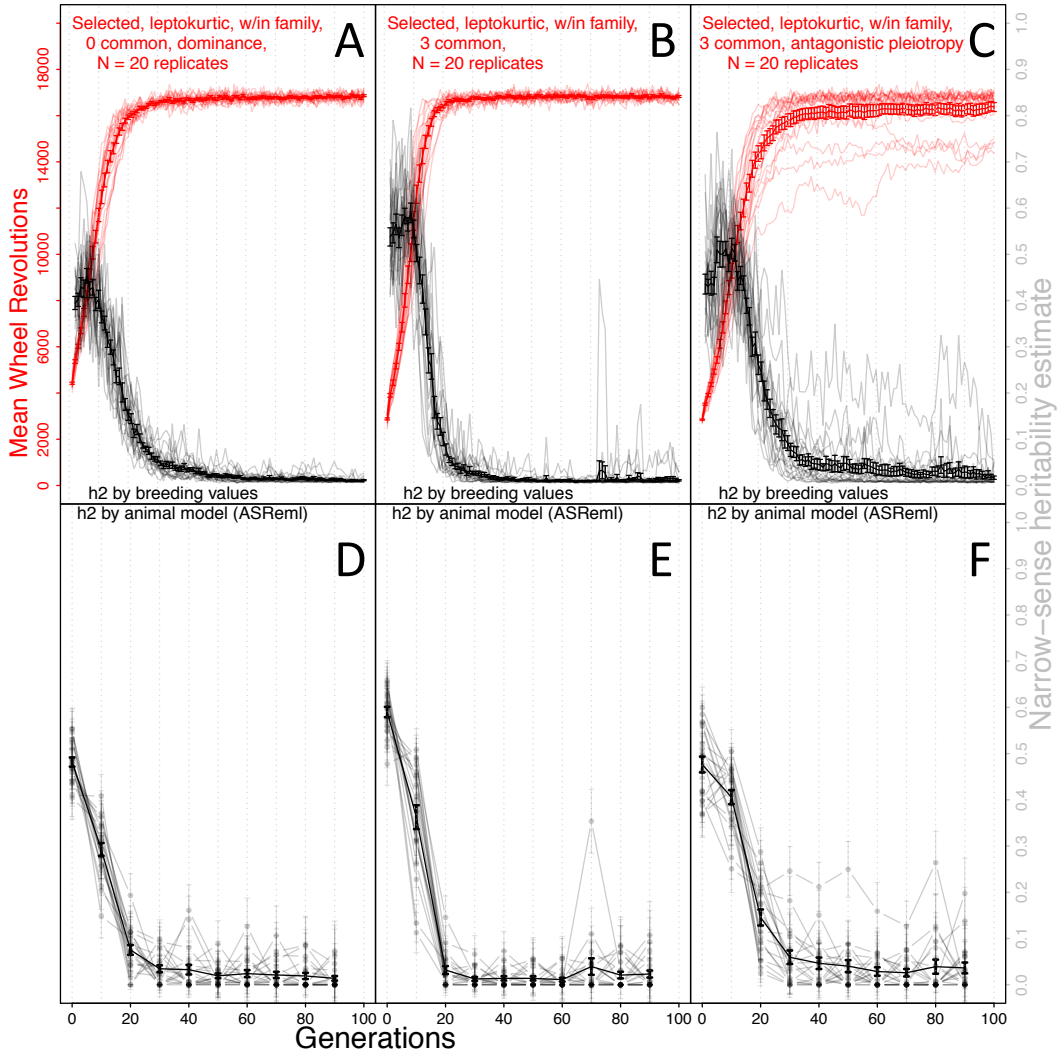


Fig. 1.6. Comparison of heritability estimates by breeding values and animal models.

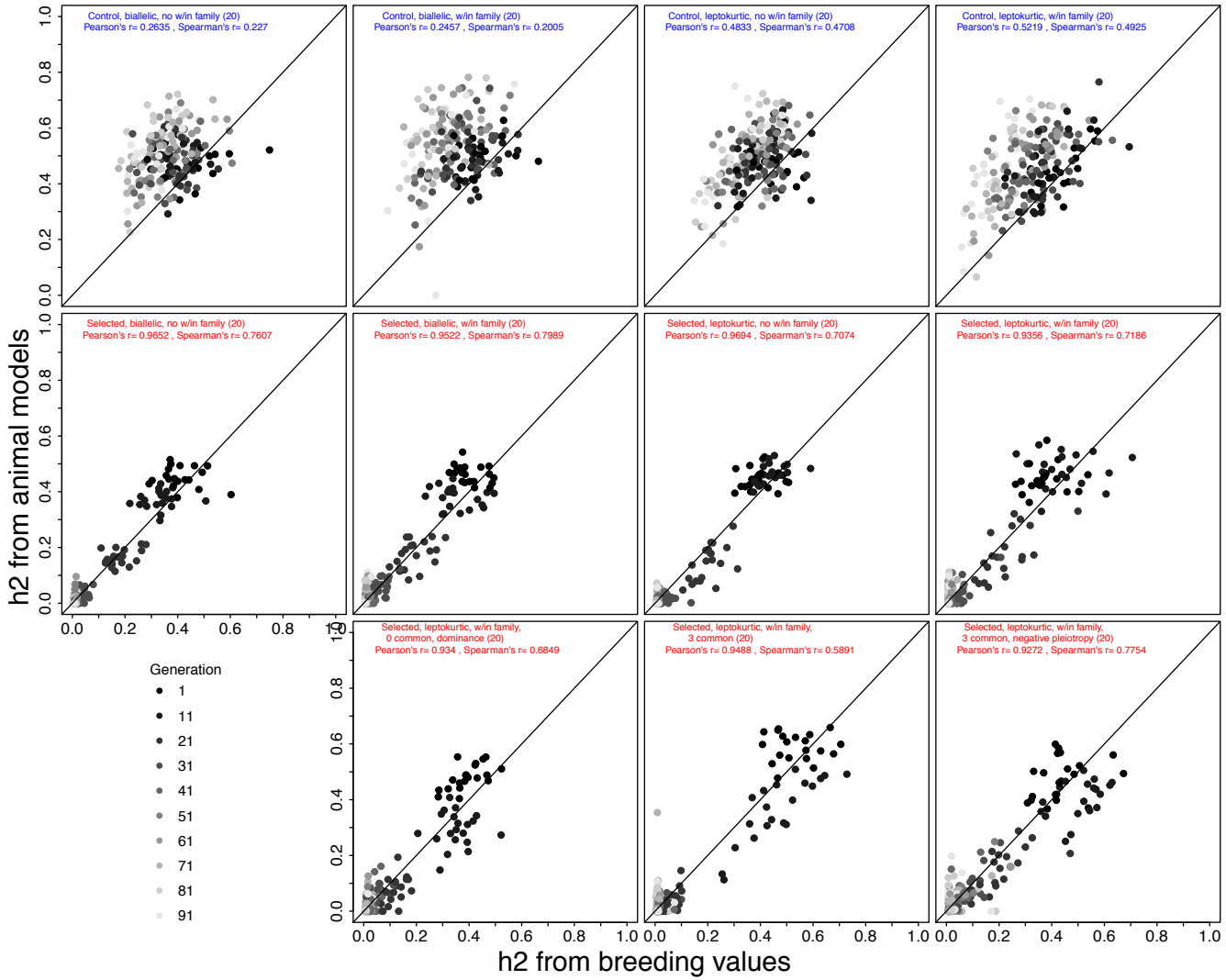


Fig. 1.7. Inbreeding coefficients (F) increasing over 100 generations.

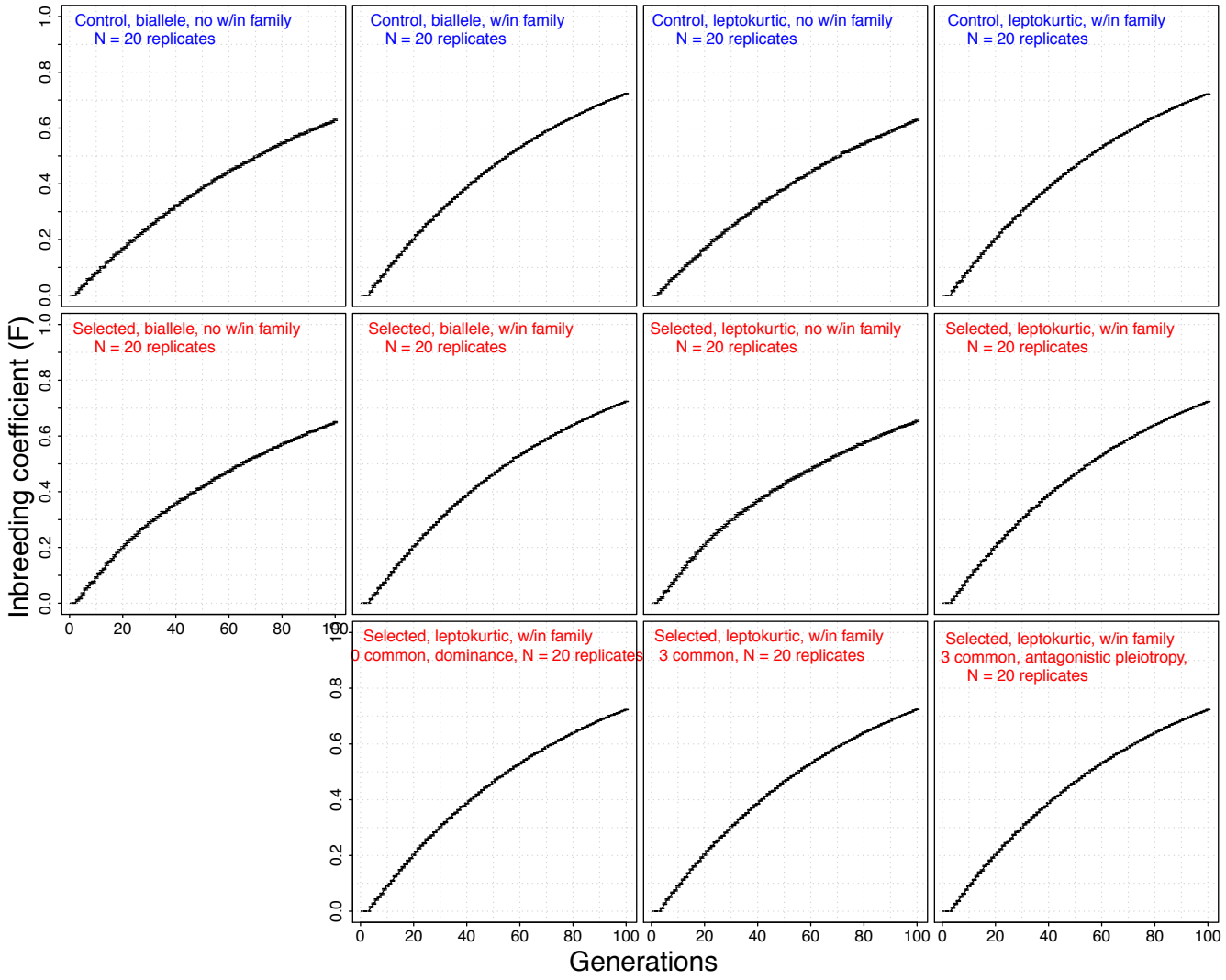
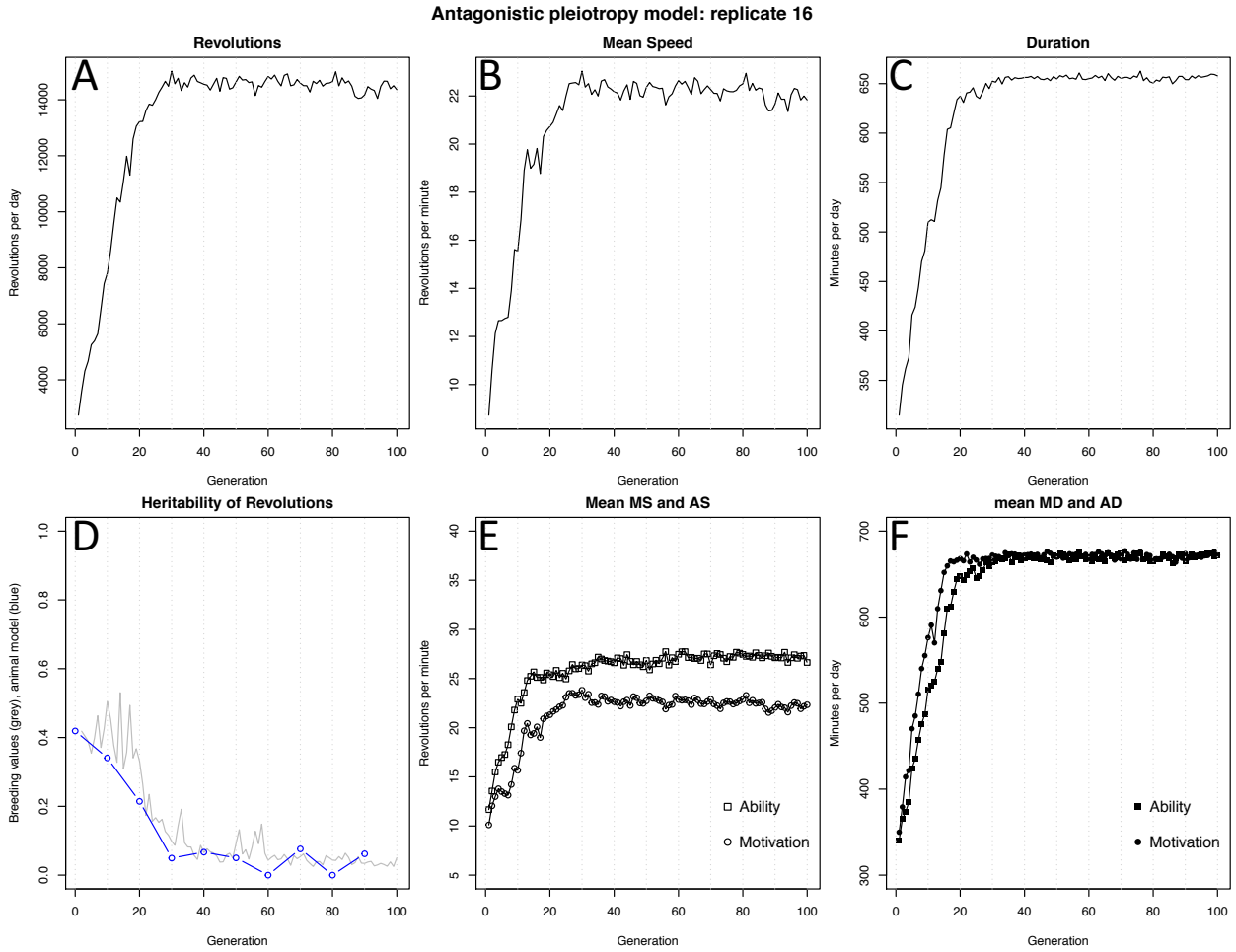


Fig. 1.8. Phenotypic values and heritability estimates for one replicate (replicate #16) of the selected antagonistic pleiotropy model.



Chapter 2

Can a hybrid line break a selection limit on voluntary wheel running in mice?

Layla Hiramatsu, Vincent Careau, and Theodore Garland, Jr.

Author Contributions: LH and TG designed the research. LH collected data. LH, VC, and TG designed the analysis. LH and VC performed the analysis. LH wrote the initial draft. LH, VC, and TG reviewed and edited the final manuscript.

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Abstract

Artificial selection yielded four replicate high runner (HR) lines of mice that voluntarily run ~3-fold more wheel revolutions per day than four non-selected control lines. HR lines and the sexes differed in the rate and magnitude of response to selection, although all four lines reached selection limits. We attempted to break the limit by crossing two HR lines at generation 68, followed by continued selection for 10 generations. Hybrid F_1 offspring showed heterosis for running distance, but this was lost in subsequent generations and the hybrid line did not break the limit. Both male and female hybrids ran faster than the parental lines for most generations, but running duration was intermediate or reduced, indicating different genetic architecture for these traits. The hybrid line had increased heritability for running speed and duration, but not total distance, compared with the parental lines. The hybrid line inherited a negative genetic correlation between speed and duration, which had evolved in the parental lines from a positive genetic correlation in the base population. The genetic trade-off between running duration and speed may explain the inability for the hybrid line to break the selection limit despite renewed additive genetic variance for component traits.

1. Introduction

Limits to selection (plateaus) are common in selection experiments and can result from various causes (Falconer and MacKay 1996; Garland and Rose 2009; Careau et al. 2013). Perhaps the most intuitive potential cause of a selection limit is simply exhaustion of additive genetic variance (i.e., the narrow-sense heritability is reduced to zero). In a population under selection, both the selection regime and genetic drift would cause fixation of alleles, with the former fixing beneficial alleles and the latter fixing alleles without regard of their relevance for the selection regime (Falconer and MacKay 1996). Fixation of alleles is especially likely under strong selection and/or with small population sizes (Weber 1996), as is the case for most artificial selection experiments with rodents (e.g., Meyer and Hill 1991; Beniwal et al. 1992; Heath et al. 1995).

Another possible cause of selection limits is counterposing natural selection (e.g., selected lines suffer a dramatic decrease in fertility), which can be viewed as a consequence of adverse pleiotropic effects of alleles under selection (Barton and Turelli 1989; Hill and Mbaga 1998). For example, two selection experiments for body mass in mice resulted in decreased fertility and postnatal survival in lines at or near plateaus (Falconer 1955; Roberts 1966). Inbreeding depression in small populations may also decrease reproductive success and other aspects of Darwinian fitness (Falconer and MacKay 1996; Birchler et al. 2006; Charlesworth and Willis 2009; Pemberton et al. 2016).

One recent example of a selection experiment that reached clear limits involves selection for high voluntary wheel running in laboratory mice (*Mus domesticus*; Hsd:ICR

strain). Replicated directional selection for wheel running produced four High Runner (HR) lines of mice that run ~3 times as much as four non-selected control (C) lines (Swallow et al. 1998; Careau et al. 2013). Although all replicate HR lines show approximately the same increase in total wheel revolutions per day, they differ in the component traits of average running speed and running duration per day (Garland et al. 2011), and an apparent trade-off between these components of running behavior has emerged (see Fig. 3 in Garland et al. 2011). The HR lines have evolved a variety of differences when compared to the control lines, including reduced body mass and length (Swallow et al. 1999), higher endurance (tested on a treadmill; (Meek et al. 2009)) and maximal aerobic capacity (Rezende et al. 2006), and larger hearts and brains (Kolb et al. 2013; Copes et al. 2015). The HR lines also show an altered brain reward system, including in the dopamine (Rhodes and Garland 2003), serotonin (Claghorn et al. 2016), and endocannabinoid pathways (Thompson et al. 2017).

The HR lines reached selection limits between generations 17-25, differing slightly based on replicate line and sex within line (Careau et al. 2013). The limit experienced in the HR lines was apparently not caused by depleted additive genetic variance or counterpoising natural selection (Careau et al. 2013). The purpose of the present study was to break the selection limit experienced by the HR lines in order to understand the genetic architecture of voluntarily wheel running and the mechanisms underlying the selection limit.

One way to break selection limits is through the use of a hybrid line of two replicate selected populations after they have reached limits. Especially in small

populations, random genetic drift would result in the loss of some favorable alleles before selection could "recruit" them, and these lost alleles would likely differ among replicate populations. Moreover, as mentioned previously, random genetic drift can potentially fix alleles with neutral or detrimental effects, and will, on average, cause populations to diverge genetically. Thus, a cross of two replicate selected lines will inherit favorable genes from both, replenishing alleles lost by genetic drift (e.g., Ehiobu and Goddard 1990; Bult and Lynch 1996; review in Lippman and Zamir 2007).

This increase in a favored trait in hybrid offspring is termed heterosis (also known as hybrid vigor), and is measured as the increase in a trait value of hybrid offspring compared with either 1) the trait value of the higher of two parental populations (Charlesworth and Willis 2009), or 2) the intermediate value of two parental populations (Shull 1914). Heterosis has been explained genetically as the result of potential dominance, overdominance, pseudo-dominance, and/or epistasis among alleles (Lippman and Zamir 2007; Charlesworth and Willis 2009; Schulthess et al. 2017). Using crosses of numerous inbred strains of mice, heterosis has been observed for multiple behavioral traits including food competition (Manosevitz 1972), motor behavior (Guttman et al. 1980), maze behavior (Wahlsten et al. 1991), and activity rhythms (Beau 1991). Wheel running has also been reported to exhibit heterosis in crosses of multiple inbred strains (Bruell 1964), a cross of wild house mice and C mice (Dohm et al. 1994), a cross of HR line 8 and C57BL/6J (Nehrenberg et al. 2009), a cross of HR line 8 and one C line (Hannon 2010), and a cross of two of the four replicate HR lines (Hannon et al. 2011).

The presence of heterosis for a trait suggests that with continued selection, hybrids may break a selection limit previously experienced by parental lines.

A selection experiment on nest building in mice gives evidence that this approach can indeed be used to break selection limits for behavioral traits. Replicate lines bred for high and low nest-building reached selection limits at around generation 20 (Lynch 1994). Replicate hybrid lines were created at generation 46 for both the high and low selected lines, and all 4 hybrid lines broke selection limits after ~8-10 generations of renewed selection (Bult and Lynch 2000). Thus, with renewed selection, hybrid populations could break the selection limit experienced in the parental populations. Because Hannon et al. (2011) observed heterosis for wheel running in male hybrids of two HR lines, continued selection on the hybrid line has the potential to break the selection limit faced in the replicate HR lines.

The purpose of the present study was to attempt the paradigm outlined above to break the selection limit reached in the HR mice. By breaking this limit, we might better understand the reason for the selection limit in the parental lines and further elucidate the underlying genetic architecture of wheel running and its component traits. We crossed two of four replicate HR lines and continued selection on this hybrid line for 10 generations.

2. Materials and Methods

All experiments were approved by the Institutional Animal Care and use Committee of the University of California, Riverside.

2.1. Original selected lines

We used laboratory mice (*Mus domesticus*) that had undergone 68 generations of directional selection for high levels of voluntary wheel running (Swallow et al. 1998; Careau et al. 2013). The base population for this long-term selection experiment was 224 unrelated mice from the genetically variable, outbred Hsd:ICR strain (Harlan-Sprague-Dawley, Institute of Cancer Research). After 2 generations of random mating, we established 4 high-runner (HR) and 4 non-selected control (C) lines. At all times throughout the experiment, mice were kept with a 12:12 light-dark cycle at 20-24 degrees Celsius, and food and water were provided *ad libitum*. As young adults (~6-9 weeks of age), all mice were placed in new, individual home cages with access to wheels for 6 days to measure their voluntary wheel running. The wheels were 1.12 m in circumference and attached externally to the home cage, accessed via a tunnel (see Fig. S1 in Kelly et al. 2017). HR lines were bred based on their total wheel revolutions on days 5 and 6. Ten pairs per line were maintained using within-family selection, so that all families were represented in each generation and inbreeding was minimized ($N_e \sim 35$ per line). That is, we selected the highest running female and male from each family and mated them to the highest running male or female from other families. We avoided pairing of siblings. Only first litters were used. The same testing and breeding protocols were followed in the current hybrid line experiment.

A sensor attached to each wheel counted every rotation, and a custom computer program recorded the number of rotations in 1-minute intervals for 23 hours/day. Once a

day before starting the next test, we checked every cage for the health of the mouse and that the wheels were turning. After 6 days, we took mice off wheels and placed them back in cages of 4. Daily metrics of wheel running were: total revolutions, number of 1-minute intervals active, average speed (total revolutions divided by number of 1-minute intervals active), and maximum speed (the highest number of revolutions in any 1-minute interval). We weighed all mice before placing them in the wheel cages on day 1 and when we took them out of the wheel cages at the end of day 6.

2.2. Hybrid line

HR replicate lines 7 and 8 were chosen for this study due to the absence of the mini-muscle allele (fixed in HR line 3 and polymorphic in HR line 6; (Kelly et al. 2013)), which affects many traits, including wheel running and organ masses (see Garland et al. 2002; Hannon et al. 2008). At generation 68, in addition to breeding the replicate HR lines as usual, we bred a subset of females and males from lines 7 and 8 to create two reciprocal hybrid crosses (7 female × 8 male and 8 female × 7 male).

In creating the next generation (F_2) of the hybrid line, we used a factorial breeding design to maximize allele mixing and retain the ability to test for grand-parental effects. We bred females from one reciprocal cross to males from the same and different crosses: i.e., females from the F_1 reciprocal 7F × 8M were bred to males from the F_1 reciprocal 7F × 8M or males from the F_1 reciprocal 8F × 7M, and the same for females from the F_1 reciprocal 8F × 7M. In subsequent generations (F_3+) of the hybrid line, we combined these crosses as one pool of breeders. We continued selection in the following

generations for the parental and hybrid lines, following the usual selection protocol with within-family selection, for a total of 10 generations of the hybrid line.

2.3. Comparison of lines and line crosses

Analyses were performed separately by sex unless otherwise noted, because of many known differences between sexes (Garland et al. 2011; Hannon et al. 2011). For each generation, we tested whether the hybrid line had diverged significantly from the parental lines using analysis of covariance (ANCOVA) in the Mixed procedure in SAS (version 23; SAS Institute, Cary, NC, USA). Analyses of body mass used age as a covariate. Analyses of wheel-running traits used age and wheel freeness as covariates. Wheel freeness was tested for each wheel by accelerating the wheel to a constant velocity and counting revolutions until the wheel stopped on its own (Copes et al. 2015). For analysis, the square-root of wheel freeness was used to obtain a more homogenous spread of values. We tested for the difference between the hybrid line and parental lines using three separate a priori contrasts: hybrid line 9 vs. HR line 7, hybrid line 9 vs. HR line 8, and hybrid line 9 vs. the average of HR line 7 and 8. We used additional contrasts for F_1 reciprocal crosses: hybrids created from line 7 females crossed with line 8 males vs. hybrids created from line 8 females crossed with line 7 males. We also used additional contrasts for F_2 reciprocal crosses.

The hybrid line exhibited greater variance than parental lines, so we considered 6 different models with (1) a single estimate for residual variance, (2) a single estimate for residual variance and a single estimate for variance among families (as a nested random

effect), (3) a single estimate for residual variance and separate estimates for family variance, (4) a separate estimate of residual variance for each cross-type and no variance among families, (5) a separate estimate of residual variance for each type and a single estimate for variance among families (see also Garland et al. 2011; Hannon et al. 2011).

2.4. Heritability estimates by offspring-on-parent regressions

We estimated the heritability of wheel running within each line as the slope of the linear regression of offspring-on-mid-parent or twice the slope of regressions of daughters-on-dams or sons-on-sires. The slopes are doubled in the latter two cases because each parent contributes half of the additive genetic effects to its offspring. Males contributed no parental care in this experiment, but they were paired with the female up to parturition, which may have conferred non-genetic effects on the offspring. Also, common environments and maternal effects were shared among offspring in the same litter, which could inflate the estimate of heritability. Thus, none of these estimates are good estimates of narrow-sense heritability *per se*.

Values used for the regressions were residuals of wheel running (mean values for days 5+6) for all individuals (all 3 lines and all 10 hybrid generations) regressed on sex, age, wheel freeness, testing batch and room (8 dummy categories), line, generation, and the interactions of line and generation, sex and line, sex and generation, and sex and line and generation. We also obtained residuals of running speed and duration (both as mean values for days 5+6) and body mass using the same regression factors, except for body mass which did not use wheel freeness and testing batch and room. The values for

offspring in a given generation were then averaged for each litter, separately by sex in the case of single-sex regressions (i.e., averaged for daughters and sons separately). These analyses included individuals pooled across all 10 hybrid generations, but we also analyzed heritability generation-by-generation (in both cases, heritabilities were estimated separately for each line).

The estimate of heritability by offspring-on-parent regressions assumes equal litter sizes and no assortative mating, both of which we did not have. To correct for unequal litter sizes, the regression was weighted by a score based on both the number of offspring in each litter and the intra-class correlation, using an iterative method described in Lynch and Walsh (1998) and the osw() function in R (Careau et al. 2013). To correct for assortative mating, we used this equation from Roff (1997 p. 199): h^2 = 2 x slope of regression / (1 + r). We calculated r as the Pearson correlation of the residual values of wheel running (as above) between dams and sires, calculated separately for each line and generation, or pooled across generations. The corresponding standard error was calculated as in Falconer (1981): se = se of regression slope x sqrt(1 + r). Lastly, when the trait had unequal variances between the sexes, the mid-parent regression was not used. Instead, heritability was calculated as the mean of the two sex-specific heritability estimates and the corresponding standard error was approximated by dividing the averages of the standard errors of the sexes by the square root of 2 (Bult and Lynch 2000).

2.5. Heritability estimates by the animal model

To estimate the heritability of wheel running and its components using an "animal model," we first obtained the pedigree of the selection experiment. We used the same pedigree for these mice as published previously up to generation 31 (Careau et al. 2013, 2015) to which we added information up to generation 78. The pedigree included data from the original 224 mice purchased from Harlan Sprague-Dawley, but no information before then (thus, these 224 mice were assumed to be unrelated (Careau et al. 2013, 2015)). Then, we obtained inbreeding coefficients (F) using the relationship matrix calculated from the pedigree (Butler et al. 2007). For the parental generation used to create the hybrid line (i.e., generation 68 of the selection experiment), the average (± standard deviation) inbreeding coefficient for HR line 7 was F =0.7087 ± 0.0105 and for HR line 8 was F =0.7198 ± 0.0106.

Because we wanted to estimate heritability of wheel running in the first generation of the hybrid experiment (generation 69 = hybrid generation 1), we subset the pedigree to only the generations relevant to the hybrid line. Thus, the pedigree used to estimate heritability did not include the first 68 generations of selection. This allowed us to estimate additive genetic variance at generation 69 instead of implicitly inferring back to the base population of the selection experiment (Careau et al. 2013). This procedure effectively assumes that individuals within a line at generation 69 are outbred, which is, of course, untrue (see above). Therefore, we specified the inbreeding coefficient of all breeders at generation 69 when calculating the A-inverse matrix used in the animal model, to account for inbreeding. The animal model assumes that individuals in the base

population of a pedigree are unrelated. Although this is a reasonable assumption for the base population of the experiment, it is certainly not at generation 69. Yet, violating this assumption is necessary to estimate additive genetic variance at a given time point in a pedigree (otherwise the animal model makes inference back to the base population of the experiment).

For each trait for which we wanted to estimate heritability, we first standardized the trait to have mean = 0 and standard deviation = 1 separately in each line within each generation. This enabled us to pool generations together and directly compare estimates of variance and regression coefficients between lines. Then, we estimated variance components for each line using linear mixed-effects models, which included fixed effects (age, sex, F coefficient, and wheel freeness) and variance components of common maternal environment (i.e., identity of the mouse's dam), additive genetic variance (i.e., the identity of the mouse linked with the pedigree), and residual variance. Narrow-sense heritability was calculated as the ratio of the additive genetic variance component divided by the sum of all variance components. Confidence intervals for the variance components were estimated using profile likelihoods with the R package nadiv (Wolak 2012).

We measured cumulative response to directional selection (i.e., selective gain) separately in the sexes as the deviation in each of the 3 lines from the mean of the four C lines, and as the deviation of line 9 from the average of lines 7 and 8. We also measured the cumulative selection differential in units of standard phenotypic deviation from hybrid generation 1.

2.6. Genetic correlation

We measured the genetic correlation between wheel-running speed and duration by using cross-covariances between offspring and mid-parent values and by use of the "animal model". In the first, we used the same residuals used in heritability estimates, with mean offspring values averaged for each family and mid-parent values averaged between the dam and sire. Genetic correlation was calculated as the mean of two cross-covariance estimates based on the equation below (Roff 1997 p. 81): 1, the covariance of offspring duration and mid-parent speed, and 2, the covariance of offspring speed and mid-parent duration.

$$r_{A\,XY} = \frac{COV_{XY}}{\sqrt{COV_{XX} \times COV_{YY}}}$$

$$r_{A\,YX} = \frac{COV_{YX}}{\sqrt{COV_{XX} \times COV_{YY}}}$$

Cov = covariances, X = duration of wheel running, Y = speed of wheel running. The first letter (X or Y) after Cov refers to the trait in offspring and the second character refers to the average trait of the parents. The standard error for the genetic correlation was calculated as (Roff 1997):

$$SE = \frac{1 - r_A^2}{\sqrt{2}} \times \sqrt{\frac{SEh_X^2 \times SEh_Y^2}{h_X^2 \times h_Y^2}}$$

We also estimated the genetic correlation between running speed and duration in the base population of the HR selection experiment (generation 0 in Swallow et al. 1998)

by cross-covariance of generations 0 (parents) and 1 (offspring). Values used were residuals of running speed and duration regressed on sex, number of toes cut for identification, age, z-transformed age squared, wheel freeness, z-transformed wheel freeness squared, testing batch and room, generation, and the interaction of sex and generation. All but the last two factors in the regressions were used in previous estimates of heritability in the base population by offspring-on-parent regressions (Swallow et al. 1998). Note, the factors are different from those used to obtain residuals in lines 7, 8, and hybrid line 9 in the present study (for example, line was not included as a factor because the base population had not yet been separated into lines).

The second method to calculate genetic correlation was by use of the "animal model" to analyze wheel-running duration, speed, and the correlation between them by fixed effects of age, sex, measurement batch, F coefficient, and wheel freeness, and variance components of common maternal environment (i.e., identity of the mouse's dam), additive genetic variance (i.e., the identity of the mouse linked with the pedigree), and residual variance. The general variance component models used were general correlation models ("corgh") or unstructured general covariance matrix models ("us"). Traits were standardized to z-scores (mean = 0, sd = 1) separately in each line within each generation. Confidence intervals for the variance components were estimated using profile likelihoods with the R package nadiv (Wolak 2012). Analyses were pooled for both sexes.

3. Results

3.1. Parental effects in the F_1 and grand-parental effects in the F_2

In the first generation of the hybrid line (F_1), no parent-of-origin effect was apparent for wheel-running distance, duration, or speed (Fig. 2.1: left panels). That is, the reciprocal crosses were not different from each other.

The F_2 revealed interesting grand-parent-of-origin effects. In particular, F_2 females had significantly increased speed ($p = 0.0155$) when the mother was from the F_1 cross 8F × 7M compared with F_2 females with mothers from the F_1 cross 7F × 8M (Fig. 2.1: Revolutions per minute, right panel). In addition, F_2 males whose mothers were the F_1 cross 8F × 7M and whose fathers were the F_1 cross 7F × 8M tended to have reduced running distance ($p = 0.0580$) and had significantly reduced running speed ($p = 0.0121$) compared with males from the other three F_2 crosses (Fig. 2.1: right panels).

Body mass had no apparent parent-of-origin or grand-parent-of-origin effects (Fig. 2.3). All reciprocal groups were intermediate to the two purebred HR lines.

3.2. Total wheel running

In the F_1 generation, the hybrid line had significantly increased total wheel revolutions per day compared to the average of lines 7 and 8, for both females and males (Figs. 1, 2). In females, the hybrid line was also significantly different from each parental line, but in males, the hybrid line was only statistically different from parental HR line 7, not line 8.

In successive generations, wheel running in the hybrid line generally declined to that of the parental lines (Fig. 2.2). Specifically, in females, the hybrid line ran significantly more revolutions/day compared to the average of the parental lines in generation 3 and 7. In males, the hybrid line ran statistically more revolutions than the average of the parental lines for the first 5 generations. In generation 3, the hybrid line was also significantly different from both parental lines. However, from generation 6 on, the males of the hybrid line did not differ in wheel running from the parental lines.

Wheel running shows considerable variation across generations, with all three lines following the same pattern (e.g., dip in generations 7 and 10). This variation is assumed to be due to intergenerational environmental fluctuations of unknown origin, as well as some amount of apparently endogenous seasonal variation, which is also present in control lines (Careau et al. 2013). One way to control for this variation is to calculate the selective gain by subtracting the average wheel running in control lines to the wheel running in each HR line, which reveal the same pattern either as function of generation (Fig. 2.4; top panels) or cumulative selection differential (Fig. 2.4; bottom panels). That is, the hybrid line starts with higher selective gain than lines 7 and 8, but that difference gradually diminishes.

3.3. Duration of wheel running

Wheel-running duration (minutes per day) was measured as the number of 1-minute intervals for which the mice showed at least one revolution (Swallow et al. 1998). Generally, the hybrid line was intermediate or lower than parental lines in running

duration (Fig. 2.2). Specifically, in females, the hybrid line ran for significantly less time compared to the average of the parental lines in 6 of the 10 generations. In generations 4 and 10, the hybrid line ran significantly fewer minutes per day compared with each parental line. In males, the hybrid line did not differ significantly from the average of the parental lines except in generation 6 (when they ran the same minutes per day as HR line 7).

3.4. Average speed of wheel running

Average wheel-running speed (revolutions per minute) was measured as the number of revolutions per day divided by the number of 1-minute intervals for which the mice were active per day. Generally, the hybrid line ran at higher speeds than the parental lines (Fig. 2.2). Specifically, in females, the hybrid line ran at significantly higher average speed for all 10 generations compared to the average of the parental lines. At 5 of those time points (generation 1, 6, 7, 9, and 10), the hybrid line also had significantly higher speeds compared with each parental line. In males, the hybrid line had higher average running speed for the first 9 generations compared with the average of the parental lines, but was intermediate in the 10th generation. For 4 of those generations (1, 2, 3, and 5), the hybrid line was had significantly higher speeds compared with each parental line.

3.5. Maximum speed of wheel running

The maximum wheel-running speed (maximum revolutions per minute) was measured as the highest number of revolutions run in any 1-minute interval, averaged between day 5 and 6. Following the trend for average speed, the hybrid line had higher maximum running speed compared to the parental lines (Fig. 2.2). In females, the hybrid line had significantly higher maximum speeds for all 10 generations compared to the average of the parental lines. At 6 of those time points (generation 1, 2, 4, 5, 6, and 10), the hybrid line also had significantly higher speeds compared with each parental line. For males, the hybrid line also had significantly higher maximum speed for all 10 generations. For the first 7 generations, the hybrid line had significantly higher speeds than each parental line.

3.6. Body mass

Adult body mass (measured before wheel access) of the hybrid line was intermediate to the parental lines for most generations in both females and males (Fig. 2.3). Specifically, in females, the hybrid line did not differ significantly in body mass compared to the average of the parental lines, except in the last 3 generations. For the last 3 generations of renewed selection, the hybrid line had higher body mass than the average of lines 7 and 8, but only differed significantly from line 7.

In males, the hybrid line did not differ significantly in body mass compared to the average of the parental lines, except in 3 generations. In generation 3, the hybrid line had lower body mass than the average of lines 7 and 8, but only differed significantly from

line 8. In generations 8 and 9, the hybrid line had higher body mass compared to the average of the parental lines, but only differed significantly from line 7.

3.7. Heritability estimates

Considering data and pedigree information for generations 69 to 78, total daily wheel running was not significantly heritable for either parental line or for the hybrid line, except in females of HR line 7 (Table 2.1, 2.2).

The two components of wheel running, duration and average speed, showed a more complicated pattern. Overall, wheel-running duration was heritable for HR line 9 (Table 2.1, 2.2; except females in Table 2.2) and line 8 males (Table 2.1, 2.2). In estimates using offspring-on-parent regressions, duration was also heritable for line 8 when sexes were pooled (Table 2.1). In addition, in estimates using the animal model, duration was heritable for HR line 7 females (Table 2.2).

Average wheel-running speed was heritable for HR line 7 females, line 8 males, and line 9 (pooled sexes; Table 2.1, 2.2). In addition, the estimates of h^2 for speed from offspring-on-mid-parent regression was significant for line 7 (pooled sexes), line 8 (pooled sexes), and line 9 females (Table 2.1).

Adult body mass prior to wheel testing was heritable for HR line 9 and line 8 when sexes were pooled (Table 2.1, 2.2). The estimate from daughters-on-dams regression was non-zero for line 8 females (Table 2.1). In addition, heritability estimates from the animal model were non-zero for pooled sexes in line 7 (Table 2.2).

3.8. Genetic correlation

Wheel-running duration and speed had a significant negative genetic correlation in hybrid line 9, estimated both by cross-covariances between offspring and mid-parent and by use of "animal models" (Table 2.3, 2.4). For HR line 7, the genetic correlation could not be estimated due to negative (Table 2.1) or low (Table 2.2) genetic variance for duration of running (Table 2.3, 2.4). For line 8, the estimated genetic correlation was negative, but the standard error was too large to bound the estimate away from zero in two of the three methodologies used (Table 2.3, 2.4). Estimates of genetic correlation in the base population were positive with a large standard error (not significantly different from zero, Table 2.3, 2.4).

4. Discussion

The purpose of the present study was to attempt to break a selection limit reached in a long-term selection experiment for high voluntary wheel running in mice. By breaking this limit, we might better understand the reason for the selection limit in the selected lines and further elucidate the underlying genetic architecture of wheel running and its component traits. After crossing two of four replicate High Runner (HR) lines, heterosis for wheel-running distance was confirmed in the hybrid F_1 for both sexes (as in Hannon et al. 2011). However, even with subsequent selection, the hybrid line did not break the prevailing selection limit of the parental HR lines 7 and 8 (line numbers are lab designations). Further examination of the component traits of wheel running, average

speed and duration, led to interesting hypotheses regarding their genetic architecture. In addition, the observed patterns of heterosis were sex-specific, indicating underlying sex and line differences in the traits affecting wheel running in the parental lines, as reported previously (Garland et al. 2011).

4.1. Parental and grand-parental effects

Analyses of parental effects on wheel running (total, duration, average speed, and maximum speed) demonstrated no parent-of-origin effects in the F_1 generation. That is, the reciprocal F_1 hybrids showed no statistical difference from one another. Previous research reported parent-of-origin effects in a reciprocal cross between HR line 8 and a control line (Hannon 2010) and in an intercross population between HR line 8 and inbred C57BL/6J (Kelly et al. 2010a). This discrepancy may be due to the fact that mice from HR line 8 were much more similar to those from HR line 7 than Control or C57BL/6J mice.

In the F_2 population, however, we found differences between reciprocal hybrids for total wheel running, speed, and maximum speed. These grand-parental effects were further mediated by sex. Specifically, F_2 female mice whose mothers were 7F×8M hybrids had lower total wheel running and speed than mice whose mothers were 8F×7M hybrids (although these mothers themselves were did not show any differences in the F_1), and this was true regardless of the father's cross-type. Reciprocal crosses of male F_2 hybrids were not different, expect for one specific cross-type (maternal 8F×7M × paternal 7F×8M) which had reduced total wheel running and speed. The mechanism for grand-

parental effects in the absence of parent-of-origin effects is unclear and beyond the scope of the current study. Some potential mechanisms to explain the sex differences in grand-parental effects (i.e., female vs. male grand-offspring of the same cross-type) is that the allelic combinations (or regulating mechanisms of these combinations), might be found on the X chromosome, mitochondrial DNA, or modulated by epigenetic mechanisms. Discussion of these sex-dependent mechanisms can be found elsewhere (Kelly et al. 2010a).

4.2. Wheel-running duration vs. speed

The heterosis observed for wheel running was apparently achieved via increase in average running speed, not the number of active minutes per day (i.e., duration). This was also observed for the previous F_1 cross (Hannon et al. 2011). Previous QTL analyses with an advanced intercross population of mice generated from HR line 8 and C57BL/6J mice revealed that running speed and duration were affected by different loci in the genome (Kelly et al. 2010b). (Others have reported co-localized QTL for running speed and duration, but they used a cross of two inbred strains (C57BL/6J and C3H/HeJ) and measured wheel running over 21 days instead of 6 days (Leamy et al. 2008; Lightfoot et al. 2008).)

Running duration was intermediate in the hybrid line, or even lower in some generations in females. The observed depression in running duration suggests separation of beneficial allele combinations via recombination (termed hybrid breakdown) and/or

that Dobzhansky-Muller incompatibilities were generated (termed outbreeding depression) (Charlesworth and Willis 2009).

On the other hand, the hybrid line had increased average running speed compared with the two parental lines. As outlined in the Introduction, this result suggests that the parental lines had some number of alleles fixed by genetic drift that were detrimental to running speed, but the two parental HR lines had different detrimental alleles. Thus, the hybrid line inherited alleles that facilitate higher running speed from both parental lines and renewed selection on the hybrid line purged the deleterious alleles found in parental lines (Charlesworth and Willis 2009). This possibility, however, should have allowed the hybrid line to break the selection limit, which was not observed. One explanation for this contradiction is that hybrid vigor for wheel-running speed was equally matched with hybrid breakdown / outbreeding depression, but this rationalization remains highly speculative at this time.

4.3. Heritability estimates

Heritability for wheel running was mostly depleted in HR lines 7 and 8 by the start of the hybrid experiment (Table 2.1). Although the estimate for females in line 7 could be bounded away from zero (Table 2.1, 2.2), this may have been inflated by non-genetic maternal effects. Low heritabilities were not unexpected because these lines had undergone 68 generations of directional selection prior to the creation of the hybrid line, although they had maintained heritability at least up until generation ~20 (Careau et al.

2013). (Bult and Lynch (2000) had also estimated non-zero heritabilities for nest building in their selected lines at selection limits.)

Contrary to our prediction, estimates from both offspring-on-parent and animal model analyses indicated that heritability for wheel running was not increased in the hybrid line, as compared with the two parental lines. The lack of heritability would appear to be an obvious explanation for the lack of response to continued selection. (Had we known heritability was not increasing above zero, we could have predicted no response, but that was not clear until after applying selection for at least a few generations.)

Despite the lack of heritability in wheel running, heritability was actually increased in the hybrid line for both wheel-running duration and average speed (Table 2.1, 2.2). As total wheel running is the product of duration and average speed, the presence of heritability for each component trait might suggest heritability for the composite trait. However, this was not found in our experiment.

4.4. Evolution of negative genetic correlation between duration and speed of running

In the base population of the selection experiment, wheel-running duration and speed were genetically uncorrelated or perhaps weakly positively correlated (Table 2.4). In the generations used in the present experiment, duration and speed in HR lines 7 and 8 were negatively uncorrelated (Tables 2.3 and 2.4). Using all three estimation methods, the genetic correlation between duration and speed was also negative for hybrid HR line 9. The negative genetic correlation could explain the presence of heritability for duration

and speed but not wheel running and thus the inability for hybrid line to break the selection limit despite renewed genetic variance. The negative genetic correlation could be caused by linkage disequilibrium or pleiotropy of alleles with opposite effects for the component traits. In both cases, alleles with positive effects for running duration and negative effects for running speed (or vice versa) would inherited together. That is, the evolution of negative pleiotropy for two traits under selection might explain a selection limit even with the maintenance of additive genetic variance (Falconer 1981).

4.5. Sex differences

As mentioned above, wheel-running duration, which did not exhibit heterosis, was differentially affected in female vs. male hybrid mice. In addition, sex-specific effects were observed in the heritability estimates, further indicating the different underlying genetic architecture of wheel running and its component traits between the two sexes. One potential interpretation is that some alleles that affect running duration may be connected to sex chromosomes. Counter to this hypothesis, no sex-specific QTL were found for wheel running and component traits in a study of an advanced intercross population of HR line 8 and C57BL/6J mice (Kelly et al. 2010b). However, interpretation is limited because they used just 30 markers on the X chromosome and no markers on the Y chromosome (Kelly et al. 2010b). Another study utilizing the same intercross at a later generation reported 10 QTL for exercise across 20 chromosomes (including the X chromosome), but none of these interacted with sex (Leamy et al. 2012).

Other potential mechanisms of sex differences (mitochondrial DNA, epigenetics, or environmental effects) are discussed elsewhere (Kelly et al. 2010). Identifying the specific mechanism of the sex-specific heterosis is outside the scope of the current study, but future analyses of the genetic samples of these mice will yield insight into potential mechanisms of sex differences.

4.6. Body mass

In the first few generations, the hybrid line had intermediate values of body mass compared with HR line 7 and 8, indicating additive inheritance (Falconer 1955). A hybrid cross study of wild house mice and ICR mice also reported intermediate values for body mass in the hybrids (only measured F_1, (Dohm et al. 1994)). After the first few generations, however, the hybrid line became more similar to HR line 8, implying net dominance of the alleles found in HR line 8 for body mass. Heritability was found for body mass in HR line 8 (pooled sexes) and line 9 (pooled sexes and males; Table 2.1).

4.7. Comparison with Bult and Lynch (2000)

The current study was inspired by the hybrid cross experiments of Bult and Lynch (2000). Similarities included long-term selection for a behavioral trait, similar population sizes and replicated selected lines, using within-family selection, selection limits being reached at generation ~20, and genetic variance being maintained at the limit. At generation 46, they created hybrid lines by crossing their selected lines and were able to break the selection limits after 10 generations of renewed selection (Bult and Lynch

2000). Despite the similarities in these studies, our hybrid line did not break the selection limit. Aside from the obvious difference between these studies in the behavior under selection (nest building vs. wheel running) and direction of selection (high and low vs. only high selected lines), other discrepancies may have contributed to the difference in outcome. Bult and Lynch (2000) used different mice for the base population of their experiment (outbred HS/Ibg stock of an eight-way cross among inbred strains vs. outbred Hsd:ICR mice) and used two replicate hybrid lines (for both high and low selected lines) once they renewed selection, whereas we only had one hybrid line.

Perhaps most importantly, Bult and Lynch (2000) allowed random mating in the first 3 generations of the hybrid line before renewing selection for 10 more generations. In our experiment, we opted to select on the hybrid line from hybrid generation 1. This was partly due to limitations based on the number of mice we could keep for this experiment while maintaining the other selected and control lines of the selection experiment. Although we did not have random mating, the factorial design in creating the F_2 allowed some allele mixing. In addition, we tested these first few generations for wheel running and were able to test for the expected increase in variance in the first 3 generations of the hybrid line, unlike Bult and Lynch (2000).

4.8. Concluding remarks

Genetic architecture (specifically negative genetic correlation between wheel-running component traits duration and speed) constrained the hybrid line from increasing voluntary wheel running beyond the selection limit experienced by the parental lines.

Even with renewed genetic variation for duration and speed of wheel running, hybrids were not able to break the selection limit on total wheel running, because hybrid vigor was countered by one or more forms hybrid depression. That is, the two benefits of a hybrid line (1. reduction of slightly deleterious homozygous alleles found in parental lines after generations of inbreeding, and 2. new, beneficial combinations of genes) may have been outweighed by breaking up good combinations (i.e., favored by past selection) that were already in each parental line or by the creation of new, harmful combinations of alleles. Aside from the issue of hybrid vigor versus depression, the possible contributions of dominance, overdominance or pseudo-overdominance to the observed heterosis for wheel running in the first few generations are unknown. Samples of breeders from all 10 generations of the hybrid line have been preserved for future genomic analyses, which may uncover these genetic mechanisms.

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Tables and Figures

Table 2.1. Heritability estimates ± standard errors from offspring-on-parent regressions, with numbers of parents and offspring in parentheses.

	HR 7	HR 8	HR 9
Total wheel running			
Both sexes	0.10 ± 0.06 (115, 934)	0.12 ± 0.08 (119, 869)	0.07 ± 0.10 (126, 1072)
Females	**0.24 ± 0.09 (114, 455)**	0.13 ± 0.10 (117, 436)	0.12 ± 0.08 (124, 529)
Males	-0.05 ± 0.08 (114, 479)	0.10 ± 0.12 (117, 433)	0.13 ± 0.10 (126, 543)
Duration			
Both sexes	-0.09 ± 0.06 (115, 934)	**0.14 ± 0.06** (119, 869)	**0.24 ± 0.06** (126, 1072)
Females	-0.09 ± 0.08 (114, 455)	0.08 ± 0.08 (117, 436)	**0.16 ± 0.07** (124, 529)
Males	-0.09 ± 0.08 (114, 479)	**0.20 ± 0.09** (117, 433)	**0.32 ± 0.09** (126, 543)
Speed			
Both sexes	**0.16 ± 0.05** (115, 934)	**0.13 ± 0.06** (119, 869)	**0.15 ± 0.06** (126, 1072)
Females	**0.22 ± 0.07** (114, 455)	0.04 ± 0.10 (117, 436)	**0.22 ± 0.09** (124, 529)
Males	0.10 ± 0.07 (114, 479)	**0.22 ± 0.09** (117, 433)	0.09 ± 0.09 (126, 543)
Adult body mass			
Both sexes	-0.21 ± 0.07 (115, 934)	**0.15 ± 0.07** (119, 869)	**0.22 ± 0.06** (126, 1072)
Females	-0.15 ± 0.10 (114, 455)	**0.22 ± 0.10** (117, 436)	**0.18 ± 0.09** (124, 529)
Males	-0.26 ± 0.09 (114, 479)	0.08 ± 0.09 (117, 433)	**0.26 ± 0.09** (126, 543)

Analyses use all generations pooled and residuals from a fixed-effects model outlined in the Methods. Analyses were done separately by sex (females = daughters and dams, males = sons and sires) or pooled for both sexes (offspring and mid-parent values; although if trait variances differed significantly by sex, the estimate given is the average of estimates from females and males). **In bold:** Estimate is greater than zero and the 95% confidence interval (estimate ± 2 x standard error) excludes zero.

Table 2.2. Narrow-sense heritability estimates ± standard errors from analyses of variance components using a pedigree-based linear mixed-model, with number of individuals in parentheses.
Data were analyzed in the "animal model" with fixed effects (age, sex, measurement batch, F coefficient, and wheel freeness) and variance components of common maternal environment (i.e., identity of the mouse's dam), additive genetic variance (i.e., the identity of the mouse linked with the pedigree), and residual variance. The "animal model" makes inference back to the starting population, so the pedigree was cut to only include hybrid generation 1. To correct for known relatedness between individuals at hybrid generation 1, each individual in that generation was given the known starting inbreeding coefficient (F) according to analysis of the entire pedigree. Traits were standardized to z-scores (mean=0, sd=1) separately in each line within each generation. Narrow-sense heritability was calculated as the ratio of the additive genetic variance component divided by the sum of all variance components. Confidence intervals for the variance components were estimated using profile likelihoods with the R package nadiv (Wolak 2012). Analyses were done separately by sex or pooled for both sexes. Total number of individuals used in the analyses are shown in parentheses. In bold: Estimate is greater than zero and the 95% confidence interval (estimate ± 2 x standard error) excludes zero.

Table 2.2.

	HR 7	HR 8	HR 9
Total wheel running			
Both sexes	0.02 ± 0.02 (863)	0.00 ± 0.02 (777)	0.07 ± 0.04 (917)
Females	**0.22 ± 0.06** (420)	Boundary (386)	0.10 ± 0.07 (455)
Males	Boundary (443)	0.09 ± 0.05 (391)	0.12 ± 0.07 (462)
Duration			
Both sexes	0.00 ± 0.02 (863)	0.05 ± 0.04 (777)	**0.16 ± 0.05** (917)
Females	**0.15 ± 0.07** (420)	0.02 ± 0.05 (386)	0.12 ± 0.08 (455)
Males	Boundary (443)	**0.17 ± 0.07** (391)	**0.28 ± 0.08** (462)
Speed			
Both sexes	0.07 ± 0.03 (863)	0.07 ± 0.05 (777)	**0.10 ± 0.05** (917)
Females	**0.14 ± 0.05** (420)	0.03 ± 0.05 (386)	0.14 ± 0.08 (455)
Males	0.01 ± 0.04 (443)	**0.19 ± 0.06** (391)	0.06 ± 0.06 (462)
Adult body mass			
Both sexes	**0.15 ± 0.06** (863)	**0.24 ± 0.06** (777)	**0.21 ± 0.07** (917)
Females	0.07 ± 0.08 (420)	0.13 ± 0.09 (386)	**0.24 ± 0.09** (455)
Males	0.04 ± 0.07 (443)	Boundary (391)	**0.21 ± 0.10** (462)

Boundary = Unable to be estimated because the additive variance component was getting pushed to be negative to fit the model, but are constrained to be positive.

Table 2.3. Genetic correlation between speed and duration from analyses of offspring and mid-parent cross-covariances.

	Covariance (Cov) between offspring and mid-parent				Additive genetic correlation (r_A)			Standard error
	Duration in offspring and speed in parents	Speed in offspring and duration in parents	Duration in offspring and duration in parents	Speed in offspring and speed in parents	Duration in offspring and speed in parents	Speed in offspring and duration in parents	Duration vs speed	
	Cov_{XY}	Cov_{YX}	Cov_{XX}	Cov_{YY}	$r_{A\,XY}$ †	$r_{A\,YX}$ †	r_A	r_A SE ‡
Base population	11.43	10.76	453.26	2.02	0.3776	0.3555	0.3666	0.2553
Line 7	-16.82	-16.58	-389.44	2.36	Cannot compute	Cannot compute	NA	NA
Line 8	-17.59	0.16	794.87	0.79	-0.7030	0.0063	-0.3483	0.3830
Line 9	-14.46	-55.30	1218.06	1.58	-0.3299	-1.2615	**-0.7957**	**0.2520**

† $r_{A\,XY} = Cov_{XY} / \text{sqrt}(Cov_{XX} \times Cov_{YY})$ and $r_{A\,XY} = Cov_{YX} / \text{sqrt}(Cov_{XX} \times Cov_{YY})$ (Roff 1997)

‡ $SE = ((1 - r^2_A)/\text{sqrt}(2)) * (\text{sqrt}(se.h^2_X * se.h^2_Y/(h^2_X * h^2_Y))$ (Roff 1997)

Genetic correlation between running speed and duration was significantly negative in line 9 (in bold; 95% confidence interval [estimate ± 2 x standard error] is bound away from zero). In line 7, r_A could not be computed because a negative Cov_{XX} forced a square-root of a negative number. The estimate for line 8 was negative, but could not be bound away from zero. The base population had a positive genetic correlation (although not bound away from zero), indicating that negative genetic correlation evolved in HR lines. Analyses used all generations pooled and residuals from a fixed-effects model outlined in the Methods. Mean offspring values were used for each family and mid-parent values were the mean value of the dam and sire. The overall genetic correlation was calculated as the average of two cross-correlations. The first character after Cov refers to the trait in offspring and the second character refers to the average trait of the parents (X = duration, Y = speed). **In bold:** Estimate is greater than zero and the 95% confidence interval excludes zero.

Table 2.4. Genetic correlation between speed and duration from animal model analyses.

Estimate of genetic correlation using general correlation models ("corgh")			
	r_A	se	Chi^2 p-value
Base population	0.4905	0.5535	0.4390
Line 7	-1.0000†	NA	0.9258
Line 8	-1.0000†	NA	0.0367
Line 9	-0.5744	0.2482	**0.0189**
Line 9 (F_3+)	-0.5664	0.3190	0.1020
Estimate of genetic correlation using unstructured general covariance matrix models ("us")			
	r_A	se	Chi^2 p-value
Base population	0.4905	0.5535	0.4390
Line 7	-0.9405†	0.8571	0.3612
Line 8	-0.8619	0.4702	0.0310
Line 9	-0.5774	0.2628	**0.0113**
Line 9 (F_3+)	-0.5653	0.3577	0.0992

†Low genetic variance in line 7 gives uncertainty to these estimates. The warning message from ASReml is: "Boundary parameter: confidence interval estimation may produce strange behavior - proceed with caution"

Genetic correlations (r_A) between speed and duration were positive in the base population and significantly negative in line 9 (significance from chi-square tests). Estimates for line 7 were unreliable due to low genetic variance. For line 8, the estimate for genetic correlation was significantly negative. Genetic correlations between speed and duration were analyzed in the "animal model" with fixed effects (age, sex, measurement batch, F coefficient, and wheel freeness) and variance components of common maternal environment (i.e., identity of the mouse's dam), additive genetic variance (i.e., the identity of the mouse linked with the pedigree), and residual variance. The general variance component models used were general correlation models (corgh) or unstructured general covariance matrix models (us). The "animal model" makes inference back to the starting population, so the pedigree was cut to only include hybrid generation 1. To correct for known relatedness between individuals at hybrid generation 1, each individual in that generation was given the known starting inbreeding coefficient (*F*) according to analysis of the entire pedigree. Traits were standardized to z-scores (mean=0, sd=1) separately in each line within each generation. Analyses were pooled for both sexes.

Figure Legends

Fig. 2.1. Wheel running and component traits for hybrid generations 1 and 2 (measured as mean of days 5+6 of a 6-day exposure to wheels). Values are least-squares means ± standard errors from analysis of covariance models in SAS Procedure Mixed, performed separately for the two sexes. 7 × 7 and 8 × 8 denote purebred mice from HR lines 7 and 8. Left panels are mice from hybrid generation 1 and show purebred mice and reciprocal hybrid crosses. Right panels are mice from hybrid generation 2 and show purebred mice and 4-way crosses of the reciprocal hybrid mice. For example, 7Fx8M × 8Fx7M denotes offspring from crosses of females from the F_1 reciprocal 7F × 8M with males from the F_1 reciprocal 8F × 7M. No parent-of-origin effect was apparent for wheel-running distance or component traits in the reciprocal hybrids ($p > 0.05$ for contrast between 7F × 8M vs. 8F × 7M in both sexes). Grand-parental effects were apparent for females whose mothers were from the F_1 cross 8F × 7M compared with females whose mothers were from the F_1 cross 7F × 8M, with significantly increased running speed ($p = 0.0155$), but no difference in total wheel-running distance ($p = 0.1376$) or duration ($p = 0.7396$). Males whose mothers were the F_1 cross 8F × 7M and fathers were the F_1 cross 7F × 8M tended to have reduced running distance ($p = 0.0580$) and had significantly reduced speed ($p = 0.0121$) compared with males from the other three F_2 crosses.

Fig. 2.2. Wheel-running activity for hybrid generations 1 through 10, measured as days 5 and 6 of a 6-day exposure to wheels attached to standard housing cages. Values are least-squares means ± standard errors from analysis of covariance models in SAS Procedure Mixed, performed separately for the two sexes. (Values for generation 1 are the same as in left panels from Figure 1; 2 markers indicate the reciprocal hybrid crosses. Values for generation 2 are the same as in right panels from Figure 1; 4 markers indicate the 2-way crosses of the reciprocal hybrids.) Parental lines are in grey (HR 7 open, HR 8 filled) and the hybrid line is in black. Asterisks (*) indicate when hybrid line 9 was significantly different ($P < 0.05$) from line 7, line 8, and the average of lines 7+8. Ampersand (&) symbols indicate when hybrid line 9 was significantly different from one parental line and the average of lines 7+8.

Fig. 2.3. Body mass at the start of wheel exposure. Values are least-squares means ± standard errors from analysis of covariance models in SAS Procedure Mixed, performed separately for the two sexes. **Top left)** Mice from hybrid generation 1, showing purebred mice and reciprocal hybrid crosses. **Top right)** Mice from hybrid generation 2, showing purebred mice and the 4-way crosses of the reciprocal hybrid mice. **Bottom panels)** Body mass, separated by sex, for hybrid generations 1 through 10. Note that values for generation 1 are the same as in top left panel of this figure and values for generation 2 are the same as in top right panel. Body mass is missing for generation 5 due to a broken scale. Parental lines are in grey (HR 7 open, HR 8 filled) and the hybrid line is in black. Asterisks (*) indicate when hybrid line 9 was significantly different ($P < 0.05$) from line

7, line 8, and the average of lines 7+8. Ampersand (&) symbols indicate when hybrid line 9 was significantly different from one parental line and the average of lines 7+8.

Fig. 2.4. Cumulative response to directional selection (i.e., selective gain) as a function of hybrid generations 1 to 10 (**top panels**) or the cumulative selection differential (in units of standard phenotypic deviation; **bottom panels**). Selective gain was measured separately by sex as the deviation of each selected line 7, 8, and 9 from the mean of the four control lines (panels "**females**" and "**males**"). Selected gain in the hybrid line 9 was additionally measured as the deviation of line 9 from the average of lines 7 and 8 (panel "**line 9 vs 7&8**").

Fig. 2.1. Wheel running and component traits for hybrid generations 1 and 2.

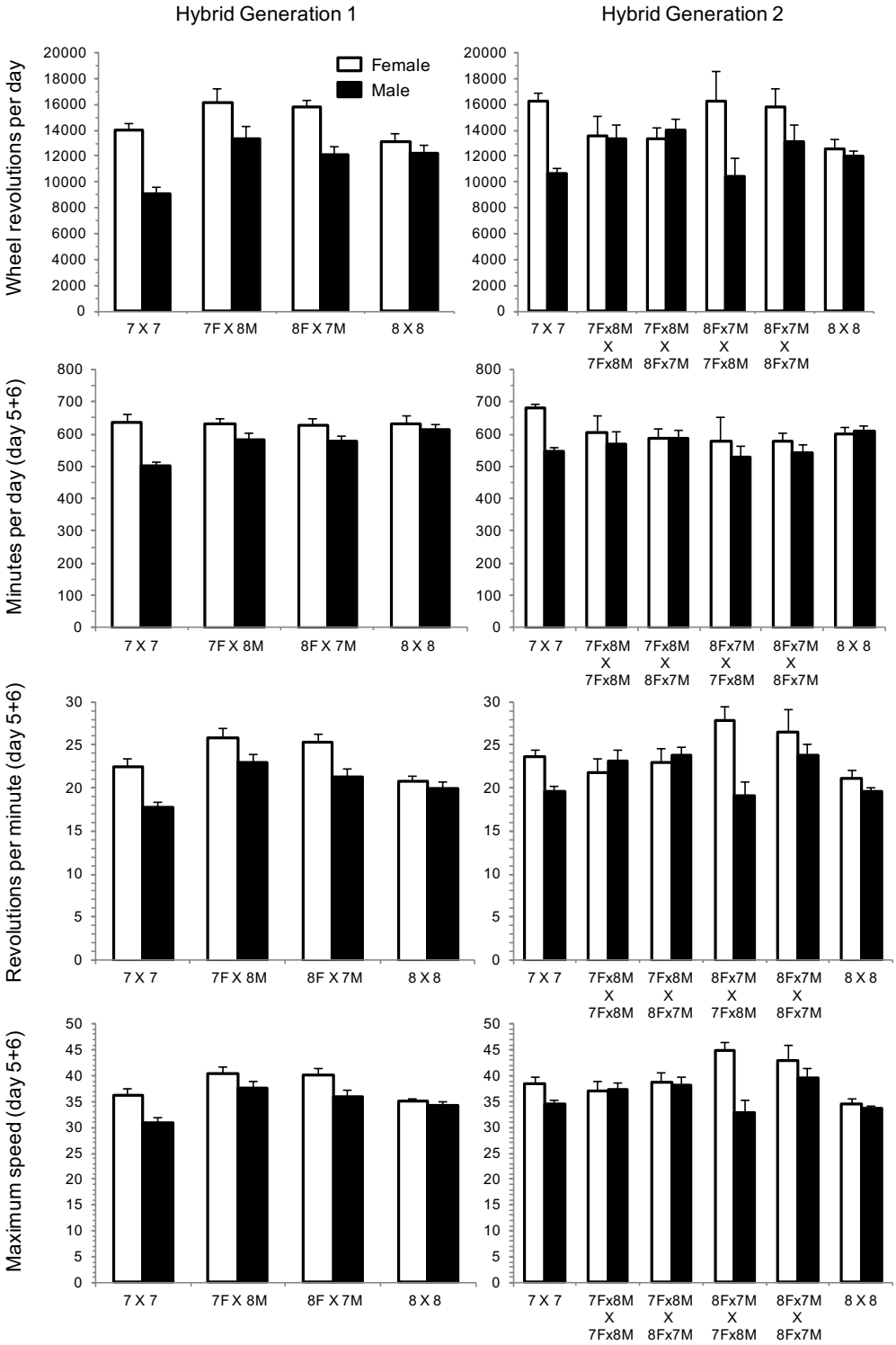


Fig. 2.2. Wheel-running and component traits for hybrid generations 1 through 10.

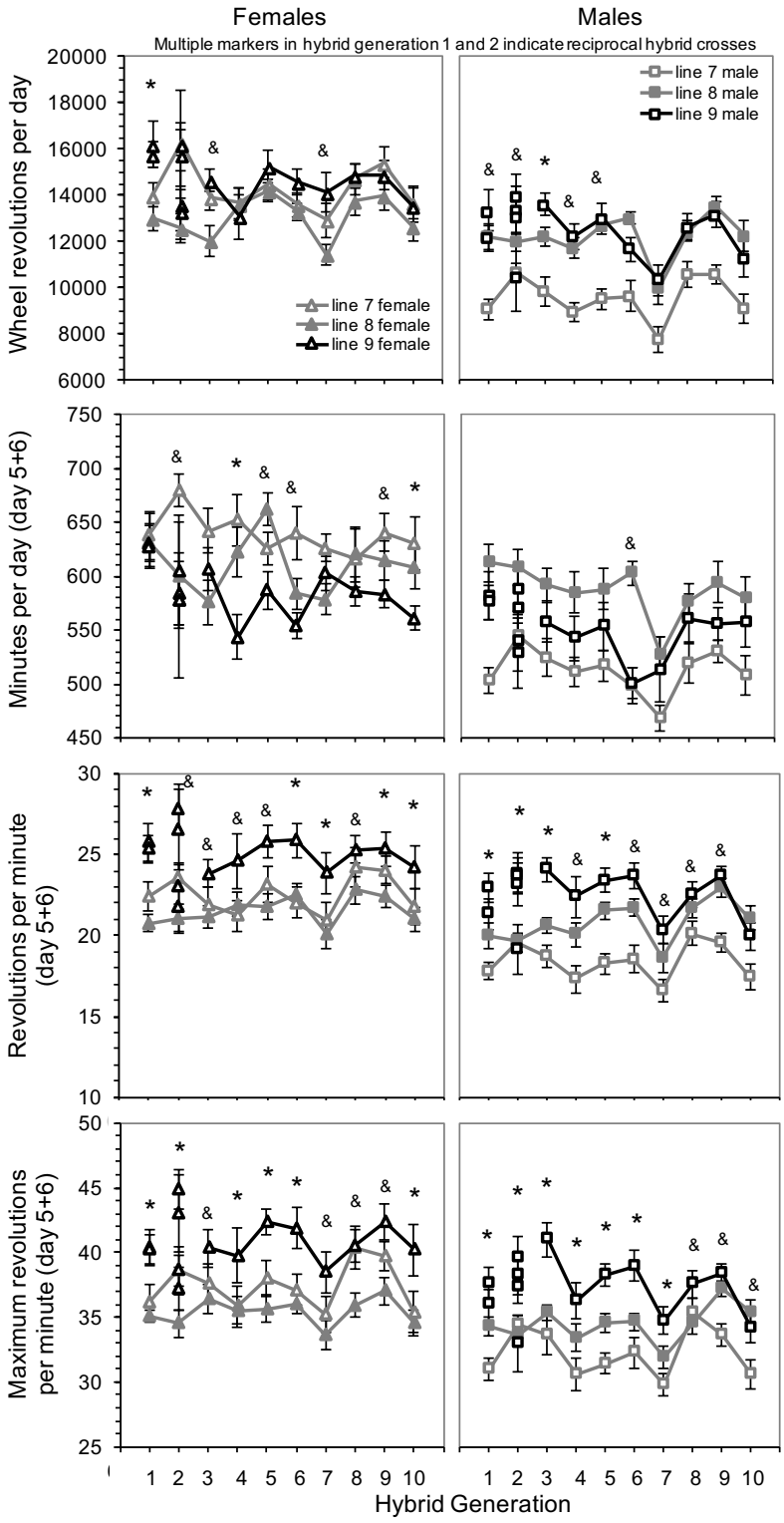


Fig. 2.3. Body mass at the start of wheel exposure.

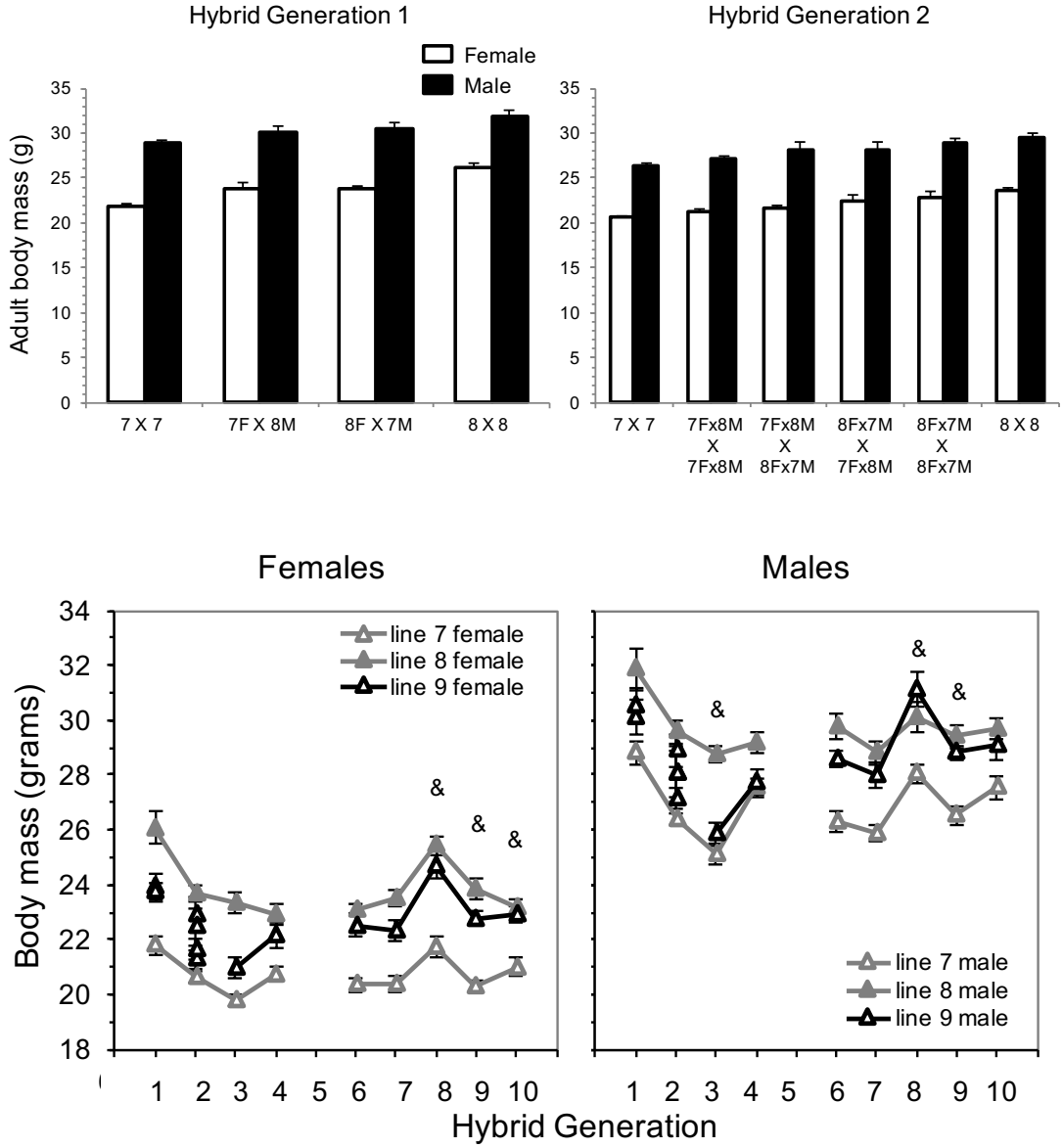
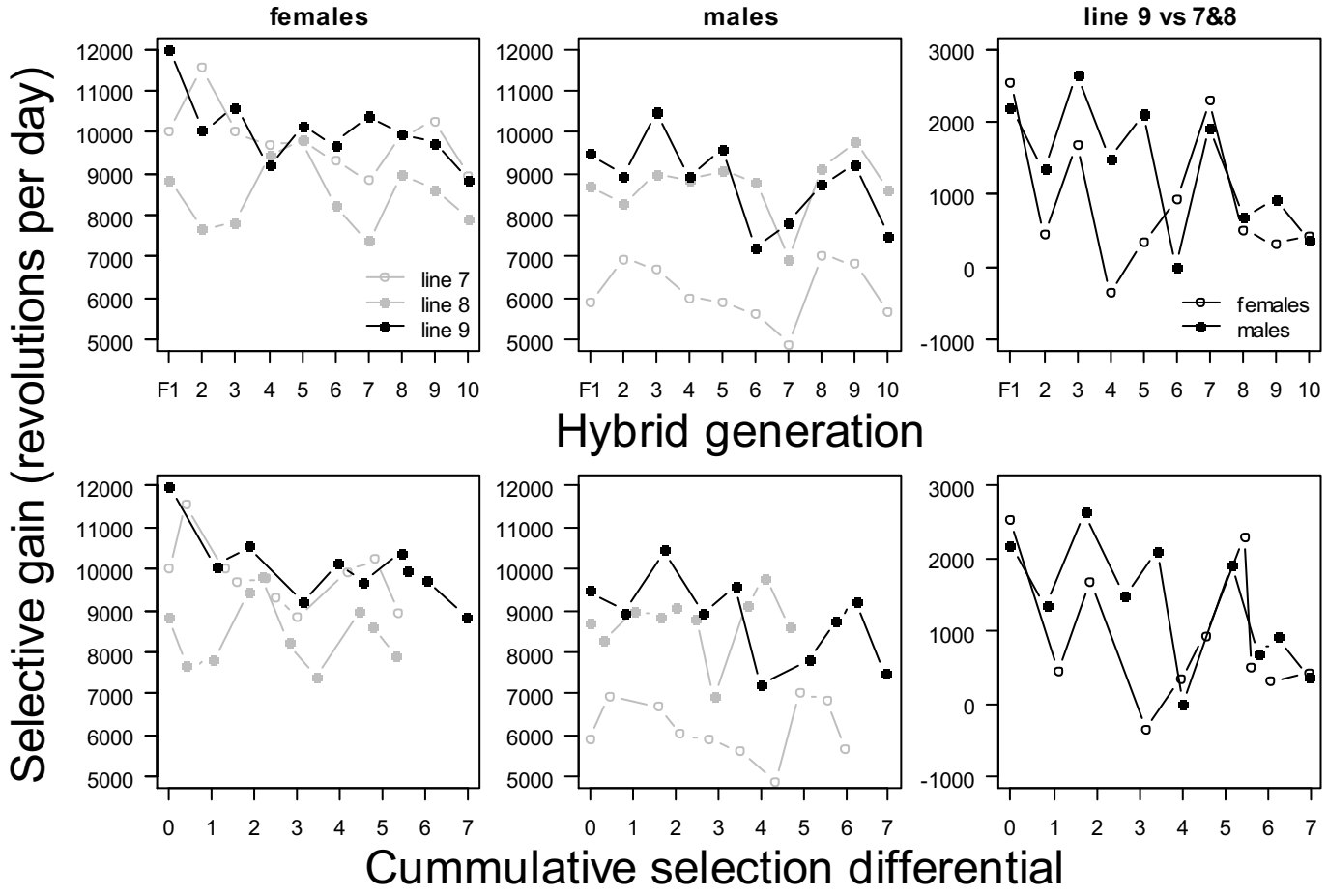


Fig. 2.4. Cumulative response to directional selection.



Chapter 3

Mice selectively bred for high wheel running conserve more fat despite increased exercise

Layla Hiramatsu and Theodore Garland, Jr.

Abstract

Physical activity is an important component of energy expenditure, and acute changes in activity can lead to energy imbalances that affect body composition, even under *ad libitum* food availability. One example of acute increases in physical activity is found in four replicate, selectively-bred High Runner (HR) lines of mice that voluntarily run ~3-fold more wheel revolution per day when given wheel access for six days, compared with four non-selected control (C) lines. Mice from the HR lines have a number of correlated responses to selection that relate to energy balance, including increased home-cage activity when wheels are absent, increased food consumption, and reduced total body and fat masses. The purpose of the present study was to (1) compare wheel running, cage activity, food consumption, and body composition between HR and C lines of both sexes (generation 77), (2) examine the interrelationships of those traits over a 6-day period of wheel access, and (3) determine if the phenotypic architecture of these traits differed between linetype and/or between the sexes. In general, we expected that voluntary exercise would increase food consumption, build lean mass, and reduce fat mass, but that these effects would likely differ between the sexes or between HR and C lines of mice. In addition, we expected the phenotypic architecture to differ among groups. Before wheel testing, HR mice weighed less than C mice, primarily due to reduced lean mass, and females were lighter than males, entirely due to lower lean mass. Over 6 days of wheel access, all groups tended to gain small amounts of lean mass, but lose fat mass, resulting in overall loss of total body mass and altered body composition. Mice from HR lines lost less fat than those from C lines, resulting in a convergence to a

fat mass of ~1.7 g for all 4 groups. HR mice lost less fat mass in spite of the fact that they were much more active on wheels (and slightly more active in home-cages). HR mice consumed significantly more food than C mice over the six days (with body mass as a covariate in statistical models), and this was true when accounting for their higher activity levels by use of wheel running and cage activity as additional covariates. No statistically significant sex-by-linetype interactions were observed for any of the foregoing traits. Structural equation models showed that the four sex-by-linetype groups differed considerably in the complex phenotypic architecture of these traits. Higher food consumption was associated with higher average running speeds in females, longer wheel-running duration in C males, and longer time spent active in home-cages in HR males. Lean change was unaffected by food consumption or physical activity (except with wheel-running duration in HR females). On the other hand, fat change was significantly increased by food consumption in all groups (all groups lost fat, so mice that ate more lost less fat) and affected by both voluntary exercise and cage activity, but these effects were different and sometimes conflicting among the four groups. Differences among groups by genetic background and sex are complex and lend support to the growing attention on personalized medicine for humans, especially in physical activity. This long-term selection experiment offers a unique model for studying the effects of voluntary exercise and spontaneous physical activity on body composition, and how these effects may depend on the overall level of activity and differ between the sexes.

1. Introduction

Imbalances between energy intake and expenditure cause changes in body mass and composition that can be mediated by body size, sex, and genetic background (Pomp et al. 2008; McAllister et al. 2009; Kelly et al. 2011). One important factor of energy expenditure is physical activity, the major components of which are voluntary exercise (VE) and spontaneous physical activity (SPA) (Garland et al. 2011b; Thompson et al. 2012; Teske et al. 2014). The definitions of VE and SPA are not always clear (review in Garland et al. 2011b). In humans, VE is generally self-evident and SPA is considered all other physical activity which is not VE, including fidgeting and pacing (although "gray areas" exist, e.g., physical education classes in primary school). In rodents, VE is recorded by wheel running (Sherwin 1998) and SPA is recorded by home cage activity (Garland et al. 2011b). The relative importance of VE and SPA as sources of energy expenditure varies among species and with environmental conditions, and also depending on whether variation in either type of activity is caused mainly by variation in frequency, duration or average intensity (e.g., Koteja et al. 1999; Copes et al. 2015).

When the level of VE or SPA increases, animals may compensate by reducing energy expenditure related to the other component or during other aspects of the daily lifecycle; alternatively or in addition, they may increase food consumption (Westerterp and Plasqui 2004; King et al. 2008; Garland et al. 2011b). Such adjustments may or may not lead to stability in body mass and composition, depending on how long the altered physical activity occurs and the availability of additional food, as well as the sophistication of the organism's homeostatic mechanisms, such as appetite (e.g., see

Blundell and King 1998; Piersma and Van Gils 2011). In general, animals that have evolved with a history of short-term changes in energy demand, as through temporarily increased levels of physical activity, would be expected to cope with those changes better than animals that are not adapted to such conditions. We tested this general proposition by comparison of lines of mice that vary genetically in levels of physical activity.

Specifically, we compared four replicate High Runner (HR) lines of mice selectively bred for increased wheel running during days 5 and 6 of a 6-day period of wheel access with four non-selected Control lines (Swallow et al. 1998). Mice from HR lines run 2.5-3 times more distance per day than C mice over the 6-day period of wheel access (e.g., Belter et al. 2004) and offer a unique model for studying the effects of acute increases in physical activity on (changes in) food consumption and body composition. Despite continued selection for increased levels of VE, all of the HR lines have been at a selection limit since generation 17-25, depending on line and sex (Careau et al. 2013). In principle, these limits could be related to an inability to maintain energy balance and body composition during the 6-day trial. Alternatively, the HR mice may have evolved mechanisms to compensate for the dramatically increased VE.

In addition to much higher levels of VE, several other differences between HR and C lines (Garland et al. 2016; Wallace and Garland 2016) suggest differences in their ability to regulate body mass or composition. For example, HR mice are more active in home-cages when wheels are not provided (Malisch et al. 2009; Copes et al. 2015), eat more as adults even when housed without wheels (Swallow et al. 2001; Copes et al. 2015), are smaller in total body mass (Koteja et al. 1999; Swallow et al. 1999), with the

difference more pronounced in males than females (Swallow et al. 1999; Garland et al. 2011a)), have reduced body fat (Swallow et al. 2001; Nehrenberg et al. 2009), reduced circulating leptin levels (Girard et al. 2007), and increased adiponectin levels (Vaanholt et al. 2007). Moreover, the amount of wheel running does not reach a plateau within six days in either HR or C mice (e.g., Swallow et al. 2001; Acosta et al. 2017), and neither does the amount of cage activity, a measure of SPA (Acosta et al. 2017), or body mass (Swallow et al. 2001; Bronikowski et al. 2006).

The purpose of the present study was to characterize the effect of sex and genetic background on the initial body composition and changes after 6 days of access to voluntary exercise. Furthermore, within each of the four groups (C male, C female, HR male, HR female), we used structural equations to model the relative importance of various paths in the complex network of activity and body composition phenotypes at the level of individual variation (cf. King et al. 2008): initial body mass, intensity and duration of VE and SPA, food consumption, and changes in body composition (lean vs. fat mass).

2. Methods

2.1. Mouse model

The High Runner (HR) and Control (C) lines of mice were established from a base population of 224 outbred Hsd:ICR mice. After two generations of random mating, we established 4 HR lines and 4 non-selected C lines (Swallow et al. 1998). For the current study, we used 348 mice (approximately half C and half HR) from generation 77 of the HR selection experiment. Mice were weaned at 3 weeks of age into standard

mouse-size cages with up to 4 mice of the same sex and given *ad libitum* food and water. All mouse rooms were maintained at 20-24 degrees Celsius with 12:12 light-dark cycles.

As young adults (range: 46-70 days old), mice were placed in new individual home cages (same size as regular cages) with access to wheels for 6 days to measure voluntary wheel running (with *ad libitum* food and water). The wheels were 1.12 m in circumference and attached externally to the home cage, accessed via a tunnel (see Fig. S1 in Kelly et al. 2017). Wheel running was recorded with an automated counting system in 1-minute increments for each day. From this we obtained daily running distance (revolutions per day), duration (minutes per day), mean speed (revolutions per minute), and maximum speed (maximum number of revolutions in any 1-minute interval). Mice were similarly monitored for activity in the home-cage by passive infrared motion-detection sensors connected to a digital I/O board (ICS 2313; ICS Electronics, Pleasanton, California, USA) interfaced with a custom software developed by Dr. Mark A. Chappell (Copes et al. 2015). The software recorded '1' (movement detected) or '0' (no movement detected) 3 times per second from the sensor and saved the mean value (between 0 and 1) every minute. From these we obtained daily activity levels (arbitrary activity units), duration, mean intensity (activity units per minute), and maximum intensity (maximum activity units in any 1-minute interval). We analyzed wheel running and home-cage activity for the last two days of the 6-day trial (mean of days 5 and 6) because those are used in the selection protocol.

In addition to weighing mice before and after wheel access (±0.01 g), we weighed food hoppers (±0.01 g) of every mouse to measure food consumption, noting obvious signs of food wasting or shredding, in order to measure food consumption.

To analyze body composition before and after wheel access, we used a non-invasive, quantitative magnetic resonance whole body composition analyzer (EchoMRI-100, Echo Medical Systems, Houston, TX), which independently determined lean and fat masses of each animal.

2.2. Conventional statistical analyses

Among-group differences were analyzed using covariance models with Type III tests of fixed effects in the Mixed Procedure in SAS 9.4M4 (SAS Institute, Cary, NC, USA). Sex, linetype (HR or C), and their interaction were included in the model as fixed effects. Random effects in the model were replicate lines nested within linetype, family identity nested within line and linetype, and sex-by-line interaction effects nested within linetype.

Total, lean, and fat masses were analyzed separately for before and after wheel access, and change in mass was calculated as mass after wheel access minus mass before wheel access. We also analyzed lean and fat masses as percent of body mass. In addition, the masses were analyzed as repeated measures before and after wheel access. Analyses of masses included age and age-squared as covariates because mice were tested over a span of 4 weeks, which resulted in a curvilinear relationship. We obtained age-

squared by standardizing age to have mean = 0 and standard deviation = 1 and then squaring those standardized values.

Analyses of food consumption used initial body mass as a covariate. We also used a model with covariates of activity levels (both intensity and duration of wheel running and home-cage activity).

Wheel running and component traits (duration, mean and maximum speed) were analyzed with age and wheel freeness as covariates. Rotational freeness was measured for each wheel by accelerating it to a constant speed for 5 rotations and counting revolutions until the wheel stopped on its own. Home-cage activity and component traits were analyzed similarly, but to obtain normality of residuals, total home-cage activity, duration, and mean intensity were $\log_{10}$-transformed and maximum intensity was raised to the 2.5^{th} power. We used covariates of age and infrared sensor sensitivity, which was calibrated by using a heating stick swung in the home-cage for 5 seconds and recording the activity reported by each sensor. Sensor sensitivity and wheel freeness were each square-rooted to obtain a normal spread of values and the mean of measurements taken before and after wheel access (with two measures per time) was used as a covariate.

2.3. Structural equation modeling analyses

To determine the complex phenotypic architecture of activity and body composition with each group, we analyzed our data using structural equation modeling in Onyx version 1.0-937 (von Oertzen et al. 2015). The variables tested were wheel-running speed and duration, home-cage activity intensity and duration, initial body mass,

food consumption, change in fat mass and lean mass, and nuisance variables of age, age-squared, square-rooted wheel freeness, and square-rooted sensor sensitivity. We ran the same model separately for the four sex-and-linetype groups: female C, female HR, male C, and male HR. To account for known differences between the replicate lines (Garland et al. 2011a), we centered every dependent variable to have the same mean among the 4 replicate lines within sex-and-linetype groups. In the model, each variable was z-transformed with a variance fixed to 1.0, and every exogenous variable pair had covariances. All paths except variances were unfixed (freed parameters). Within each group, we used the parameter estimate and standard error (SE) for each path to obtain 95% confidence intervals (estimate ± 2 x SE) and significance was determined by the confidence interval being bound away from zero.

3. Results

3.1. Body, lean, and fat mass

Before 6 days of wheel testing, body mass was significantly lower in HR mice than C mice ($p = 0.0489$, Table 3.1). This reduction was due mostly to reduced lean mass ($p = 0.0631$) as opposed to reduced fat mass ($p = 0.1185$, Table 3.1 and Fig. 3.1). Females also had significantly reduced body mass compared to males ($p < 0.0001$), which was entirely due to lower lean mass ($p < 0.0001$) and not fat mass ($p = 0.3234$, Table 3.1 and Fig. 3.1). Analyzed as percent body mass, lean mass was significantly lower ($p = 0.0041$) and fat mass was significantly higher ($p = 0.0007$) in females compared to males (Table 3.1 and Fig. 3.2).

All groups lost body mass after 6 days of wheel access ($p = 0.0342$) due to a significant loss in fat mass ($p < 0.0001$) and despite a tendency for increased lean mass (Table 3.2 and Fig. 3.1). The loss in body mass and the gain in lean mass were not significantly affected by sex, linetype, or their interaction ($p > 0.05$, Table 3.1). Wheel minutes, speed, and home-cage minutes were significant predictors of total body mass change ($p < 0.05$, Table 3.1), but using them as covariates did not change the main effects of sex and linetype.

HR lost significantly less fat mass than C mice ($p = 0.0133$ in Table 3.1 and $p = 0.0141$ in Table 3.2). After accounting for activity levels, the effect of linetype was not significant ($p = 0.2916$), but females tended to lose less fat ($p = 0.0518$, Table 3.1). Higher wheel-running duration resulted in greater fat loss ($p < 0.0001$) while higher running speed and minutes spent in home-cage activity resulted in decreased fat loss ($p < 0.0001$ and $p = 0.0002$, Table 3.1; see section 3.4. below for more detailed explanation of these effects).

3.2. Food consumption

Adjusting for initial body mass before wheel access, HR mice consumed significantly more food than C mice ($p < 0.0001$), with no effect of sex. Running speed, duration, and home-cage activity duration were significant positive predictors of food consumption, but adding them as covariates did not change the main effect of linetype ($p = 0.0031$) or sex (Table 3.3 and Fig. 3.3).

3.3. Activity levels

HR mice ran for significantly more distance (revolutions per day) than C mice ($p < 0.0001$) on days 5+6 of wheel access by running more minutes per day ($p = 0.0480$) at higher mean ($p < 0.0001$) and maximum speeds ($p < 0.0001$, Table 3.4 and Fig. 3.4). Females ran more than males ($p = 0.0061$) by running more minutes per day ($p = 0.0005$) but not at significantly higher speeds compared with males (Table 3.4 and Fig. 3.4).

Total home-cage activity during days 5+6 of wheel access was not different among groups (Table 3.4 and Fig. 3.5). Interestingly, females were active more minutes per day ($p = 0.0013$) but at lower intensities ($p = 0.0321$) compared with males (Table 3.4 and Fig. 3.5).

3.4. Structural equation models

We used structural equation models to determine the relative importance of different types of activity, initial body mass, and food consumption on lean and fat mass change. The model was analyzed separately for each sex-by-linetype group (i.e., C females, C males, HR females, and HR males) in order to detect differences in phenotypic architecture.

For all 4 groups, the intensity and duration of home-cage activity were positively related and food consumption decreased amount of fat lost over 6 days of wheel access (Fig. 3.6, note that all groups lost fat mass, so a positive relationship indicates reduced fat loss). The only other paths shared by all groups were non-significant effects (e.g., wheel speed did not predict change in lean mass in any group, Fig. 3.6). As expected, some

paths were linetype-specific (e.g., in C but not HR lines, wheel-running speed was positively related to wheel-running duration, Fig. 3.6) while other paths were sex-specific (e.g., wheel-running speed predicted food consumption in females but not males, Fig. 3.6).

Lean change was only affected by wheel-running duration and this effect was only significant in HR females (HR females with higher running duration gained less lean mass, Fig. 3.6). On the other hand, change in fat mass was affected by running speed, decreased by running duration, and increased by home-cage duration in males, and decreased by running duration in HR females (note, decreased means more fat lost and increased means less fat lost; Fig. 3.6). Interestingly, the effect of running speed on fat change was opposite in sign for C and HR males. That is, C males that ran faster lost more fat, but HR males that ran faster lost less fat (Fig. 3.6 and Fig. 3.7-3.10 for parameter estimates).

Food consumption was significantly increased by initial body mass for all groups except HR females (Fig. 3.6; but HR females also had a positive estimate, see Fig. 3.7-3.10) and the effect was greater in males than females (higher parameter estimates in males in Fig. 3.7-3.10). Food consumption was also increased by wheel speed (females), running duration (C males), and home-cage duration (HR males; Fig. 3.6). Intensity of home-cage activity did not affect food consumption in any group (Fig. 3.6).

4. Discussion

Body mass and composition are affected by physical activity. Individuals might compensate for increased activity by increasing food consumption or decreasing other aspects of physical activity. However, these effects may be different in genetic backgrounds predisposed to increased activity levels, and may differ between sexes. Because duration and intensity of activity might affect body composition differently, we partitioned voluntary exercise (wheel running) and spontaneous activity (home-cage activity) as minutes spent doing the activity per day and intensity of activity per minute.

4.1. Among-group differences

Mice from HR lines ran more and had lower total body mass and tended to have lower lean and fat mass compared with C lines, even before wheel access (Table 3.1). These findings are consistent with multiple previous studies on these mice (Swallow et al. 1999, 2001; Copes et al. 2015).

Over 6 days of wheel access, all groups lost total mass and tended to gain lean mass, and these effects were not statistically different between the sexes or linetypes. On the other hand, HR mice lost significantly less fat than C mice despite their higher activity in wheels. At the end of 6 days of wheel access, all groups had converged to approximately 1.7 grams of fat (Fig. 3.1), or 6% body fat for males and 7.5% body fat for females (Fig. 3. 2). This amount of fat is potentially a lower limit to healthy adult fat

mass in these mice. A previous study also reported fat mass of ~2 grams in C and HR mice after 6 days of wheel access (Hiramatsu et al. 2017).

Food consumption was higher in HR than C mice (adjusted for their smaller body mass; Fig. 3.3; (also found in Koteja et al. 1999; Swallow et al. 2001)), suggesting that HR mice compensated for increased energy expenditure by increasing energy intake. Rodent and human studies often report increased food intake to compensate increased voluntary exercise (review in Garland et al. 2011b), although in humans, some individuals are "compensators" and others not (see King et al. 2008). The increase in food consumption in HR mice was statistically significant even in models that used four separate metrics of physical activity as covariates (Table 3.3). Thus, HR mice are eating more than C even after compensating for their increased physical activity, but they still lose fat. Our finding conflicts with a previous study that reported no difference in food consumption between HR and C mice when using activity metrics (the same 4 as ours) as covariates (only females tested, Copes et al. 2015; our results did not change when we analyzed the sexes separately [results not shown]). The discrepancy is likely due to the fact that the mice they studied were given wheel access for 8 weeks prior to one week of food consumption measurements (Copes et al. 2015), which is well after stabilization of wheel running which occur after about two weeks (Acosta et al. 2017). The mice we studied were younger and were not acclimated to having access to wheels, which likely resulted in short-term physiological differences for HR vs. C. These differences between HR and C in short-term energy balance could be related to previously reported

differences in circulating leptin and adiponectin hormone levels (Girard et al. 2007; Vaanholt et al. 2007; Garland et al. 2016).

If a minimum amount of body fat is required to sustain high levels of physical activity over the 6 days of wheel access, then HR mice may be at a limit for activity because of their low body fat. That is, despite compensatory eating, HR mice still lose fat, so they may be unable to increase their activity beyond current levels. This limit in energy balance could be a general explanation for the selection limits experienced in HR lines (Careau et al. 2013).

It is important to note that the mice do not seem to be limited in how much food they can consume over these 6 days. Food is available *ad libitum* and time is available each day for eating, even in HR lines which spend more minutes on wheels (Fig. 3.4). (In fact, wheel-running duration is a positive predictor of food consumption when used as a covariate; Table 3.3). HR and C mice (of both sexes) did increase food consumption during cold exposure (over 3-6 days) to an average of ~10 g per day, which was sufficient to maintain body mass even in ambient temperatures at -15°C (Koteja et al. 2001). In comparison, food consumption during 6 days of wheel access was ~ 4 g in C mice and ~ 6 g in HR mice and all groups lost fat mass (Fig. 3.6).

4.2. Structural equation modeling

At the level of individual variation within each of the four sex-by-linetype groups, food consumption was positively related to initial body mass and the duration and intensity of both VE and SPA. The four sex-by-linetype groups differed in which type of

activity (duration or intensity, in wheels or home-cages) significantly predicted the increase in food consumption, but overall the path estimates from activity to food consumption were always positive (Fig. 3.6; and also reported by Copes et al. 2015).

We expected that both duration and intensity of physical activity (especially VE) would affect both lean and fat masses. However, we found that lean mass was only affected by the duration of exercise on wheels, and only in HR females (Fig. 3.6). Furthermore, the effect of wheel duration was negative, i.e., HR females that exercised for more minutes per day gained less lean mass over 6 days of wheel access (Fig. 3.6). Among groups, HR females also tended to run the most minutes on wheels (Fig. 3.4) and to gain the least mass (Fig. 3.1) although these results were not statistically significant (Tables 1 and 4).

We expected that the change in fat mass would be negatively affected by activity duration and intensity (or not affected at all, if mice were compensating by increased food intake). Fat mass was affected by activity metrics in males (but only in one case in females), but not always negatively (Fig. 3.6). For example, HR males lost more fat mass with increased wheel duration (as expected), but lost less fat with increased wheel speed and increased duration of home-cage activity (Fig. 3.6). The conflicting results might be explained by a negative relationship between wheel duration and home-cage duration (i.e., mice with increased home-cage duration may be spending less time on wheels, thus losing less fat), but the relationship was positive in HR males (Fig. 3.6).

Although we tried to account for multiple factors affecting lean and fat change, the structured equation models could be further improved. For example, had we

measured body lengths, we could have used it in the model to account for body size as a separate metric from body mass. Also, as stated above, differences in circulating hormone levels could be mediating changes to body fat.

4.3. Future directions

We chose to do a short-term exposure to voluntary exercise in the present study because that was used in the selection experiment and may answer questions about the selection limit. However, day-to-day increases in wheel running and simultaneous decreases in home-cage activity are still occurring during and after 6 days, with neither measure of activity reaching a plateau until approximately two weeks (Acosta et al. 2017). Thus, an interesting future direction would be to give access to wheels for several weeks and measure changes in body composition when activity levels stabilize. Compensation behaviors may be more or less efficient when given longer-term exercise, and may differ between the sexes or linetypes. A related question would be how the starting age of exercise regimes might affect compensation behaviors and changes in body mass and composition.

4.4. Concluding remarks

Overall, the results of this study suggest that the complex relationships between mass, activity levels, food consumption, and body composition are differentially controlled in the sexes and heavily dependent on genetic background. These potential differences in biological and genetic regulation need to be incorporated into studies of the

effects of physical activity, especially in human studies where environmental determinants are more commonly assumed (Lightfoot et al. in press). In HR lines selectively bred for increased exercise, changes to the regulation of energy balance have resulted in less fat lost despite increased activity by compensatory increases in energy intake. However, HR mice still lost fat over 6 days, suggesting that the compensation by increased food consumption is not adequate to regulate energy balance. Furthermore, this need to conserve fat mass may explain the selection limit reached in the HR mice. As reported in the present study, elucidating the phenotypic architecture governing complex traits requires detailed analysis of different genetic backgrounds and sexes. This study lends support for personalized medicine for humans, especially in prescribing physical activity as duration and intensity of VE and SPA.

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Tables and Figures

Table 3.1. Body mass and composition before and after 6 days of wheel access. Significance levels (P values) from statistical analyses with main effects of sex (female or male), linetype (C or HR), and their interactions ("Sex x C vs HR"). Covariates used were age and age-squared (first standardized and then squared). For analyses of change in masses, additional covariates indicating activity metrics were used. All statistically significant P values (<0.05) are in bold and signs following the value indicate direction of effect. Note, since all mice lost fat, the + sign after the linetype effect (C vs HR) means that HR lines lost less fat mass.

Table 3.1.

Trait	N	Sex	C vs HR	Sex x C vs HR	Age	Age^2	Wheel minutes	Wheel speed	Home-cage minutes	Home-cage intensity
Total mass										
Before wheel access	333	**<0.0001** +	**0.0489** -	0.1587	**<0.0001** +	0.3841				
After wheel access	334	**<0.0001** +	0.0585	0.1191	**<0.0001** +	**0.0276** -				
Change	321	0.8304	0.3174	0.2134	0.4897	0.0840				
Change with activity	321	0.5402	0.8687	0.4028	0.3412	**0.0122** -	**<0.0001**-	**0.0005** +	**0.0009** +	0.8732
Lean mass										
Before wheel access	333	**<0.0001** +	0.0631	0.1763	**<0.0001** +	0.5333				
After wheel access	333	**<0.0001** +	0.0507	0.1125	**<0.0001** +	**0.0039** -				
Change	320	0.5397	0.2146	0.2816	**<0.0001** +	**<0.0001** -				
Change with activity	320	0.5620	0.1577	0.3584	0.0001	**<0.0001** -	**0.0052**-	0.2098	**0.0473** +	0.9948
Fat mass										
Before wheel access	333	0.3234	0.1185	0.9132	0.1597	0.1207				
After wheel access	333	0.3455	0.7954	0.3410	0.1472	0.1523				
Change	320	0.1412	**0.0133** +	0.3428	**0.0050** -	**0.0044** +				
Change with activity	320	0.0518	0.2916	0.8877	**0.0122** -	**0.0167** +	**<0.0001**-	**<0.0001** +	**0.0002** +	0.3545
Lean mass as percent of body mass										
Before wheel access	333	**0.0003** +	0.5488	0.4773	0.6379	0.3190				
After wheel access	326	**<0.0001** +	0.5517	0.4920	**<0.0001** +	**0.0054** -				
Change	320	0.8582	**0.0162** -	0.3975	**<0.0001** +	**0.0004** -				
Fat mass as percent of body mass										
Before wheel access	333	**0.0041** -	0.2772	0.3745	0.2529	0.1795				
After wheel access	332	**0.0007** -	0.8106	0.4769	**0.0004** -	**0.0290** +				
Change	322	0.9312	**0.0139** +	0.2705	**0.0233** -	**0.0006** +				

Table 3.2. Repeated-measures analyses of body mass and composition before and after wheel access.
Main effects were sex (female or male), linetype (C or HR), wheel access (before vs after access), and all possible interactions of the three main effects. Covariates used were age and age-squared (first standardized and then squared). For lean mass, analyses were run separately be sex and analysis of females did not include age in the model. All statistically significant P values (<0.05) are in bold and signs following the value indicate direction of effect. Sample sizes (N) are approximately doubled because each mouse had two measurements of mass (before and after wheel access).

							Interactions			
Trait	N	Sex	C vs HR	After wheels	Age	Age^2	Sex x C vs HR	Sex x After wheels	C vs HR x After wheels	Sex x C vs HR x After wheels
Total mass	640	**<0.0001** +	0.0567	**0.0342** -	**<0.0001** +	0.1955	0.2262	0.8373	0.3261	0.1919
Total mass – males	315		**0.0397** -	0.1219	**<0.0001** +	0.5600			0.8459	
Total mass – females	325		0.1324	0.0515	†	†			0.0587	
Lean mass†										
Lean mass – males	314		**0.0468** -	**0.0411** +	**<0.0001** +	0.2768			0.0902	
Lean mass – females	325		0.1371	0.1355	†	†			0.6902	
Fat mass	637	0.7679	0.3510	**<0.0001** -	0.9104	0.8507	0.8649	0.1160	**0.0141** -	0.3068
Fat mass – males	314		0.3697	**<0.0001** -	0.4825	0.1854			**0.0043** -	
Fat mass – females	331		0.3557	**0.0013** -	0.6957	0.5284			**0.0464** -	

†Unable to estimate due to infinite likelihood. Models with repeated measures for lean mass could not be analyzed for both sexes pooled. Female-specific models for total and lean mass repeated measures could not be analyzed when age or age^2 was included. Because total mass and lean mass were significantly affected by age, these estimates in the females may be unreliable.

Table 3.3. Food consumption, measured over 6 days of wheel access.
Main effects were sex (female or male), linetype (C or HR), and their interactions ("Sex x C vs HR"). Covariates used were age and age-squared (first standardized and then squared). An additional model included covariates of activity metrics. Statistically significant P values (<0.05) are in bold and signs following the value indicate direction of effect.

Trait	N	Sex	C vs HR	Sex x C vs HR	Age	Age^2	Wheel minutes	Wheel speed	Home-cage minutes	Home-cage intensity	Mean mass
Food consumption	312	0.1493	**<0.0001** +	0.3836	**0.0267** -	0.2274					**<0.0001** +
With activity	306	0.8089	**0.0031** +	0.3946	0.2325	0.1586	**0.0469** +	**<0.0001** +	**0.0033** +	0.9975	**<0.0001** +

Table 3.4. Activity level metrics on as means of days 5+6 of a 6-day test.
Home-cage total activity, duration, and mean intensity were log_{10} transformed and maximum intensity was square-rooted prior to analyses to obtain normality of residuals. Main effects were sex (female or male), linetype (C or HR), and their interactions ("Sex x C vs HR"). Covariates used were age and a measure of wheel freeness (for wheel traits) or sensor sensitivity (for home-cage traits). All statistically significant P values (<0.05) are in bold and signs following the value indicate direction of effect.

Trait	N	Sex	C vs HR	Sex x C vs HR	Age	Wheel freeness	Sensor sensitivity
Total wheel running	341	**0.0061** -	**<0.0001** +	0.5188	**<0.0001** -	**0.0002** +	
Duration	338	**0.0005** -	**0.0480** +	0.0287	**0.0262** -	**0.0041** +	
Mean speed	340	0.0819	**<0.0001** +	0.5339	**<0.0001** -	0.1154	
Maximum speed	337	0.1155	**<0.0001** +	0.7363	**0.0005** -	0.0834	
Total home-cage activity	343	0.5049	0.2651	0.7191	0.9082		**0.0272** +
Duration	346	**0.0013** -	0.1889	0.3753	0.4836		0.6169
Mean intensity	344	**0.0321** +	0.6639	0.1587	0.9935		**0.0014** +
Maximum intensity	346	0.2222	0.5421	0.6252	0.8929		**0.0005** +

Figure Legends

Fig. 3.1. Total, lean, and body mass measured for each mouse before and after 6 days of wheel exposure. Males had higher total and lean mass before and after the 6 days of wheel access. On average, mice lost body mass, gained lean mass, and lost fat mass over 6 days. Values are least-squares means ± standard errors from analyses of covariance in SAS Procedure Mixed. Corresponding *P* values are in Table 3.I. Analyses included covariates of age and age-squared. Each point represents ~80 mice. Markers are males = square, females = circle, C = grey and HR = black.

Fig. 3.2. Lean and fat as percent of total body mass, before and after 6 days of wheel exposure. Males had higher lean % and females had higher fat % before and after the 6 days of wheel access. On average, mice gained lean % and lost fat % over 6 days. Values are least-squares means ± standard errors from analyses of covariance in SAS Procedure Mixed. Corresponding *P* values are in Table 3.1. Analyses included covariates of age and age-squared. Each point represents ~80 mice. Markers are males = square, females = circle, C = grey and HR = black.

Fig. 3.3. Food consumption over 6 days of wheel access, adjusted for body mass and activity metrics. HR mice ate more food than C mice (top panel), even accounting for their increased activity levels (bottom panel). Activity metrics used as covariates were duration and intensity of wheel running and home-cage activity. Values are least-squares means + 1 standard error from analyses of covariance in SAS Procedure Mixed. Corresponding *P* values are in Table 3.3. Analyses included covariates of age and age-squared. Each point represents ~80 mice. Linetype is represented as C = grey and HR = black.

Fig. 3.4. Wheel running and component traits on days 5+6 of a 6-day wheel test. HR mice ran 2.6-3.1 times more than C by running for more minutes per day and at higher speeds. Females ran more than males by running for more minutes per day. Values are least-squares means + 1 standard error from analyses of covariance in SAS Procedure Mixed. Corresponding *P* values are in Table 3.4. Analyses included covariates of age and wheel freeness. Each point represents ~80 mice. Linetype is represented as C = grey and HR = black.

Fig. 3.5. Home-cage activity and component traits on days 5+6 of a 6-day wheel test. HR were not more active in their home-cages than C. Males had reduced duration but increased intensity of home-cage activity. Total activity, duration, and intensity were $\log_{10}$-transformed and maximum activity was square-rooted before doing analyses of covariance in SAS Procedure Mixed. The values presented here were back-transformed. Error bars represent the back-transformed upper 95% confidence interval calculated from the mean and standard error of transformed values. Corresponding *P* values are in Table 3.4. Analyses included covariates of age and sensor sensitivity. Each point represents ~80 mice. Linetype is represented as C = grey and HR = black.

Fig. 3.6. Structural equation model of activity levels and body mass effects on food consumption and lean and fat mass changes, compiled for 4 groups separated by sex and genetic background. As shown in Fig. 3.1, all groups on average gained lean mass and lost fat mass. However, within each group, the predictors of the lean and mass change varied. Only two relationships were consistently significant in all 4 groups: the positive relationship between intensity and duration of home-cage activity and the positive effect of food consumption on fat change. Since all mice lost fast, this positive effect means that mice that ate more food lost less fat. Analyses were run in the structural equation modeling software Onyx. Line color (black, blue, red, or grey) and style (solid, dotted, or dashed) represent significant paths for different combinations of groups. Significance was determined by the 95% confidence interval being bound away from zero, which was calculated from the parameter estimates and corresponding standard errors obtained in Onyx. Significant paths had positive effects unless otherwise indicated on path with a minus (-) sign. Where two signs (- +) are indicated, C in blue and HR in red had opposite effects. Traits with known or possible differences among replicate lines (activity levels, body mass, food consumption, and mass changes) were centered to have the same mean among the 4 replicate lines within each group. Nuisance variables (age, age2, wheel freeness, and sensor sensitivity) were included in the models but are not shown here (but shown in Figs. 3.7-10). Each group was represented by ~80 mice. The actual parameter estimates for each group can be found in Figs. 3.7-10.

Figs. 3.7. Structural equation model of activity levels and body mass effects on food consumption and lean and fat mass changes for females from C lines. Analyses were run in the structural equation modeling software Onyx. Thicker lines indicate stronger paths (positive or negative). N ~80 mice.

Figs. 3.8. Structural equation model of activity levels and body mass effects on food consumption and lean and fat mass changes for females from HR lines. Analyses were run in the structural equation modeling software Onyx. Thicker lines indicate stronger paths (positive or negative). N ~80 mice.

Figs. 3.9. Structural equation model of activity levels and body mass effects on food consumption and lean and fat mass changes for males from C lines. Analyses were run in the structural equation modeling software Onyx. Thicker lines indicate stronger paths (positive or negative). N ~80 mice.

Figs. 3.10. Structural equation model of activity levels and body mass effects on food consumption and lean and fat mass changes for males from HR lines. Analyses were run in the structural equation modeling software Onyx. Thicker lines indicate stronger paths (positive or negative). N ~80 mice.

Fig. 3.1. Total, lean, and body mass before and after 6 days of wheel exposure.

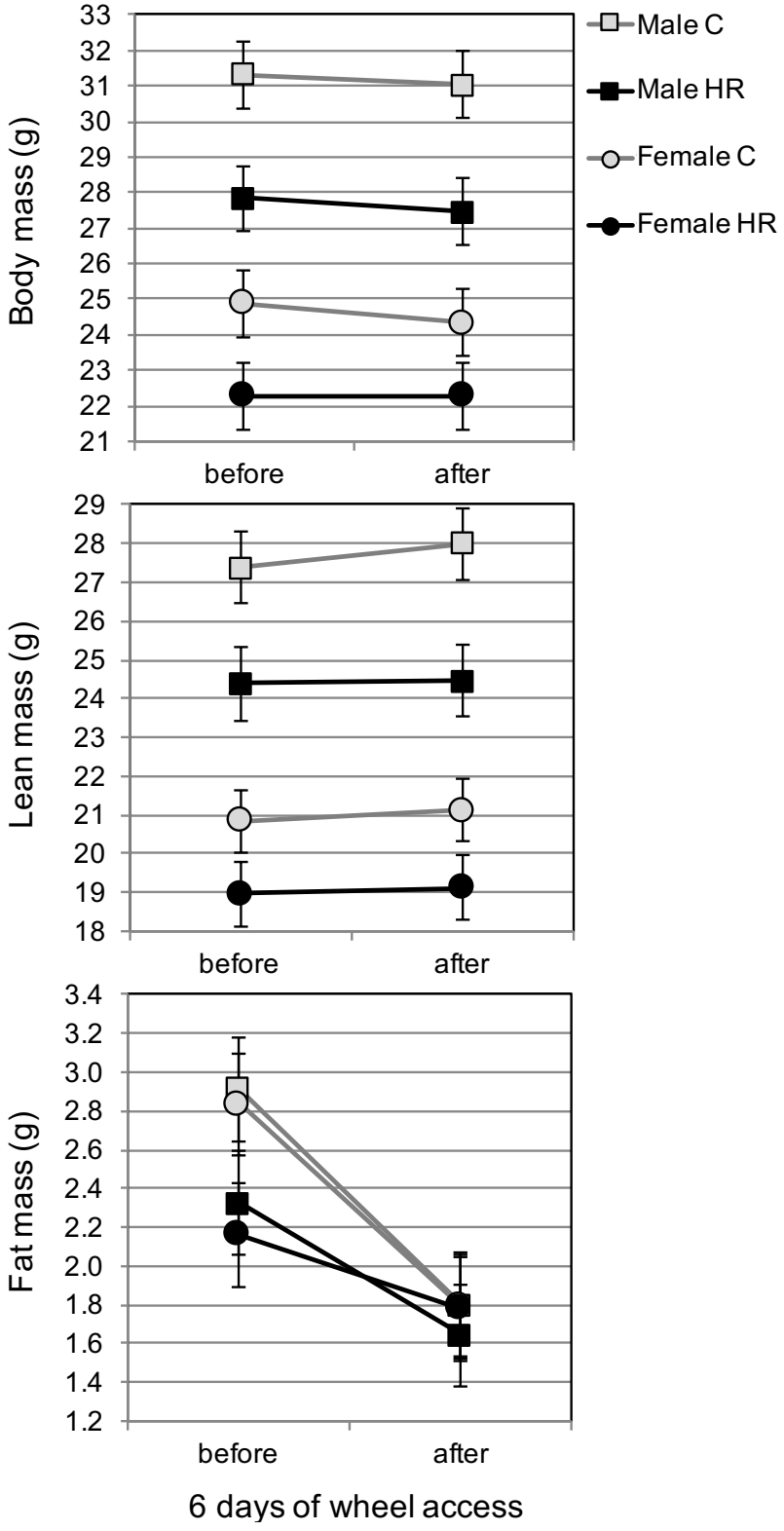


Fig. 3.2. Lean and fat as percent of total body mass, before and after 6 days of wheel exposure.

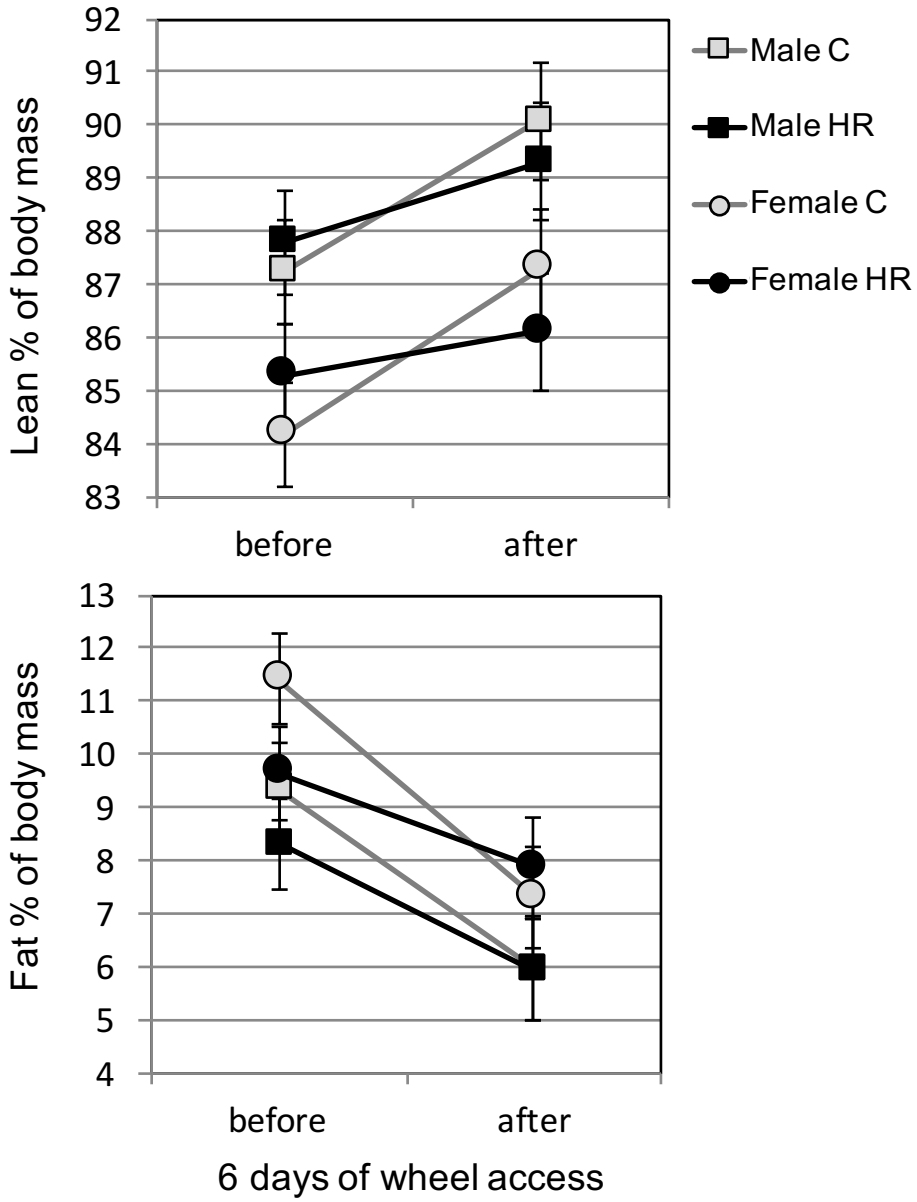


Fig. 3.3. Food consumption over 6 days of wheel access.

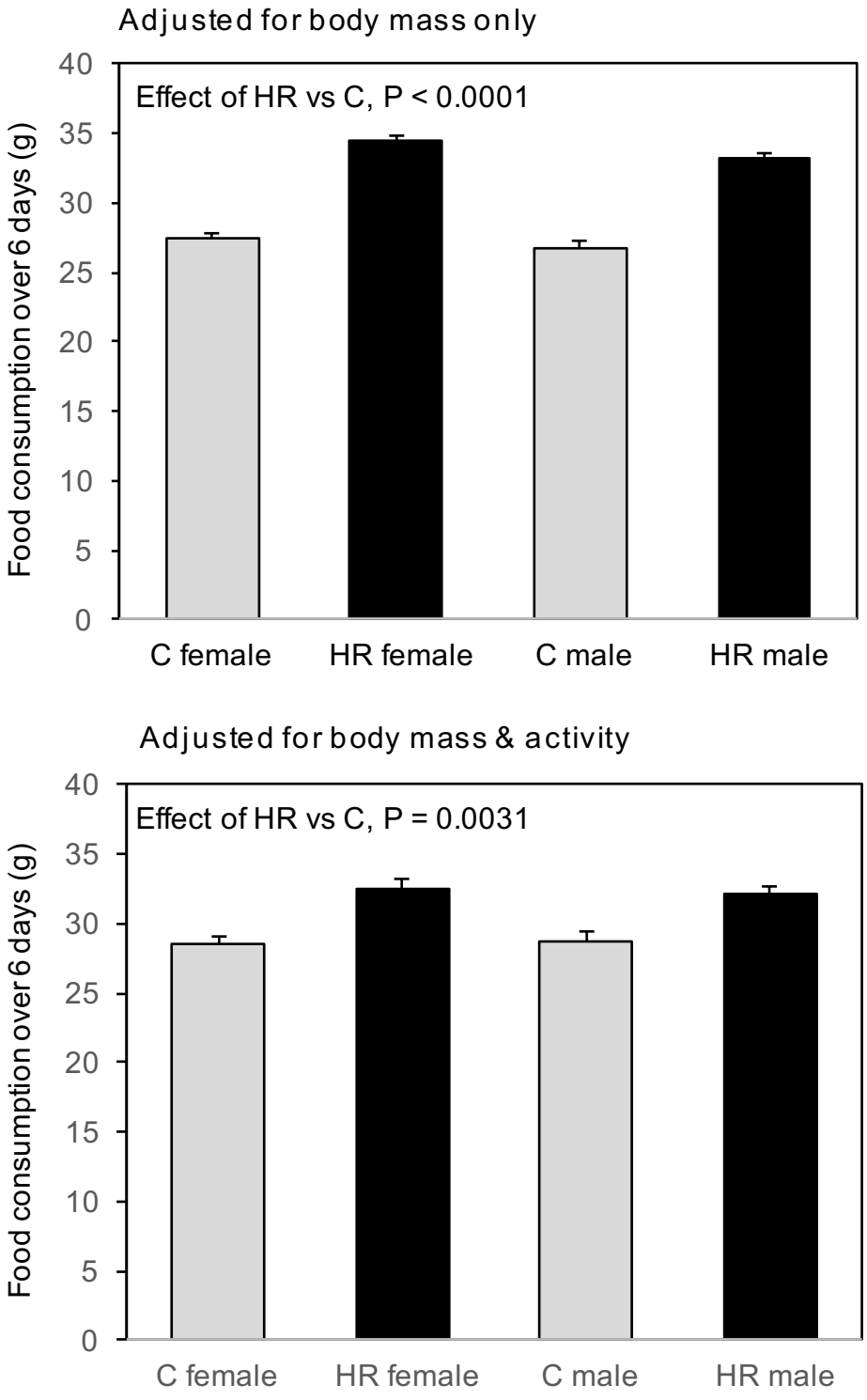


Fig. 3.4. Wheel running and component traits on days 5+6 of a 6-day wheel test.

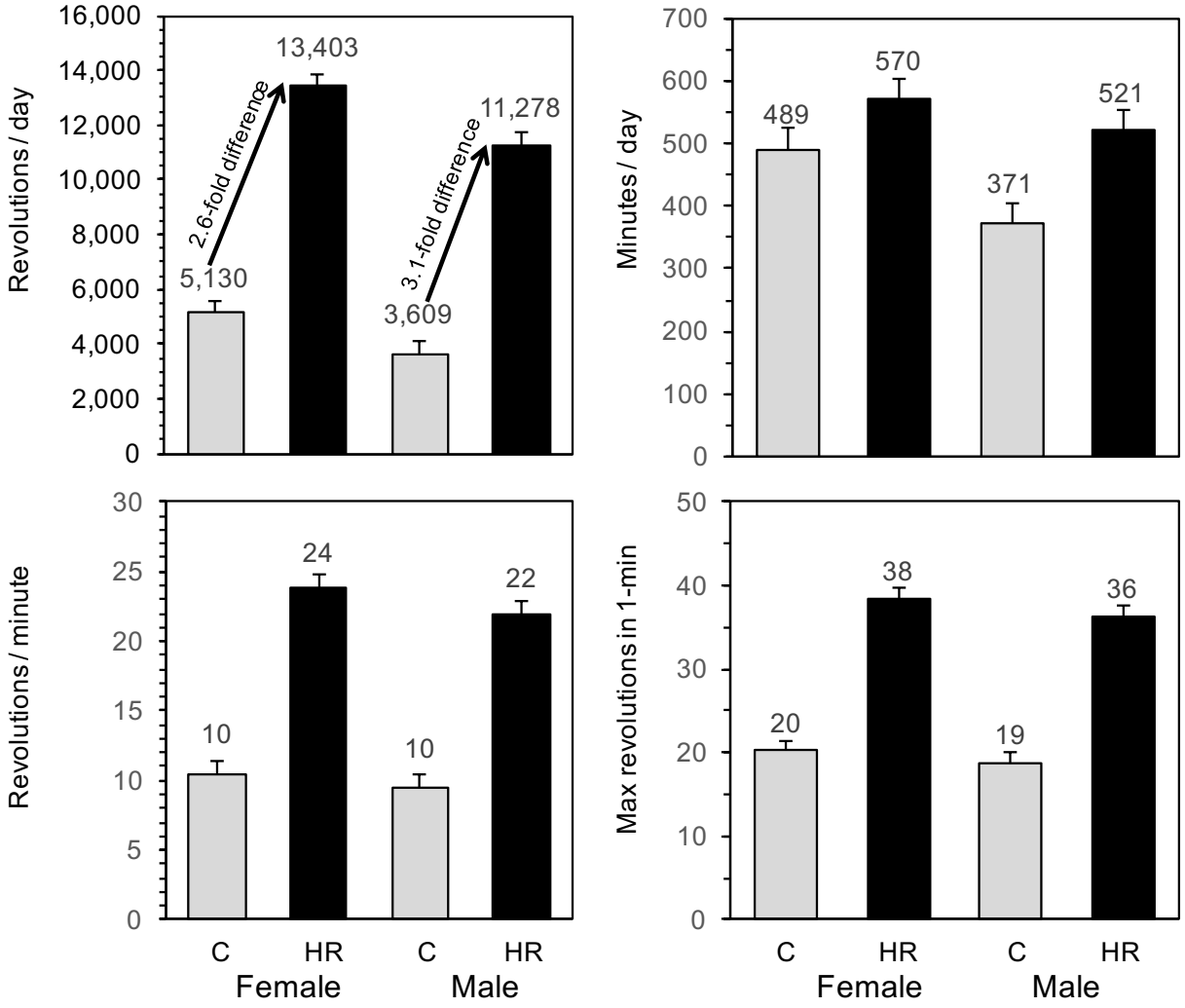


Fig. 3.5. Home-cage activity and component traits on days 5+6 of a 6-day wheel test.

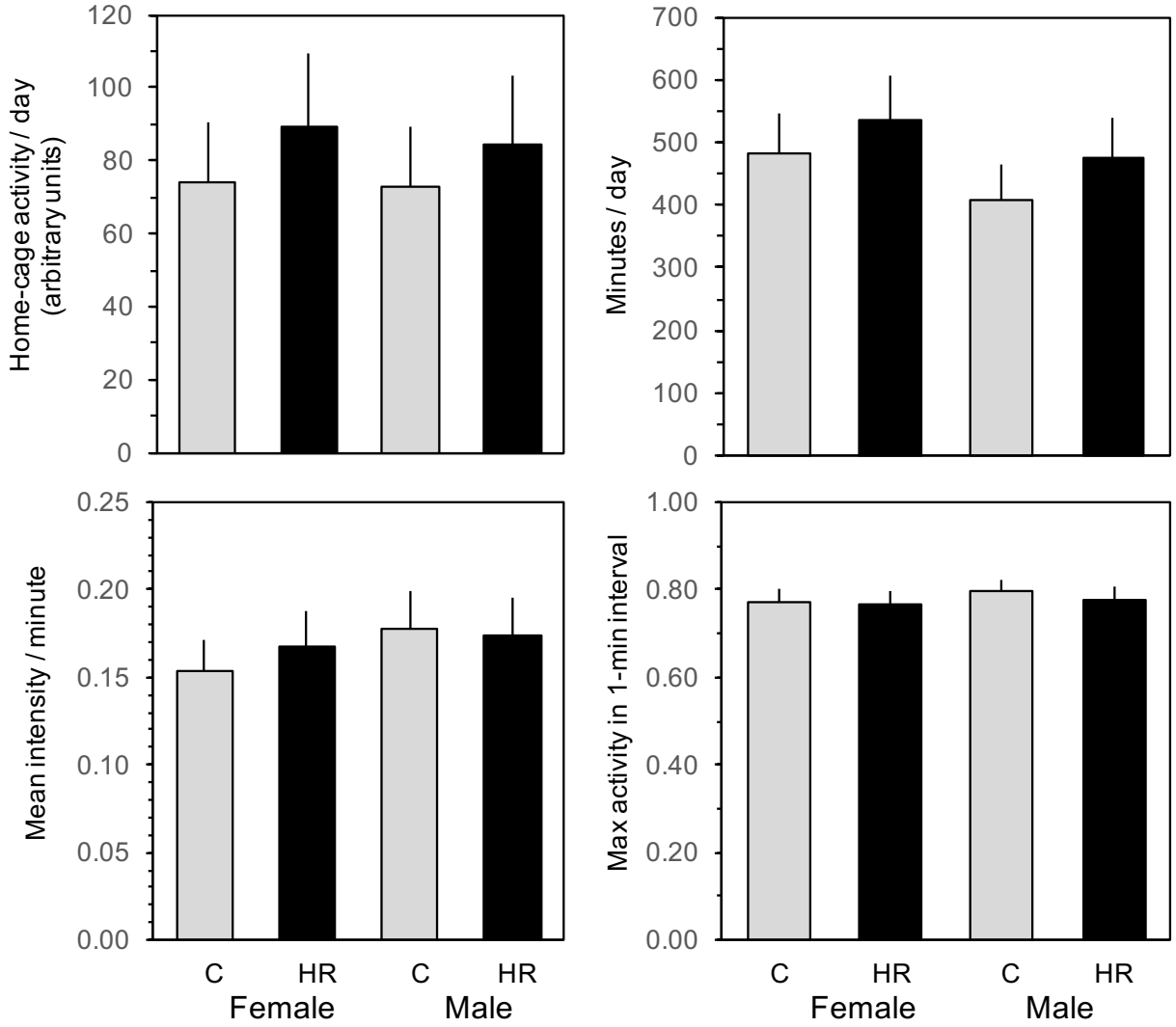


Fig. 3.6. Structural equation model of activity levels and body mass effects on food consumption and lean and fat mass changes, compiled for 4 groups separated by sex and genetic background.

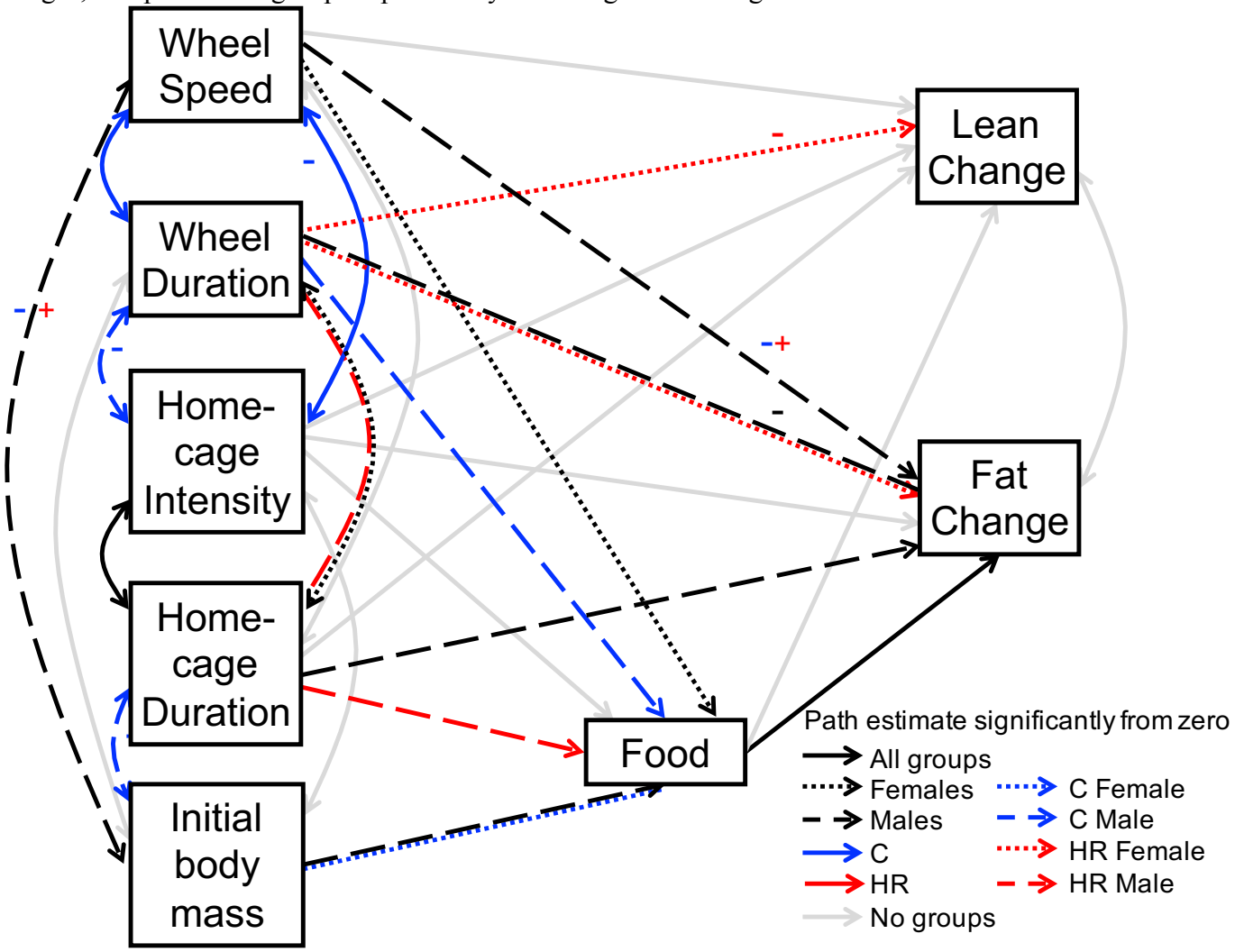
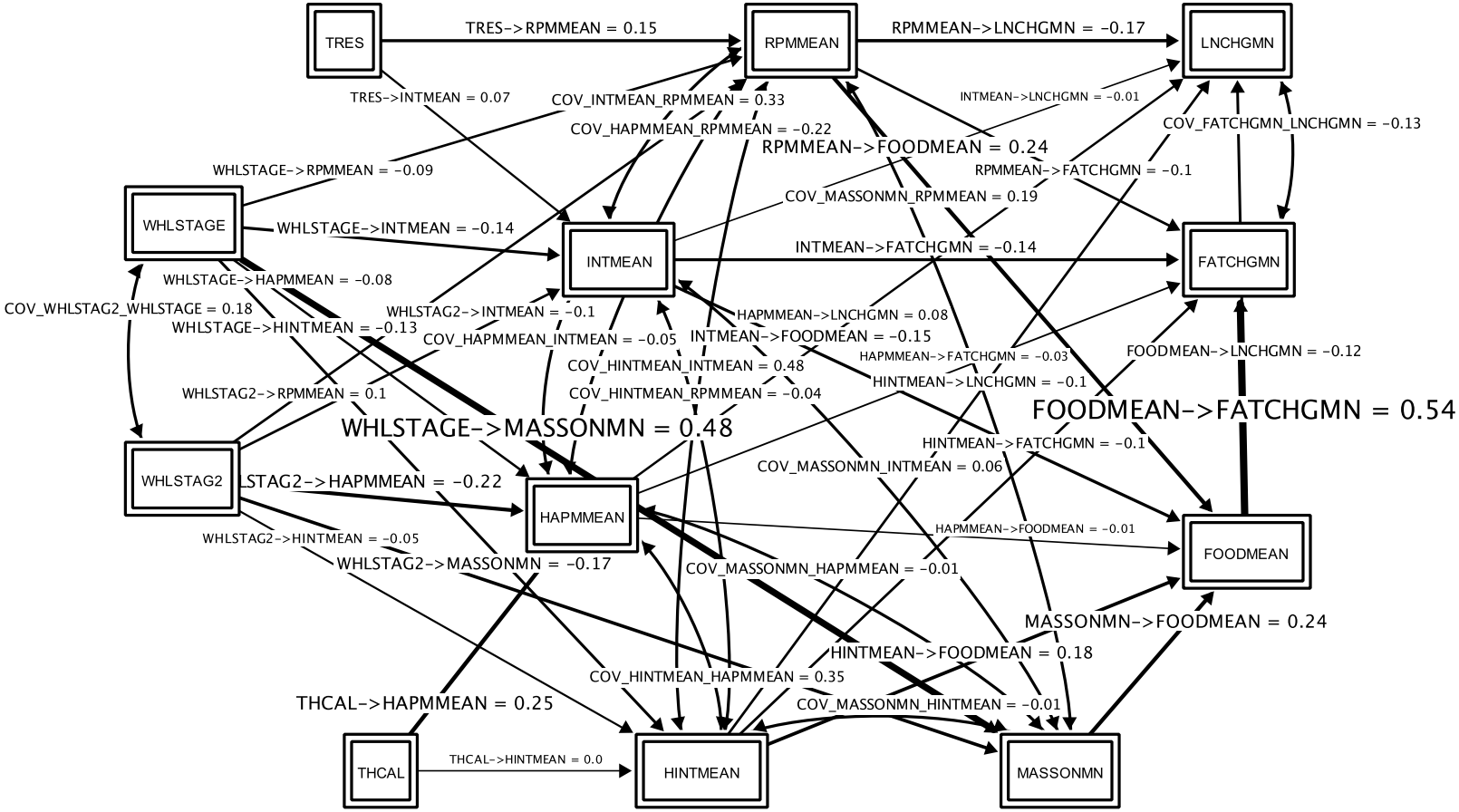


Fig. 3.7. Structural equation model results from Onyx – Control females

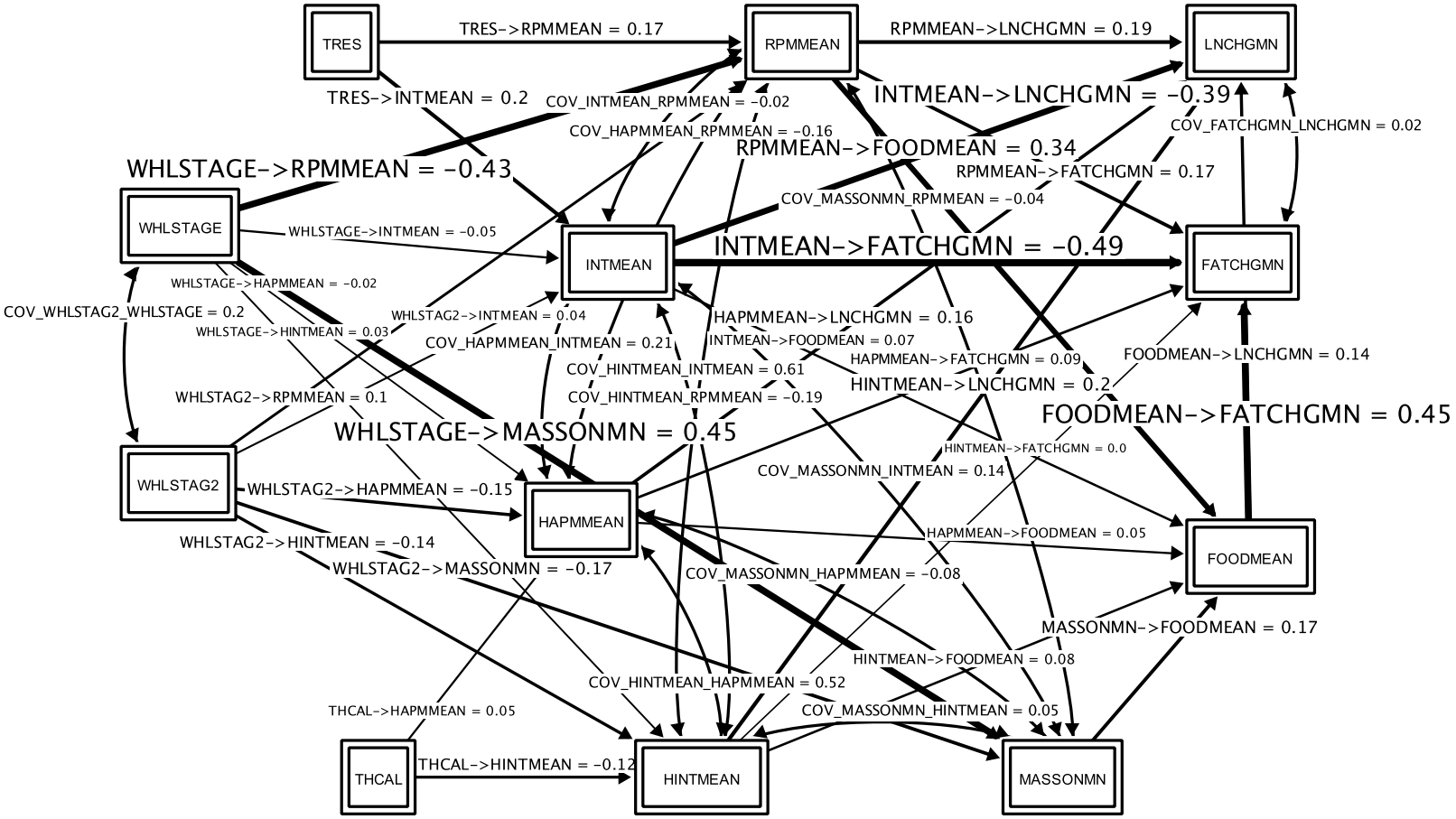


TRES: wheel freeness
WHLSTAGE: age at start of wheel access
WHLSTAG2: standardized and squared age
THCAL: home-cage sensor sensitivity

RPMMEAN: average wheel speed
INTMEAN: wheel duration
HAPMMEAN: average home-cage intensity
HINTMEAN: home-cage duration

LNCHGMN: change in lean mass over 6 days
FATCHGMN: change in fat mass over 6 days
FOODMEAN: food consumed over 6 days
MASSONMN: mass at start of wheel access

Fig. 3.8. Structural equation model results from Onyx – High-Runner females

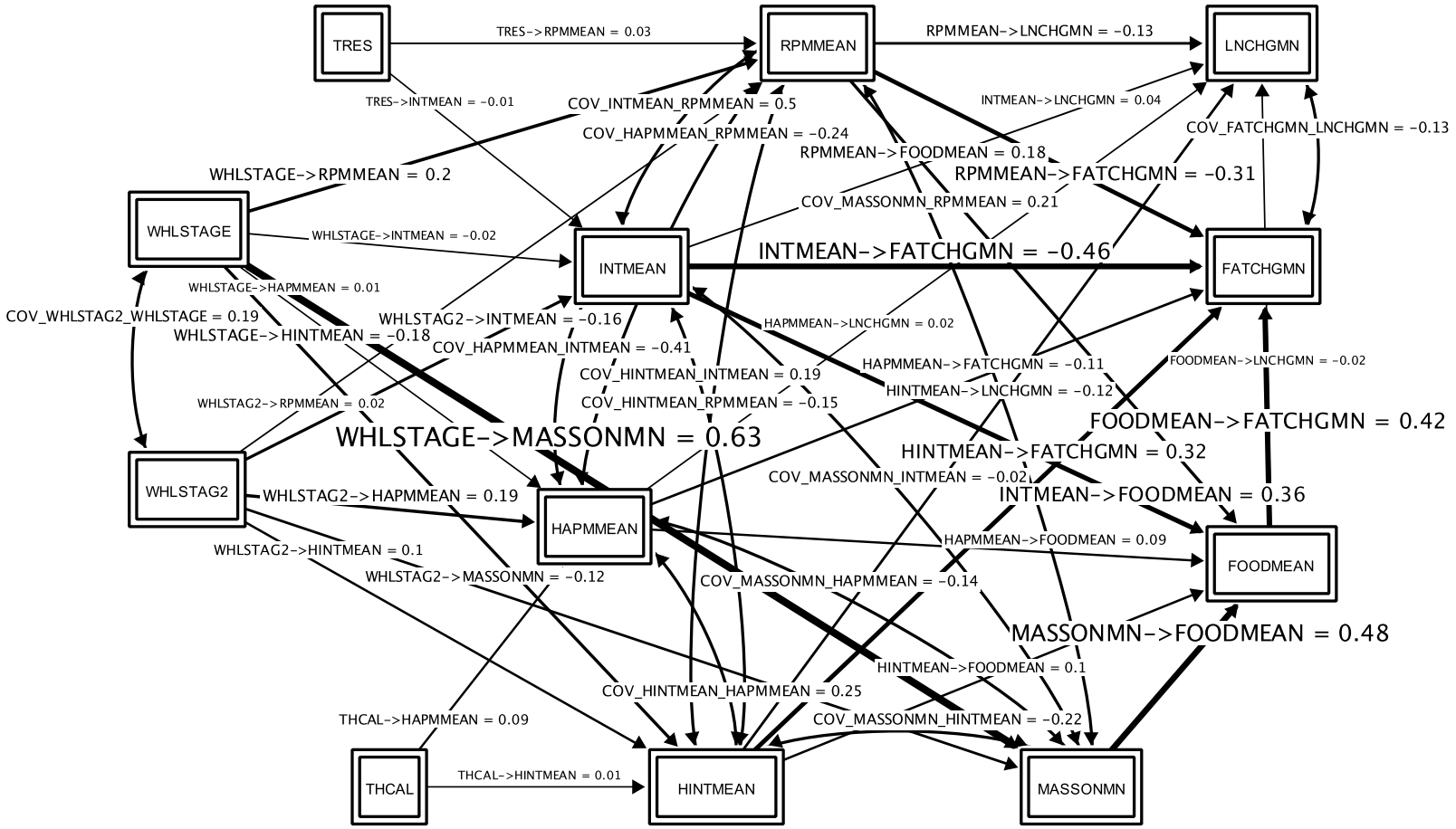


TRES: wheel freeness
WHLSTAGE: age at start of wheel access
WHLSTAG2: standardized and squared age
THCAL: home-cage sensor sensitivity

RPMMEAN: average wheel speed
INTMEAN: wheel duration
HAPMMEAN: average home-cage intensity
HINTMEAN: home-cage duration

LNCHGMN: change in lean mass over 6 days
FATCHGMN: change in fat mass over 6 days
FOODMEAN: food consumed over 6 days
MASSONMN: mass at start of wheel access

Fig. 3.9. Structural equation model results from Onyx – Control males

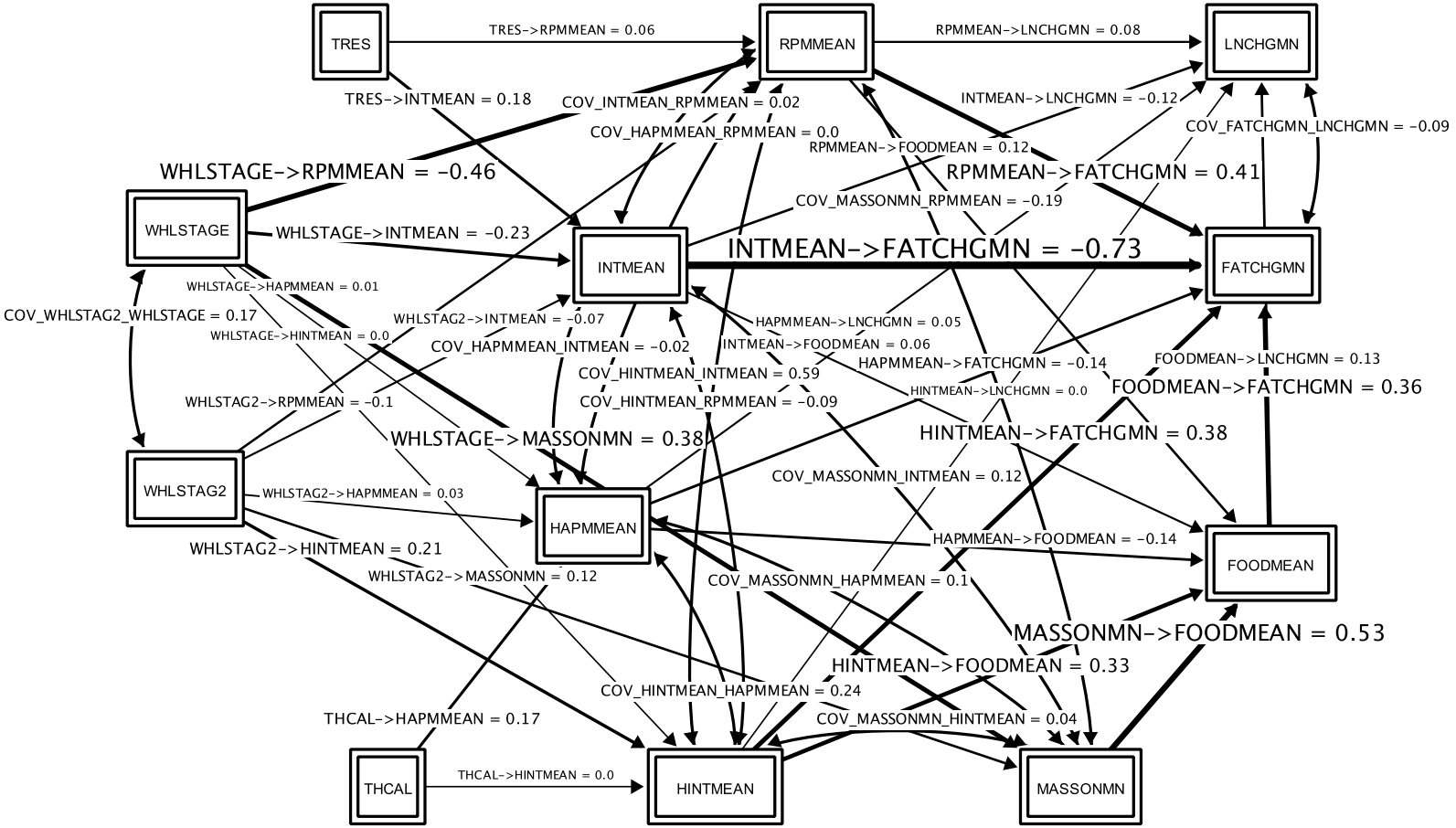


TRES: wheel freeness
WHLSTAGE: age at start of wheel access
WHLSTAG2: standardized and squared age
THCAL: home-cage sensor sensitivity

RPMMEAN: average wheel speed
INTMEAN: wheel duration
HAPMMEAN: average home-cage intensity
HINTMEAN: home-cage duration

LNCHGMN: change in lean mass over 6 days
FATCHGMN: change in fat mass over 6 days
FOODMEAN: food consumed over 6 days
MASSONMN: mass at wheel access

Fig. 3.10. Structural equation model results from Onyx – High-Runner males



TRES: wheel freeness
WHLSTAGE: age at start of wheel access
WHLSTAG2: standardized and squared age
THCAL: home-cage sensor sensitivity

RPMMEAN: average wheel speed
INTMEAN: wheel duration
HAPMMEAN: average home-cage intensity
HINTMEAN: home-cage duration

LNCHGMN: change in lean mass over 6 days
FATCHGMN: change in fat mass over 6 days
FOODMEAN: food consumed over 6 days
MASSONMN: mass at wheel access

Chapter 4

Maternal exposure to Western diet affects adult body composition and voluntary wheel running in a genotype-specific manner in mice

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Author Contributions: LH, JK, ZT, JS, GC, RLA, and TG designed the research. LH, JK, ZT, JS, GC, RLA, BH, BH, and GS collected data. LH, JK, and TG designed the analysis. LH, JK, and TG performed the analysis. LH wrote the initial draft. All authors reviewed and edited the final manuscript.

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Abstract

Some human diseases, including obesity, Type II diabetes, and numerous cancers, are thought to be influenced by environments experienced in early life, including *in utero*. Maternal diet during the perinatal period may be especially important for adult offspring energy balance, potentially affecting both body composition and physical activity. This effect may be mediated by the genetic background of individuals, including, for example, potential "protective" mechanisms for individuals with inherently high levels of physical activity or high basal metabolic rates. To examine some of the genetic and environmental factors that influence adult activity levels, we used an ongoing selection experiment with 4 replicate lines of mice bred for high voluntary wheel running (HR) and 4 replicate, non-selected control lines (C). Dams (half HR and half C) were fed a high-fat, high-sugar "Western" diet (WD) or a standard diet (SD) from 2 weeks prior to mating until their pups could feed on solid food (14 days of age). We analyzed dam and litter characteristics from birth to weaning, and offspring mass and physical activity into adulthood. One male offspring from each litter received additional metabolic and behavioral tests. Maternal WD caused pups to eat solid food significantly earlier for C litters, but not for HR litters (interaction of maternal environment and genotype). With dam mass as a covariate, mean pup mass was increased by maternal WD but litter size was unaffected. HR dams had larger litters and tended to have smaller pups than C dams. Home-cage activity of juvenile focal males was increased by maternal WD. Juvenile lean mass, fat mass, and fat percent were also increased by maternal WD, but food consumption (with body mass as a covariate) was unaffected (measured only for focal

males). Behavior in an elevated plus maze, often used to indicate anxiety, was unaffected by maternal WD. Maximal aerobic capacity (VO_2max) was also unaffected by maternal WD, but HR had higher VO_2max than C mice. Adult lean, fat, and total body masses were significantly increased by maternal WD, with greater increase for fat than for lean mass. Overall, no aspect of adult wheel running (total distance, duration, average running speed, maximum speed) or home-cage activity was statistically affected by maternal WD. However, analysis of the 8 individual lines revealed that maternal WD significantly increased wheel running for females in one of the 4 HR lines. On average, all groups lost fat mass after 6 days of voluntary wheel running, but the absolute amount lost was greater for mice with maternal WD, resulting in no effect of maternal WD on absolute or % body fat after wheel access. All groups gained lean and total body mass during wheel access, regardless of maternal WD or linetype. Measured after wheel access, circulating leptin, adiponectin, and corticosterone concentrations were unaffected by maternal WD and did not differ between HR and C mice. With body mass as a covariate, heart ventricle mass was increased by maternal WD in both HR and C mice, but fat pads, liver, spleen, and brain masses were unaffected. As found previously, HR mice had larger brains than C mice. Body mass of grand-offspring was unaffected by grand-maternal WD, but grand-offspring wheel running was significantly increased for females of one HR line and decreased for females of another HR line by grand-maternal WD. In summary, maternal Western diet had long-lasting and general effects on offspring adult morphology, but effects on adult behavior were limited and contingent on sex and genetic background.

1. Introduction

The obesity epidemic has been expanding at an alarming rate. Poor diet and lack of physical activity are generally viewed as key components contributing to the increase, but recent research highlights the importance of several other factors, including environmental experiences in early life (McAllister et al. 2009). Specifically, maternal overnutrition during the perinatal stage (gestation + lactation) has been associated with increased risk for childhood obesity, cardiovascular diseases, and other ailments, with effects often lasting into adulthood. Changes to epigenetic regulation of these traits might be passed on to further generations, compounding the epidemic.

Rodent models have reported that maternal high-fat diet induces offspring leptin insensitivity and altered hypothalamic and hippocampal function through direct and epigenetic effects (Peleg-Raibstein et al. 2012; Sun et al. 2012; Williams et al. 2014; review in Moody et al. 2017). Maternal high-fat diets have also been reported to decrease offspring birth mass in mice (Sasson et al. 2015) and rats (Howie et al. 2009; Cunha et al. 2015), but increase offspring mass (with increase in fat and lean mass) from weaning into adulthood (Howie et al. 2009; Chambers et al. 2016; Guidotti et al. 2016). Studies of laboratory rats found that dams fed a high-fat diet have greater fat content in their milk after the first week of lactation (no difference in the first week) and increased milk production (Rolls et al. 1986; Purcell et al. 2011).

Maternal high-fat diet alters the glucocorticoid pathway of the dam, affecting offspring *in utero* and in later development in mice (Sasaki et al. 2014). Offspring of dams receiving a high-fat diet may have higher corticosterone levels as adults (Grissom

et al. 2017). Maternal high-fat diets may also lead to increased adult leptin (Masuyama and Hiramatsu 2012; Guidotti et al. 2016; Taylor 2016) and decreased adiponectin (Masuyama and Hiramatsu 2012). Maternal overnutrition during the perinatal stage has also been reported to alter neurobiological processes, including dietary preferences, reward signaling, learning, and memory (e.g., Frazier et al. 2008; Teegarden et al. 2009; Vucetic et al. 2010; Ozanne and Siddle 2011). Changes in maternal care have been reported to impact stress responses of offspring as juveniles to adults. Maternal high-fat diet increased anxiety-related behaviors of offspring in an elevated plus maze in mice (Peleg-Raibstein et al. 2012) and rats (Bilbo and Tsang 2010). In another rat study, maternal high-fat diet decreased adolescent offspring anxiety, but increased adult offspring anxiety (Sasaki et al. 2014), suggesting potential differences in the effect of maternal diet at various offspring ages.

High levels of physical activity reduce risks for various diseases and promote mental health and physical fitness. Levels of physical activity are likely influenced by maternal diet. In mice, a cross-fostering study found decreased spontaneous physical activity (SPA) and increased obesity in female pups of genetically obese (Avy/a) dams, even when they were fostered from birth to lean (a/a) dams (Baker et al. 2015). Similarly, cross-fostering mice to smaller litters at birth increased body mass and adiposity and decreased physical activity and energy expenditure, apparently related to sex-specific alterations in hypothalamic DNA methylation and gene expression (Li et al. 2013). Another study of mice found that maternal Western diet (WD; various formulae, but high in fat and sugar compared with standard chow) decreased adult offspring activity in their

home cage (Samuelsson et al. 2008), although a similar study of rats observed no significant effect (Samuelsson et al. 2010). Maternal diet with added sunflower oil elevated SPA of offspring at 20 and >65 days of age in one rat study (Brenneman and Rutledge 1982). Another study found that adult offspring of mice given high carbohydrate diets were hyperactive in their home cage (Samuelsson et al. 2008; Roghair et al. 2009). Voluntary exercise was decreased by maternal WD in a study of mice (Johnson et al. 2016). Clearly, maternal diet (and potentially other environmental factors, (Wahlqvist et al. 2015; Sutton et al. 2016)) can affect offspring physical activity and related traits. (At least one other study found effects of maternal over-nutrition on measures of locomotor behavior that have little to do with habitual spontaneous physical activity in home cages or voluntary exercise on wheels (Khan et al. 2003).)

Effects of maternal diet are likely dependent on the genetic background and associated behavioral and physiological traits of both the mother and her offspring. For example, individuals with inherently high levels of physical activity or high basal metabolic rates might experience some degree of "protection" from the adverse effects of maternal Western diet. This possibility could be addressed in various ways, such as through comparisons of strains of rodents that vary in activity levels (e.g., Lightfoot et al. 2004). Here, we used the high runner (HR) mouse lines that have been bred for increased voluntary wheel running for 70+ generations and compared them with non-selected control lines. HR lines run 2.5 to 3 times more revolutions per day when given wheel access (review in Swallow et al. 2009) and are more active in their home-cage without wheels (Malisch et al. 2009). The HR lines have also been reported to increase adult

wheel running when given Western diet from weaning through adulthood, indicating changes in energy balance as compared with the C lines (Meek et al. 2010). HR lines show changes in other relevant lower-level traits, including increased heart mass, increased VO_2max, increased circulating corticosterone and adiponectin concentrations, but reduced leptin levels, and an altered brain reward system (Swallow et al. 2005; Rezende et al. 2006; Garland et al. 2016; Thompson et al. 2017).

We tested the overarching hypothesis that the early-life environment can affect both spontaneous physical activity and voluntary exercise of adults, but that these effects would be different for HR and control lines of mice. We hypothesized that the genetic predisposition for high voluntary wheel running in HR mice would be protective against negative consequences of a maternal high-fat, high-sucrose diet. We further predicted that these effects could be mediated by changes in epigenetic regulation of genes as described above, in which case we could see the effects in the grand-offspring of dams fed WD. The replication of selected HR lines (N = 4) and non-selected C lines (N = 4) in this selection experiment helps to mimic the polygenic nature of human population differences in such complex traits as physical activity.

2. Materials and methods

All experiments and methods were approved by the Institutional Animal Use and Care Committee of the University of California, Riverside.

2.1.1 Experimental animals

Mice used for this experiment were from generation 73 of an ongoing, long-term artificial selection experiment that breeds for high voluntary wheel running (for reviews, see Rhodes et al. 2005; Swallow et al. 2009). A base population of 224 outbred individuals from the Hsd:ICR strain of house mice was randomly bred for 2 generations, then separated into 8 closed lines, each starting with 10 breeding pairs. The 8 lines were randomly designated into 4 lines bred for high voluntary wheel running (HR: lab designated as lines 3, 6, 7, 8) and 4 control lines bred without regard to wheel running (C: lab designated as 1, 2, 4, 5). Each generation, 2 males and 2 females were saved for C litters and up to 5 males and 5 females were saved for HR litters for effective within-family selection. At 6-8 week of age, mice from HR and C lines were individually housed for 6 days in cages attached to a Wahman-type activity wheel (1.12 m circumference, 35.7 cm diameter, 10 cm wide wire mesh running surface) with a recording device to count revolutions of the wheel in 1-min intervals for the duration of the experiment. In the HR lines, the highest running female and male from each family were chosen as breeders for the next generation based on their wheel running on days 5 and 6. Breeders were chosen randomly for C lines. All families were represented in the breeders to the next generation (termed "within-family selection") and sibling pairs were disallowed. Room temperatures were maintained at approximately 22°C, with lights on at 0700 for a 12:12 photoperiod, and *ad libitum* water and standard diet (SD: Teklad Rodent Diet W-8604, 14% kJ from fat, 54% kJ from carbohydrates, and 32% kJ from protein, no added sugars [less than ~9% naturally occurring sugars by weight, mostly from grains]).

2.1.2 Litter characteristics

In the present study, we manipulated the diet of dams (from generation 72 of the selection experiment) from 2 weeks before mating until pups were observed attempting to feed on solid food (~14 days after birth). All dams were given wheel access as young adults prior to diet manipulation as a normal selection generation. Dams were 14-16 weeks old when they gave birth. 100 dams (50 HR, 50 C) were fed a "Western" diet (WD: Harlan Teklad TD.88137, 42% kJ from fat, 42.7% kJ from carbohydrates, 15.2% kJ from protein, 34.1% added sucrose by weight) and another 100 dams (50 HR, 50 C) stayed on SD. The source of fat in WD was anhydrous milk fat, the source of protein was casein, and the sources of carbohydrates were sucrose and cornstarch (34.1 and 15.0 g/100 g, respectively). In addition, the high-fat diet contained 0.15% cholesterol.

Starting from birth and continuing until the pups were weaned at 3 weeks of age, 100 families (50 WD, 50 SD) were observed twice daily for developmental markers of the pups (i.e., first day for eye opening, moving, and feeding on solid food). Each cage was observed with a quick look ("spot check"). When pups were 15 days old, dams and litters were all switched to SD. Pups were weaned at 21 days of age and weighed. Since these mice were used as breeders as usual for the selection experiment, 2 males and 2 females were saved for C litters and up to 5 males and 5 females were saved for HR litters. One additional male pup from 50 WD dams and 50 SD dams (half C and HR in each group) were considered focal mice and received various additional tests (Fig. 4.1).

Males were chosen as focal mice in this study to avoid variation related to the estrus cycle (e.g., Gomes et al. 2009).

2.2. Body mass and body composition

All mice were weighed at weaning (3 weeks old) and before and after 6 days of wheel access (7-11 weeks old).

Focal males were additionally weighed at ~4.5 weeks and ~5.5 weeks of age. Focal male body composition was also measured by non-invasive quantitative magnetic resonance (EchoMRI-100; Echo Medical Systems LLC, Houston, Texas, USA). The body composition scanner independently calculated fat mass and lean mass in grams. Fat mass was analyzed as such and as a percentage of total body mass.

Change in body mass after wheel access was calculated as an absolute change (post-exercise minus pre-exercise mass) and as percent change according to the equation:

$$\frac{post\text{-}exercise\ mass - pre\text{-}exercise\ mass}{pre\text{-}exercise\ mass} \times 100$$

Similarly, change in lean mass was calculated as an absolute change and using the equation above. Change in fat mass was calculated as an absolute change in grams, change in percent fat mass, and using the equation above with fat mass in grams and percent fat mass.

2.3. Food consumption

For focal males, juvenile food consumption of SD (grams/day) was measured from 3 to 6 weeks of age. Food hoppers were weighed and any obvious shredding or

wasting of food was noted. Food consumption was calculated as the absolute change in grams from 3 to 4.5 weeks of age and again from 4.5 to 6 weeks of age.

2.4. Maximal aerobic capacity (VO_2max*)*

VO_2max was measured during forced exercise in a 900 ml enclosed wheel metabolic chamber as described previously (Dlugosz et al. 2012; Claghorn et al. 2017). Each trial lasted 5 min and each mouse was tested twice, with a rest day in between. Trials started by placing a mouse in the wheel chamber and manually spinning the wheel, slowly increasing the spinning speed over the trial. Air was pumped into the wheel at 2000 ml per min. A subsample of air (150ml) was pumped out, ran through Drierite and soda lime to remove moisture and CO2, and then the volume of O2 was measured in an oxygen analyzer (S-3A Applied Electrochemistry INC. Sunnyvale, CA). Outputs from the instruments were digitized by an analog-to-digital converter (ADAM-4017 data Acquisition Module) and recorded every second on a computer using LabHelper software (Warthog Systems, www.warthog.ucr.edu). The highest 1-min interval of oxygen consumption in either trial was used to measure VO_2max per mouse. The wheel apparatus for measuring VO_2max was chosen over the more traditional treadmill-based test because they obtain equivalent values and the wheel apparatus closely mimics the behavior for which HR mice have been bred (Dlugosz et al. 2013).

2.5. Behavior in the elevated plus maze

As adults, focal males were tested for their behavior in an elevated plus maze. The maze consists of a plus-sign shaped platform, 1 m off the ground, with two exposed and two enclosed arms (length: 100 cm, width: 9 cm) joined to a central square platform (9 cm x 9 cm). Each trial lasted 5 min. The percentage of time a mouse spends in the enclosed arms is often used as a measure of anxiety (Mitra and Sapolsky 2008). Behavior in the maze was obtained at 1000 h-1400 h (3-7 h after lights on) in a lit room, recorded with HD Webcam C525 (Logitech International S.A., Lausanne, Switzerland) and analyzed using TopScan LITE software (Clever Sys, Inc., Reston, Virginia, USA). The surface of the maze was cleaned before each trial and mice were placed in the center square at the beginning of the trial.

2.6. Spontaneous physical activity

From weaning to 6 weeks of age, focal males were housed individually and monitored daily for home-cage activity using passive infrared motion-detector sensors (Acosta et al. 2015; Copes et al. 2015). A computer with custom Activity Recording Software (developed by Dr. Mark A. Chappell, UC Riverside) measured activity per 1-min intervals for 23 h (Thompson et al. 2017). Activity in home cages was also measured during wheel testing (described in 2.1.1.).

2.7. Organ masses and plasma hormone concentrations

Focal males were sacrificed by decapitation 6-16 days post wheel access (counterbalanced by diet and linetype). Analyses of organs and hormones used as a covariate the number of days between the end of wheel access and sacrifice. Blood samples and various organs and tissues were dissected and weighed: posterior subcutaneous fat pads, caudal portions of the abdominal pelvic fat pad (Cinti 2007), heart ventricles, livers, spleens, and brains. Heparinized blood samples were spun at 13,000 RPM for 12 min and collected plasma was stored at -20°C.

Plasma leptin was measured using a Crystal Chem Enzyme-linked Immunosorbent Assay (ELISA) kit (Mouse Leptin Assay Catalog #90030), without dilution and measured in duplicate in 96-well plates. Absorbances were read at 450 nm in an EPOCH2 microplate reader, using GEN5 2.07 reading software (microplate and reading software: BioTek Instruments, Inc., Winooski, VT, USA) and compared with a standard curve generated individually for each plate. Plasma adiponectin was measured similarly with an AssayPro ELISA kit (Mouse Adiponectin ACRP30 Catalog #EMA 2500-1), diluted 400-fold and measured in duplicate. Plasma corticosterone was measured similarly with an Arbor Assays ELISA kit (Corticosterone EIA kit Catalog #K014-H1), diluted 150-fold and measured in duplicate.

Leptin and adiponectin hormone concentrations were analyzed with covariates of fat percent of body mass.

2.8. Grand-offspring characteristics

To measure the effects of grand-maternal diet, males and females whose dams had the same diet (SD or WD) were paired to breed following the same protocol as the normal selection experiment. That is, for each HR line, we chose the highest-running male and female from each family and paired them to the highest runners from other families, but in this case only to mice whose mothers had the same diet. In each C line, we chose breeders without regard to their wheel running, but also paired based on maternal diet and disallowed mating between siblings. Then, the offspring of these pairings (i.e., grand-offspring of dams fed SD or WD) were given 6 days of wheel access as adults and weighed before and after.

2.9. Statistical analyses

All analyses were performed using the Mixed Procedure in SAS 9.1.3 (SAS Institute, Cary, NC, USA) to apply analysis of covariance models with Type III tests of fixed effects and REML estimation. Linetype (HR or C) and maternal diet were fixed effects; replicate lines were nested within linetype as a random effect. Effects of linetype, maternal diet, and their interaction were tested relative to the variance among replicate lines, and degrees of freedom were always 1 and 6 for these effects. The foregoing description applies to both the 100 focal males and the ~560 male and female offspring for which we obtained data on body mass at weaning, before and after adult wheel access, as well as adult wheel running for 6 days (Fig. 4.1). For the latter set of mice, we

also used dam as a random effect nested within linetype to allow for possible litter effects.

Covariates depended on the trait being analyzed and included age, body mass, wheel freeness (inverse measure of rotational resistance), home cage sensor sensitivity (Copes et al. 2015), total wheel running (revolutions), and/or time from end of wheel access to sacrifice. In the results when we refer to traits being “adjusted for” by various variables, we mean that these variables were used as covariates in ANCOVA. Dependent variables were transformed as necessary to improve normality of residuals. All P values are 2-tailed unless otherwise indicated.

Body mass and voluntary wheel running were further analyzed by line to measure differences among replicate populations. Although HR lines experienced the same directional selection, each replicate line (and sexes within lines) differed somewhat in the rate and magnitude of response to selection (Careau et al. 2013), as well as other phenotypes (e.g., Rhodes et al. 2005; Swallow et al. 2009; Wallace and Garland 2016). Thus, the replicate HR lines have had "multiple solutions" (Garland et al. 2011) and may be expected to differ in response to manipulation of maternal diet. When the interaction between diet and line was statistically significant, we checked the P value for differences of least-squares means for effect of diet on each line (SAS Procedure Mixed).

Mini-muscle status was also included as a cofactor in some analyses for focal males. Mini-muscle is a simple Mendelian genetic trait (Garland et al. 2002; Kelly et al. 2013) that causes ~50% reduced hindlimb muscle mass in two HR lines (all mice in line 3 and a subset in line 6). Mini-muscle status was determined for focal males at the end of

the study by inspection of triceps surae muscle mass regressed on body mass. All 10 focal males in line 3 (4 SD and 6 WD) and 3 of the 15 focal males in line 6 (1 SD and 2 WD) were mini-muscle individuals. Among several other phenotypic effects observed in adult mice, mini-muscle individuals were reported previously to have enlarged internal organs (e.g., Swallow et al. 2005; Kolb et al. 2010; Templeman et al. 2012) and an elevated cost of transport during voluntary wheel running (Dlugosz et al. 2009).

3. Results

Western diet had a variety of effects on the behavior and body mass of both dams and their offspring, and some of these effects were specific to mice from replicate lines (genotype-by-environment interaction).

3.1. Litter characteristics

All analyses of litter characteristics used age of the dam as a covariate. Analyses of pup behaviors were performed with and without litter size as a covariate. Litter size was not significant ($P > 0.05$) when used as a covariate, so the results without litter size are presented here (Fig. 4.2 A, B, and C; see Table 4.2 for results when litter size was included). The timing of the first occurrences for any pup of a litter to move on their own or open their eyes were not significantly affected by maternal WD and did not differ between HR and C lines (Fig. 4.2A and B, respectively). The first sighting for a pup to eat solid food was earlier for C litters with maternal WD (Diet-by-linetype interaction; Fig. 4.2C).

Adjusted for dam age, dam mass at weaning was not significantly affected by maternal WD and did not differ between HR and C (Table 4.2). Analyses of litter size, total litter mass, and mean litter mass are shown in Fig. 4.2 (D, E, and F). Adjusted for dam mass and age, the number of pups per litter at weaning was not significantly affected by maternal WD but was greater for HR than C dams (Fig. 4.2D). Total litter mass was increased by maternal WD but did not differ between HR and C families (Fig. 4.2E). Mean pup mass was increased by maternal WD and tended to be lower for HR than C (Fig. 4.2F). Adjusting for dam age, time from pairing to birthing pups was not affected by maternal WD and did not differ between HR and C mice (Table 4.2). Sex ratio of pups (measured as number of female pups divided by total number of pups) was also not affected by maternal WD and did not differ between HR and C mice (Table 4.2).

3.2. Body mass

Body mass at weaning was increased by maternal Western diet for both female and male offspring (Fig. 4.3A and B), and HR males were smaller than C males (Fig. 4.3B). Juvenile male mass was also increased by maternal WD at ~5 weeks of age and ~6 weeks of age (Fig. 4.4). Females were not weighed as juveniles.

Body mass at the start of wheel testing (~10 weeks of age) was increased for both females and males by maternal WD (Fig. 4.3C and D), and HR males were significantly smaller than C males (Fig. 4.3D). All groups significantly gained body mass during wheel access, regardless of maternal WD or linetype. However, the amount of increase in

body mass after 6 days of wheel access was not affected by maternal WD or linetype (Table 4.2). Analyses of body mass by replicate lines are presented in (Fig. 4.12).

3.3. Focal male body composition

Both lean and fat masses were increased by maternal WD at all measurements from weaning to adulthood (Fig. 4.4). The increase for fat mass was greater than for lean mass in terms of both absolute and relative (% increase) values (results not shown). All groups gained lean mass during wheel access (all $P < 0.05$ except HR whose mothers had SD, for which $P = 0.0841$). However, the amount of increase in lean mass after 6 days of wheel access was not affected by maternal WD or linetype (Table 4.2). Absolute and percent change in lean mass after 6 days of wheel access was not affected by maternal WD and did not differ between HR and C mice (Table 4.2).

Regardless of maternal diet or linetype, mice lost fat after 6 days of wheel access. The decrease in absolute fat mass was significantly greater for mice whose mothers had WD ($P = 0.0340$), though percent change in fat percentage was not affected by maternal WD and did not differ between HR and C mice (Table 4.2).

3.4. Food consumption

Food consumption of SD was measured for juvenile focal males from 3-6 weeks of age. Using body mass as a covariate, food consumption was not affected by maternal WD or linetype, with no interaction (Table 4.2).

3.5. Maximal aerobic capacity (VO_2max)

For focal male mice, mass- and age-adjusted VO_2max was unaffected by maternal WD, but HR had higher VO_2max than C mice (Fig. 4.5). Mini-muscle individuals had elevated VO_2max.

3.6. Behavior in the elevated plus maze

Total duration in the maze varied slightly (range = 293 - 314 seconds), so trial duration was used as a covariate in all analyses. Mice spent 54.4% ± 13.6% (mean ± standard deviation) of the 5-min test in the closed arms of the maze, compared with 23.7% ± 12.5% in the open arms and 21.7% ± 9.2% in the center. They moved on average 15.9 m total (range: 4.00 – 20.66 m). Distance moved in each section was 11.2 ± 3.2 m in the closed arms, 2.6 ± 2.1 m in the open arms, and 2.1 ± 0.9 m in the center square.

We analyzed measures putatively related to anxiety, including percent entries into open arms and percent of time spent in open arms (Mitra and Sapolsky 2008), as well as number of fecal pellets and urine pools at the end of the trial. We also analyzed, for each zone of the elevated plus maze (closed arms, open arms, and the center): latency to enter from the start of the test, number of entries, time spent, distance moved, and velocity. None of the above measurements of behavior were significantly affected by maternal WD or linetype, with no interaction and no effect of mini-muscle status (Table 4.2 presents a subset of these analyses).

3.7. Spontaneous physical activity

Maternal WD significantly increased home-cage activity of focal HR and C males at 3-4 and at 4-5 weeks of age, but by 5-6 weeks of age the effect was no longer statistically significant (Fig. 4.6). At 5-6 weeks of age, HR mice tended to be somewhat more active than C mice, but this effect was not statistically significant. Adding body mass to the models did not change statistical results in any important way (results not shown).

Adult focal male home-cage activity (measured during the 6 days of wheel access) was statistically unaffected by maternal WD, linetype, or their interaction. Additional analyses with amount of wheel running on days 5 and 6 as covariates did not change results (Table 4.2).

3.8. Voluntary exercise

Wheel running of adult females was 3-fold higher in HR than C and 3.1-fold higher in adult male HR than C (Fig. 4.7 A and B). Average number of minutes spent running per day was not significantly different between HR and C females, but HR males ran for more minutes than C males (Fig. 4.7 C and D). Average speed of wheel running was higher in HR females than C females and higher in HR males than C males (Fig. 4.7 E and F). No aspect of adult wheel running (total distance, duration, average running speed, maximum speed in any one-min interval [results not shown]) was statistically affected by maternal WD, with no interaction between linetype and WD. Analysis of wheel running by replicate lines are presented in section 3.11.

3.9. Organ masses

Adjusting for body mass and age, heart ventricle mass was increased by maternal WD, with no effect of linetype and no interaction (Table 4.1). Posterior subcutaneous fat pad, abdominal pelvic fat pad, liver, spleen, and brain masses were unaffected by maternal WD (Table 4.1). As reported previously (Kolb et al. 2013), HR mice tended to have larger brains than C mice. Mice with the mini-muscle phenotype had larger hearts, livers, spleens, and both fat pads (Table 4.1), with several of these effects being reported previously (see Discussion).

3.10. Plasma hormone concentrations

With age and days from the end of wheel access to sacrifice as covariates, plasma leptin, adiponectin, and corticosterone concentrations were unaffected by maternal WD and did not differ between HR and C lines, with no interaction (Table 4.3).

3.11. Line analyses of wheel running

Analyses of variation among replicate lines were performed separately by sex and linetype (Fig. 4.8). These analyses revealed that replicate lines of C (N = 4) and HR (N = 4) mice responded differently to maternal WD, and that these responses were influenced by sex.

For wheel running (mean revolutions/day on days 5 & 6) by C females, maternal WD had no statistical effect and replicate lines did not differ significantly (Fig. 4.8). In HR females, however, maternal WD significantly increased wheel running in one line

(HR line 7). In C males, maternal WD increased wheel running, and lines differed significantly. In HR males, maternal WD did not significantly affect wheel running, but lines differed significantly.

We analyzed the number of intervals run per day (1-min intervals containing at least one revolution), the mean wheel-running speed (revolutions/intervals), and the maximum speed (single highest 1-min interval), as averages of days 5 & 6 (Figs. 9-11). We also analyzed body mass by replicate line (Fig. 4.12).

3.12. Effects on grand-offspring

To measure the effects of grand-maternal diet, males and females whose dams had the same diet (SD or WD) were paired to breed. Grand-offspring body mass and wheel running as adults were unaffected by grand-maternal WD when analyzed by linetype (Table 4.4).

Line analyses of grand-offspring wheel running revealed that replicate lines of HR mice were affected by grand-maternal WD, but only in female mice (Fig. 4.13). Specifically, maternal WD significantly increased wheel running for females in HR line 7 and significantly decreased it for females in HR line 3 (differences of least squares means from SAS Procedure Mixed). C females were not affected by grand-maternal WD and replicate lines did not differ significantly. In C and HR males, maternal WD did not affect wheel running but replicate lines differed significantly. We also analyzed grand-offspring body mass by sex and replicate line, but found no differences due to grand-maternal diet (Fig. 4.14).

4. Discussion

We tested the general hypothesis that early-life exposure to maternal Western diet (beginning prior to conception and lasting through ~2/3 of the lactation period) would affect adult offspring body composition, levels of physical activity, and associated subordinate traits (e.g., organ masses and hormone levels). We expected that any such effects would be mediated by genetic background; in particular, we predicted that the four selectively bred High Runner (HR) lines of mice would be somewhat protected against negative consequences of maternal overnutrition as compared with the four non-selected Control lines. For most measures, we obtained data on ~100 focal adult males, but for adult wheel running and body mass we also obtained data for ~200 additional males and ~300 total females.

4.1. Maternal diet changes offspring body composition

Maternal WD had long-lasting effects on the morphology of offspring, increasing fat mass, lean mass, and percent fat of body mass well into adulthood (Fig. 4.4). Fat % has been found to be increased by maternal high-fat diets in rats (Howie et al. 2009; Chambers et al. 2016), but to our knowledge, our study is the first to report increased lean mass. As illustrated in Fig. 4.4 and in Table 4.2, maternal WD significantly increased absolute fat mass at weaning, at 4.5 weeks of age, at 5.5 weeks of age, and at ~10 weeks of age (all $P < 0.01$), which was immediately prior to the start of wheel access. However, the effect on fat mass was no longer statistically significant after wheel access ($P = 0.28$). Moreover, the absolute amount of fat lost during wheel access was significantly greater

for mice whose dams had experienced WD (Table 4.2). Thus, even acute exercise can reverse the effect of maternal WD on fat mass, and this occurred for both HR and C mice. In future studies, it would be of considerable interest to determine if the effect of acute adult exercise persists.

Maternal WD increased adult heart ventricle mass, even after adjusting for variation in body mass by ANCOVA. Although we have not tested whether this increase was beneficial (physiological) or detrimental (pathological), other studies of mice have reported that maternal high-fat, high-sugar diet caused pathological increases in ventricle mass (Fernandez-Twinn et al. 2012; Blackmore et al. 2014). In any case, maternal WD did not increase offspring maximal aerobic capacity, which should generally correlate positively with heart size (e.g., Rezende et al. 2006). Interestingly, we found that individuals with the mini-muscle phenotype (found only in two of the HR lines in the present sample of mice) had statistically higher VO_2max. Previous studies have generally not found this to be the case (Kolb et al. 2010; Dlugosz et al. 2013), although mini-muscle individuals did have higher VO_2max when tested in hypoxia (Rezende et al. 2006) and in a comparison of only one control and two HR lines (Templeman et al. 2012).

4.2. Maternal diet apparently changes offspring energy balance

Maternal WD increased juvenile (3-6 week old) home-cage activity. All mice became more active with age, and at 5-6 weeks HR tended to be more active than C, as expected from previous studies when these mice are housed without wheels (Rhodes et

al. 2001; Malisch et al. 2009; Acosta et al. 2015; Copes et al. 2015). However, consumption of standard chow, measured only during the juvenile stage for focal males and analyzed using body mass as a covariate, was not affected by maternal WD and did not differ between HR and C mice (Table 4.2). This result is consistent with a previous study of mice which reported that maternal over-nutrition increased offspring preference for high-fat food but did not change intake of control food (Sasaki et al. 2016). Given that offspring spontaneous physical activity was increased by maternal WD, coincident with increased offspring body mass (Fig. 4.4, weeks 3-6) but no change in juvenile food consumption (adjusted for variation in body mass), it is possible that basal or resting metabolic rate was reduced by maternal WD.

In general, maternal Western diet did not affect voluntary exercise on wheels by adult offspring. However, we did find line- and sex-specific effects. Specifically, maternal WD increased wheel running for female offspring of HR line 7, which emphasizes our previous findings that the replicate HR lines and the two sexes have undergone somewhat different evolutionary paths under the same selection regime (Garland et al. 2002, 2011; Wallace and Garland 2016). A sex-specific effect was also found in a study of rats with maternal high-fat diet, which reported less active male and more active female offspring when given free wheel access for one week (Cunha et al. 2015). Furthermore, grand-offspring of the dams fed WD had significantly altered wheel running in two HR lines (Fig. 4.13). Maternal WD significantly increased wheel running in HR line 7 offspring and grand-offspring. In HR line 3, maternal WD only tended to decrease running in the offspring, but significantly decreased running in the grand-

offspring, showing an "amplifying" effect through two generations. (Interestingly, line 3 is fixed for the mini-muscle allele (Kelly et al. 2013).) Transgenerational amplification of obesity in mice can be mediated by epigenetic alterations via DNA methylation (Waterland et al. 2008), which could be the case for activity levels as well.

Two previous studies on these lines of mice have reported that cage activity is reduced when rodents are housed with wheels (Acosta et al. 2015; Copes et al. 2015). In the present study, we only measured home-cage activity of adults with wheel access and found no statistical effect of maternal WD and no difference between HR and C mice (Table 4.2), the latter result consistent with previous reports (Acosta et al. 2015; Copes et al. 2015). Another study of mice, housed without wheels, reported that a maternal high-fat, high-sugar diet decreased spontaneous physical activity of adult offspring measured over one week via telemetry (Samuelsson et al. 2008).

4.3. No apparent effects of maternal diet on offspring hormone levels

Hormone concentrations of offspring were measured 6-16 days after 6 days of adult wheel access, which may have influenced results. As stated in section 4.1, after 6 days of wheel access, fat mass no longer differed between groups. Additionally, we found no effect of maternal WD on fat pad masses measured at dissection (Table 4.1). In general, body fat is a positive predictor of leptin levels in mice, including in previous studies of the lines we studied (Girard et al. 2007; Acosta et al. 2015). As expected, we found that leptin levels were positively associated with the masses of both posterior subcutaneous fat pads and abdominal pelvic fat pads (Table 4.3). Therefore, leptin levels

may have been affected by maternal WD before adult wheel access, but been returned to "baseline" values due to exercise-related fat loss. Alternatively, the lack of effect of maternal WD might be a common outcome, as a review of rat studies with maternal high-fat diet reported that 4 of 8 studies did not find significant effects on plasma leptin levels (Ainge et al. 2011).

One previous study of ICR mice (the base population for our selection experiment) found that maternal WD decreased circulating adiponectin concentrations in adult offspring (Masuyama and Hiramatsu 2012), but we did not observe such an effect. Circulating adiponectin concentration is expected to be strongly negatively related with body fat (e.g., Matsubara et al. 2002; Stefan et al. 2002), which, as noted in the previous paragraph, was not affected by maternal WD after 6 days of adult wheel access. Furthermore, we did not replicate the finding of a previous study that showed higher plasma adiponectin in HR versus C males (Vaanholt et al. 2007).

Maternal high-fat diet has been reported to lower offspring circulating corticosterone levels in mice (Sasaki et al. 2014; Grissom et al. 2017), but we did not observe this effect. Previous studies have reported that HR mice have higher circulating corticosterone levels than C mice (e.g., Malisch et al. 2009, 2016), but we did not obtain this result (Table 4.3), possibly because mice were measured after 6 days of wheel access, followed by several days of sedentary housing.

Physical activity is frequently a confounding factor in measurements of circulating hormone levels. Ideally, we would have taken blood samples prior to adult wheel testing, but we chose not take any blood samples at that time because we did not

want to risk affecting the wheel-running phenotype that was a crucial outcome variable for this study. Therefore, we cannot definitively conclude the lack of endocrine effects due to maternal WD. (Similar cautions would apply to organ masses, although we did find an effect of maternal WD on heart size.) Future studies should examine hormone levels at different stages of development for offspring of mothers with high-fat, high-sugar diets while including treatments of sedentary vs. active. Another approach would be to examine the density of central leptin receptors in the hypothalamus, given the differences in body fat mass resulting from maternal diet (Fig. 4.4, Table 4.2).

4.4. Concluding remarks

In summary, we found that maternal Western diet can have long-lasting effects on offspring adult morphology and behavior, although these effects can be mediated by sex and genetic background. However, some effects, such as that observed for body fat (Fig. 4.4), can be reversed by as little as 6 days of wheel access. Use of the polygenic high runner mouse lines and their control counterparts allows for a wide scope of exploration of variation in the complex phenotypes related to activity levels, including the importance of population differences and effects of genetic background in interacting with environmental factors. To our knowledge, ours is the first study to characterize effects of maternal WD on mouse strains with inherent propensity for high levels of physical activity.

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Tables and Figures

Table 4.1. Adult internal organ masses of mice after 6 days of wheel access. Log_{10} body mass, age, and days from the end of wheel access to sacrifice were used as covariates (results not shown). The grand mean of body mass was 29.90 g with standard deviation = 3.16 g (N = 92). Groups were approximately equal HR and C, with maternal SD or WD. Signs following P values indicate direction of effect: for diet, "+" indicates Western diet > standard diet; for linetype, "+" indicates high runner lines > control lines.

		P values of effects				
Organ mass (log_{10} transformed)	N	Body mass	Diet	Linetype	Interaction	Mini
Abdominal pelvic fat pad	92	<0.0001+	0.8054-	0.7513-	0.4243	**0.0380+**
Posterior subcutaneous fat pad	91	<0.0001+	0.6195+	0.9624-	0.4475	**0.0371+**
Heart ventricle	92	<0.0001+	**0.0050+**	0.2327+	0.4932	**0.0005+**
Liver	92	<0.0001+	0.1638-	0.9518-	0.9902	**0.0070+**
Spleen	89	<0.0001+	0.0578-	0.6383-	0.0932	**0.0014+**
Total brain	91	<0.0001+	0.3560+	0.0680+	0.3159	0.1455+
Cerebellum	91	0.0101+	0.6049-	0.2192+	0.2955	0.9785+
Non-cerebellar brain	91	0.0001+	0.2636+	0.1516+	0.7370	0.0825+

Table 4.2. Various physiological and behavioral tests on mice whose dams were given Western.
Main effects were maternal diet (WD or SD), linetype (HR or C), and their interaction. Signs indicate direction of effect (indicated only for $P < 0.1$) and all statistically significant P values (<0 .05) are in bold. All analyses included age as a random variable, and additional covariates were used where appropriate, including body mass, mini-muscle status, litter size, and sensitivity of home-cage sensors. All analyses included covariates of age (not shown).

			P values of effects					
Trait	Sex	N	Diet	Linetype	Interaction	Body mass	Mini	Other
Litter characteristics								
First pup move on own	Both	97	0.9411	0.9547	0.9096			litter size 0.0713-
First pup feed on own	Both	95	**0.0010+**	**0.0155-**	**0.0054**			litter size 0.0983+
First pup with eyes open	Both	93	0.0588+	0.3041	0.9398			litter size 0.2845
Dam mass at weaning	Dams	95	0.2052	0.6342	0.6527			litter size **0.0026+**
Pair-to-birth interval	Dams	100	0.9639	0.0941+	0.2730			
Pup sex ratio	Dams	119	0.1274	0.7159	0.4033			
Focal male body composition								
Body mass at weaning	Male	97	**0.0134+**	0.1629	0.9492		0.9817	
4.5 weeks	Male	96	**0.0018+**	0.1383	0.5199		0.7189	
5.5 weeks	Male	97	**0.0021+**	0.0828-	0.7811		0.2379	
Start of wheel access	Male	95	**0.0017+**	0.1403	0.8796		0.0704-	
After wheel access	Male	94	**0.0026+**	0.1592	0.5201		0.2391	
Sacrifice	Male	93	**0.0027+**	0.1386	0.5675		0.1219	
Lean mass at weaning	Male	95	**0.0088+**	0.0738-	0.8130		0.7121	
4.5 weeks	Male	96	**0.0021+**	0.1856	0.9002		0.5158	
5.5 weeks	Male	95	**0.0027+**	0.0822-	0.8247		0.0780-	
Start of wheel access	Male	94	**0.0059+**	0.1589	0.8463		**0.0221-**	
After wheel access	Male	93	**0.0031+**	0.1574	0.9127		**0.0472-**	

Table 4.2 continued.

Fat mass at weaning	Male	97	**0.0097+**	0.3084	0.5841		0.7222
4.5 weeks	Male	93	**0.0003+**	0.7204	0.6346		0.0560+
5.5 weeks	Male	96	**0.0023+**	0.3663	0.8681		0.1207
Start of wheel access	Male	94	**0.0098+**	0.5263	0.3842		0.8503
After wheel access	Male	93	0.2839	0.3912	0.9503		**0.0018+**
Fat percent at weaning	Male	91	**0.0003+**	0.5160	0.5333		0.4877
4.5 weeks	Male	94	0.0889+	0.4018	0.9746		**0.0155+**
5.5 weeks	Male	94	**0.0168+**	0.6965	0.9106		0.0655+
Start of wheel access	Male	94	0.0566+	0.7649	0.2822		0.4864
After wheel access	Male	93	0.9614	0.6383	0.9524		**<0.0001+**
Change in body composition before vs. after 6 days of wheel access							
Total mass (absolute)	Female	308	0.7503	0.6913	0.7943		
Total mass (% change)	Female	305	0.8806	0.8680	0.7911		
Total mass (absolute)	Male	341	0.1537	0.7070	0.8513		
Total mass (% change)	Male	341	0.1308	0.7254	0.8372		
Lean mass (absolute)	Male	93	0.3164	0.4462	0.9463		0.2976
Lean mass (% change)	Male	93	0.4140	0.4625	0.9470		0.1654
Fat mass (absolute)	Male	93	**0.0340+**[$]	0.3683	0.3728		**0.0297-**
Fat mass (% change)	Male	93	0.0911+	0.6388	0.1649		**0.0047-**
Fat % (absolute)	Male	93	0.0636+	0.4480	0.3038		0.1019
Fat % (% change)	Male	93	0.0938+	0.5944	0.1835		**0.0091-**
Juvenile food consumption							
3-4.5wks	Male	92	0.5656	0.1443	0.3487	**<0.0001**	0.6453
4.5-6wks	Male	91	0.4173	0.2233	0.5197	**<0.0001**	0.1410

Table 4.2 continued.

Elevated plus maze							
Total distance	Male	94	0.4745	0.2298	0.4452	0.6977	
% time in center	Male	94	0.8482	0.6350	0.7149	0.1451	
% time in open arms	Male	94	0.5962	0.8614	0.2095	0.4891	
% entries into open arms	Male	93	0.5828	0.6667	0.5474	0.4763	
% distance in open arms	Male	93	0.5752	0.9566	0.2927	0.7151	
Velocity in open arms	Male	92	0.2388	0.6103	0.7097	0.4973	
Velocity in closed arms	Male	93	0.3473	0.2542	0.4156	0.7114	
Fecal pellets	Male	93	0.7894	0.0784	0.9984	0.8283	
Fecal pellets + urine pools	Male	93	0.8721	0.3054	0.7111	0.9174	
							Sensor sensitivity
Adult home-cage activity	Male	89	0.5770	0.8249	0.2431	0.1219	0.5178
with wheel revolutions[&]	Male	65	0.5474	0.5538	0.1175	0.1325	0.2757

[$] All mice lost fat, and mice with maternal WD lost more fat.

[&]Wheel revolutions per day was an additional covariate (P = 03795), missing for a quarter of the mice.

Table 4.3. Plasma concentrations of three hormones: leptin, adiponectin, and corticosterone.
All analyses used age and number of days from the end of wheel access to sacrifice as covariates (results not shown). Bleed delay time was less than 2 minutes, and did not significantly affect corticosterone concentrations (results not shown). Sample size was N = 91-93, approximately equally distributed among experimental groups. Sign after significant P values indicates direction of effect.

	P values of effects				
Hormone	Diet	Linetype	Interaction	Mini	Other
Leptin (square root ng/ml)	0.2811	0.3424	0.4349	0.1012	
with body mass	0.7169	0.8420	0.2817	**0.0150+**	**Mass P<0.0001+**
with posterior subcutaneous (PS) fat pad	0.5476	0.6213	0.2284	0.2548	**PS P<0.0001+**
with abdominal pelvic (AP) fat pad	0.9944	0.9385	0.4316	0.1530	**AP P<0.0001+**
with PS and AP fat pads	0.9899	0.8781	0.2367	0.2558	**PS P=0.0002+**
					AP P=0.0023+
Adiponectin (square root mg/ml)	0.6008	0.3579	0.3873	0.1829	
with body mass	0.8277	0.5793	0.4354	0.2620	Mass P=0.1361
with posterior subcutaneous (PS) fat pad	0.7617	0.3486	0.3335	0.1982	PS P<0.7426
with abdominal pelvic (AP) fat pad	0.5159	0.3146	0.3951	0.2042	AP P<0.5999
with PS and AP fat pads	0.6288	0.3107	0.3602	0.1888	PS P=0.4643
					AP P=0.3296
Corticosterone (log_{10} ng/ml)	0.7406	0.1504	0.2525	0.3257	

Table 4.4. Grand-offspring body mass and wheel running as adults.
Grand-offspring were produced by mating males and females within each line whose dams had the same diet. All analyses used age as a covariate and wheel-running analyses also used wheel freeness. Groups were approximately equally HR and C, with maternal SD and WD.

		P values of effects				
Trait	N	Diet	Linetype	Interaction	Age	Wheel Freeness
Female grand-offspring						
Wheel running (day 5&6)	276	0.8083-	**<0.0001+**	0.3742	0.1758-	**0.0015+**
Adult body mass	283	0.9777-	0.0772-	0.8924	**<0.0001+**	
Adult body mass after wheel access	282	0.3610-	0.2118-	0.9095	**0.0027+**	
Male grand-offspring						
Wheel running (day 5&6)	267	0.6984-	**0.0002+**	0.8705	**0.0079-**	0.6541+
Adult body mass	331	0.7675-	**0.0460-**	0.2688	**<0.0001+**	
Adult body mass after wheel access	270	0.3022-	**0.0242-**	0.3968	**<0.0001+**	

Figure Legends

Fig. 4.1. Timeline of experimental design. Above the line are procedures given to all mice (N = 560), which include maternal diet manipulation (SD = standard diet, WD = Western diet), weaning at 3 weeks and 6 days of wheel access in adulthood. Below the line are procedures for 100 "focal" families and one male offspring from each of those families, which includes twice-daily behavior checks during the first 3 weeks, monitoring of home-cage activity and food consumption, behavioral testing on an elevated plus maze, and maximal aerobic capacity (VO_2max). Each tick mark represents one week and asterisks indicate when mice were weighed (in black) and measured for fat and lean composition (in grey).

Fig. 4.2. Litter characteristics. A-C: for each litter, the first day that developmental markers were observed for at least one pup. D-F: number of pups per litter and total and mean pup mass at weaning (3 weeks after birth), adjusted for dam mass (grand mean = 29.34g, standard deviation = 3.38g). N = 25 per group. Bars represent least-squares means (LSM) + standard error (SE). Striped bars indicate maternal standard diet, solid bars indicate maternal Western diet, grey bars are control mice, and black bars are high runner (HR) mice. Results of statistical analyses (fixed effects) are shown above each graph. All analyses included dam age as a covariate (*P* values not shown).

Fig. 4.3. Body mass at weaning (3 weeks of age) and as adults (just prior to wheel testing, 7-11 weeks of age) of C and HR mice given maternal SD or WD, with separate analyses for females and males. Bars are age-adjusted LSM + SE, and N were approximately equal for each group. Striped bars indicate maternal standard diet, solid bars indicate maternal Western diet, grey bars are control mice, and black bars are high runner (HR) mice. Results of statistical analyses (fixed effects) are shown above each graph. All analyses included age as a covariate (*P* values not shown).

Fig. 4.4. Focal male body composition (total body mass, lean mass, and fat mass), adjusted for age. 3 weeks of age corresponds to weaning and 10 weeks of age represents measurements when mice were first granted adult wheel access (though this ranged from 9-10 weeks old), and 11 weeks represents measurements after 6 days of wheel access. Age at approximately 11.5 weeks represents measurements at sacrifice (top panel only). Each point represents a LSM for ~25 males and error bars are standard errors. Open points and dashed lines indicate maternal standard diet, solid points and lines indicate maternal Western diet, control mice are in grey, and high runner mice are in black. 2-tailed $P < 0.05$ for effect of maternal diet at the time points indicated by an asterisk (*).

Fig. 4.5. Maximal aerobic capacity (VO_2max) measured at ~8 weeks of age for focal males. N = 91, approximately equal number in each group. Open points indicate maternal standard diet, solid points indicate maternal Western diet, control mice are in grey, and high runner (HR) mice are in black. Results of statistical analyses (fixed effects) are shown on the right. All analyses included covariates of age (not shown).

Fig. 4.6. Juvenile home-cage activity (measure of spontaneous physical activity, in arbitrary units, averaged across days in the week) for focal males. Panels show 3 consecutive weeks. Bars are age-adjusted LSM + SE, N = 25 in each group. Striped bars indicate maternal standard diet, solid bars indicate maternal Western diet, grey bars are control mice, and black bars are high runner (HR) mice. Results of statistical analyses (fixed effects) are shown above each graph. All analyses included age as a covariate (*P* values not shown).

Fig. 4.7. Total voluntary wheel running revolutions, duration (number of 1-min intervals with at least one revolution), and average speed of days 5+6 of 6 days of wheel access of adult C and HR mice given maternal SD or WD, with separate analyses for females and males. Bars are age- and wheel-freeness-adjusted LSM + SE and N were approximately equal for each group. Striped bars indicate maternal standard diet, solid bars indicate maternal Western diet, grey bars are control mice, and black bars are high runner (HR) mice. Results of statistical analyses (fixed effects) are shown above each graph. All analyses included age as a covariate (*P* values not shown).

Fig. 4.8. Total wheel revolutions of adult mice given maternal standard diet (striped bars) or Western diet (solid bars), with separate analyses for sex and replicate control (in grey) and high runner (in black) lines. Bars are LSM + SE (age and wheel freeness used as covariates). Total N is shown in the upper left-hand corner, and Ns were approximately equal for each line within linetype and diet within line. Results of statistical analyses (fixed effects) are shown above each graph. Asterisks indicate differences of LSM within each line between diets ($P < 0.05$) from SAS Procedure Mixed.

Fig. 4.9. Wheel running intervals per day, line analyses. Maternal WD did not overall affect C and HR females and males, but lines were significantly different in each group ($P < 0.007$), and HR females had an interaction between diet and line, with maternal WD significantly increasing intervals in HR line 7 ($P < 0.05$). LSM + SE adjusted for age and wheel freeness. Asterisks indicate differences of LSM within each line between diets ($P < 0.05$) from SAS Procedure Mixed. All analyses included covariates of age (not shown).

Fig. 4.10. Average wheel running speed (revolutions per minute), line analyses. Average speed in C males was unaffected by diet or line but C female, HR female, and HR male lines differed significantly within each group ($P < 0.05$). HR females had a significant interaction between diet and line ($P = 0.0323$), with maternal WD significantly decreasing average speed in HR line 3 ($P < 0.05$). LSM + SE adjusted for age and wheel freeness. Asterisks indicate differences of LSM within each line between diets ($P < 0.05$) from SAS Procedure Mixed. All analyses included covariates of age (not shown).

Fig. 4.11. Maximum wheel running speed (revolutions per minute), line analyses. Maximum speed in C males was unaffected by diet or line, but C female, HR female, and

HR male lines differed significantly within each group ($P < 0.05$). LSM + SE adjusted for age and wheel freeness. All analyses included covariates of age (not shown).

Fig. 4.12. Body mass of adult mice given maternal standard diet (striped bars) or Western diet (solid bars), with separate analyses for sex and replicate control (in grey) and high runner (in black) lines. Bars are LSM + SE (age used as covariate). Total N is shown in the upper left-hand corner, and Ns were approximately equal for each group. Results of statistical analyses (fixed effects) are shown above each graph. Asterisks indicate differences of LSM within each line between diets ($P < 0.05$) from SAS Procedure Mixed. All analyses included covariates of age (not shown).

Fig. 4.13. Total wheel revolutions of grand-offspring of dams given standard diet (striped bars) or Western diet (solid bars), with separate analyses for sex and replicate control (in grey) and high runner (in black) lines. Grand-offspring were produced by mating males and females within each line whose dams had the same diet. Bars are LSM + SE (age and wheel freeness used as covariates). Total N is shown in the upper left-hand corner, and Ns were approximately equal for each subgroup. Results of statistical analyses (fixed effects) are shown above each graph. Asterisks indicate differences of LSM within each line between diets ($P < 0.05$) from SAS Procedure Mixed.

Fig. 4.14. Adult body mass of grand-offspring of dams given standard diet (striped bars) or Western diet (solid bars), with separate analyses for sex and replicate control (in grey) and high runner (in black) lines. Grand-offspring were produced by mating males and females within each line whose dams had the same diet. Bars are LSM + SE (age used as a covariate). Total N is shown in the upper left-hand corner, and Ns were approximately equal for each group. Results of statistical analyses (fixed effects) are shown above each graph. Asterisks indicate differences of LSM within each line between diets ($P < 0.05$) from SAS Procedure Mixed. All analyses included covariates of age (not shown).

Fig. 4.1. Timeline of experimental design.

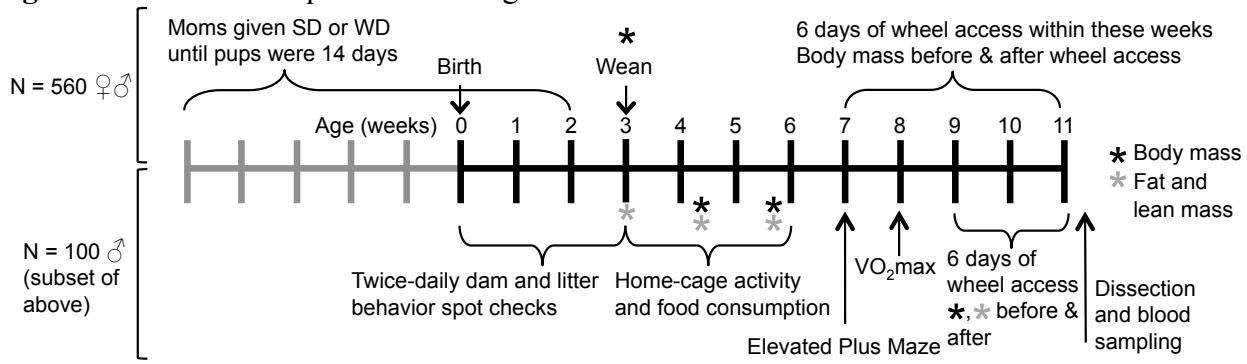


Fig. 4.2. Litter characteristics.

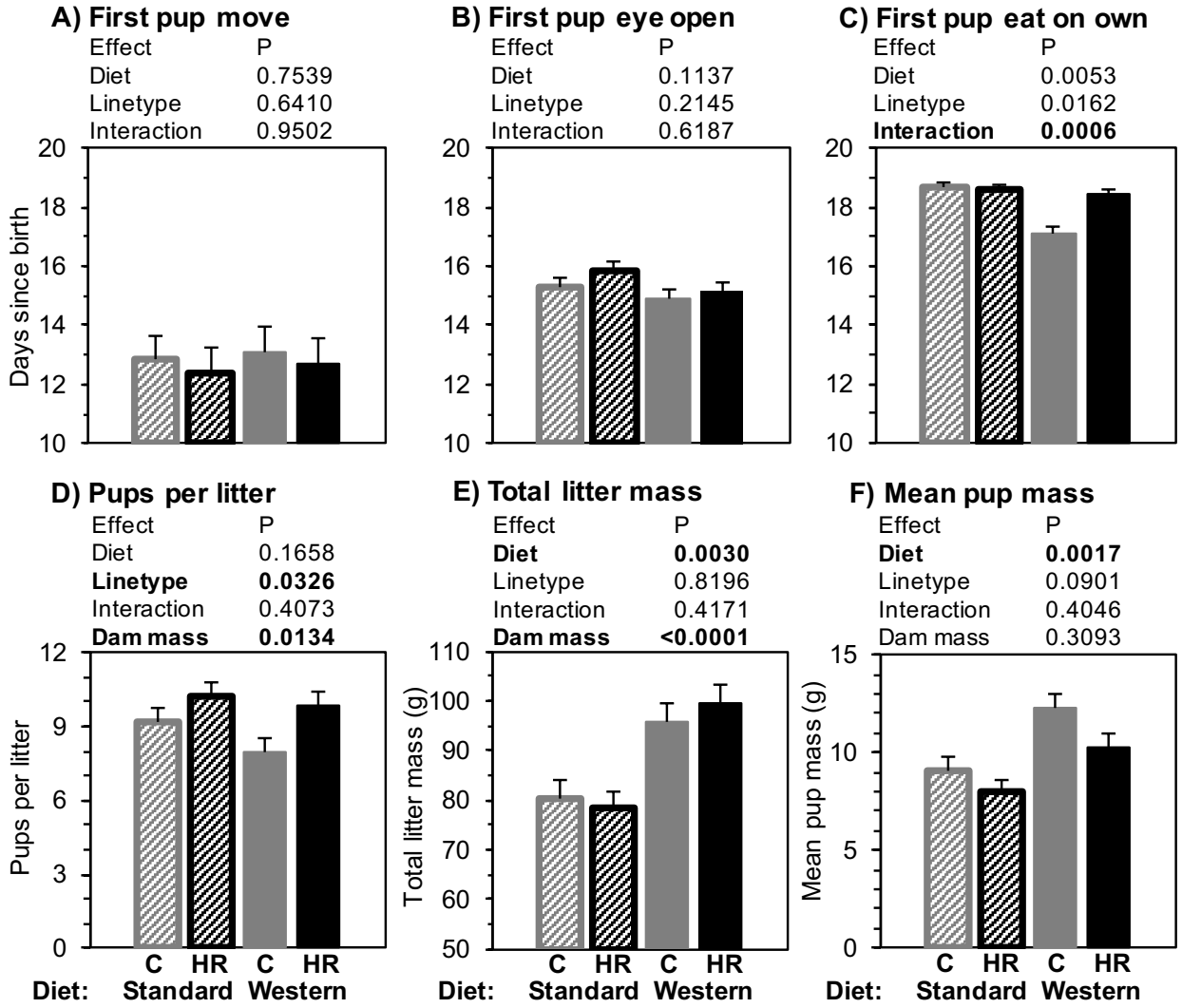


Fig. 4.3. Body mass at weaning and as adults.

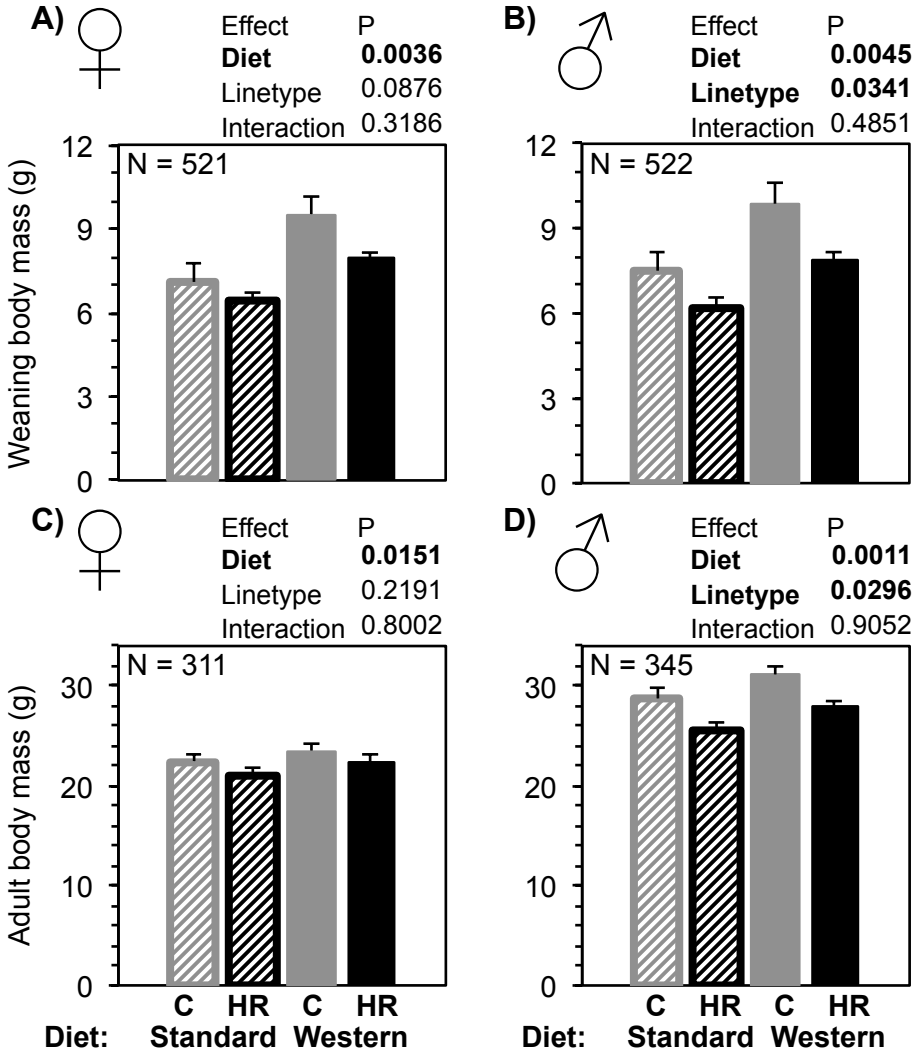


Fig. 4.4. Focal male body composition.

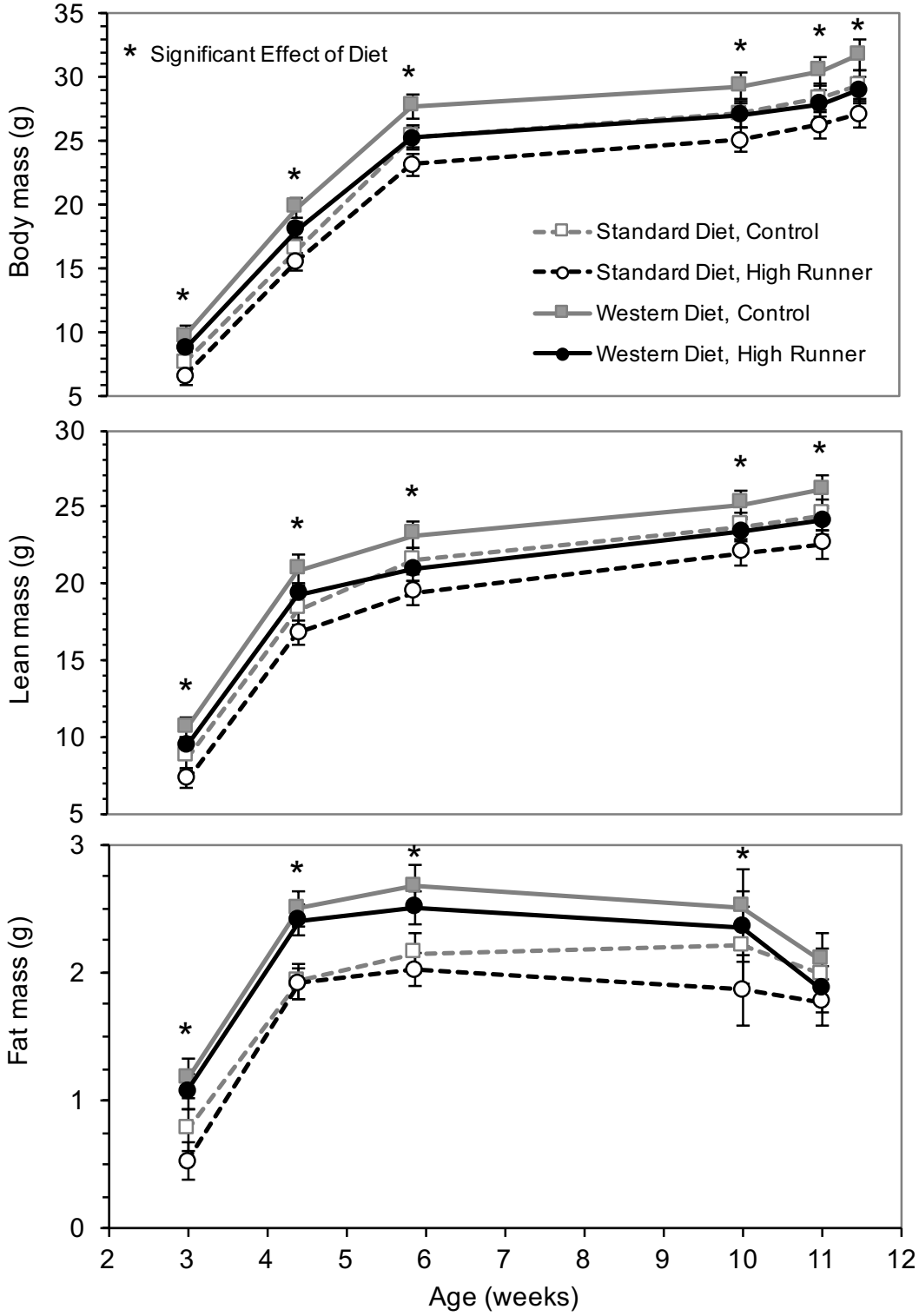


Fig. 4.5. Maximal aerobic capacity (VO_2max).

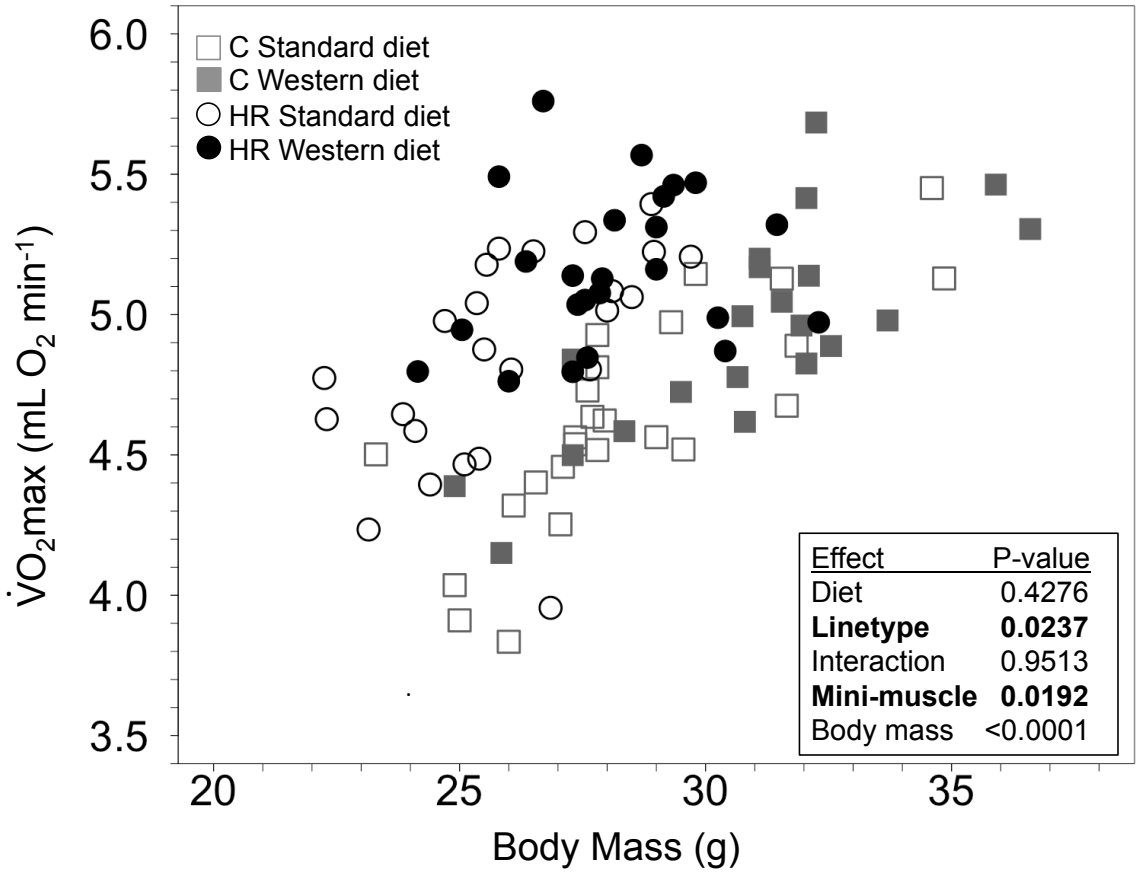


Fig. 4.6. Juvenile home-cage activity.

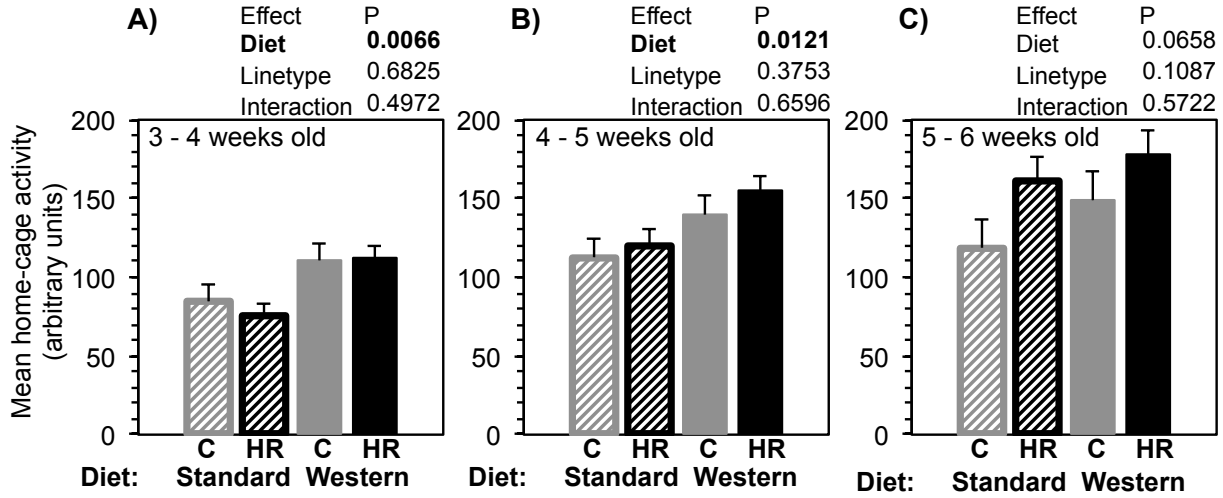


Fig. 4.7. Wheel running and component traits.

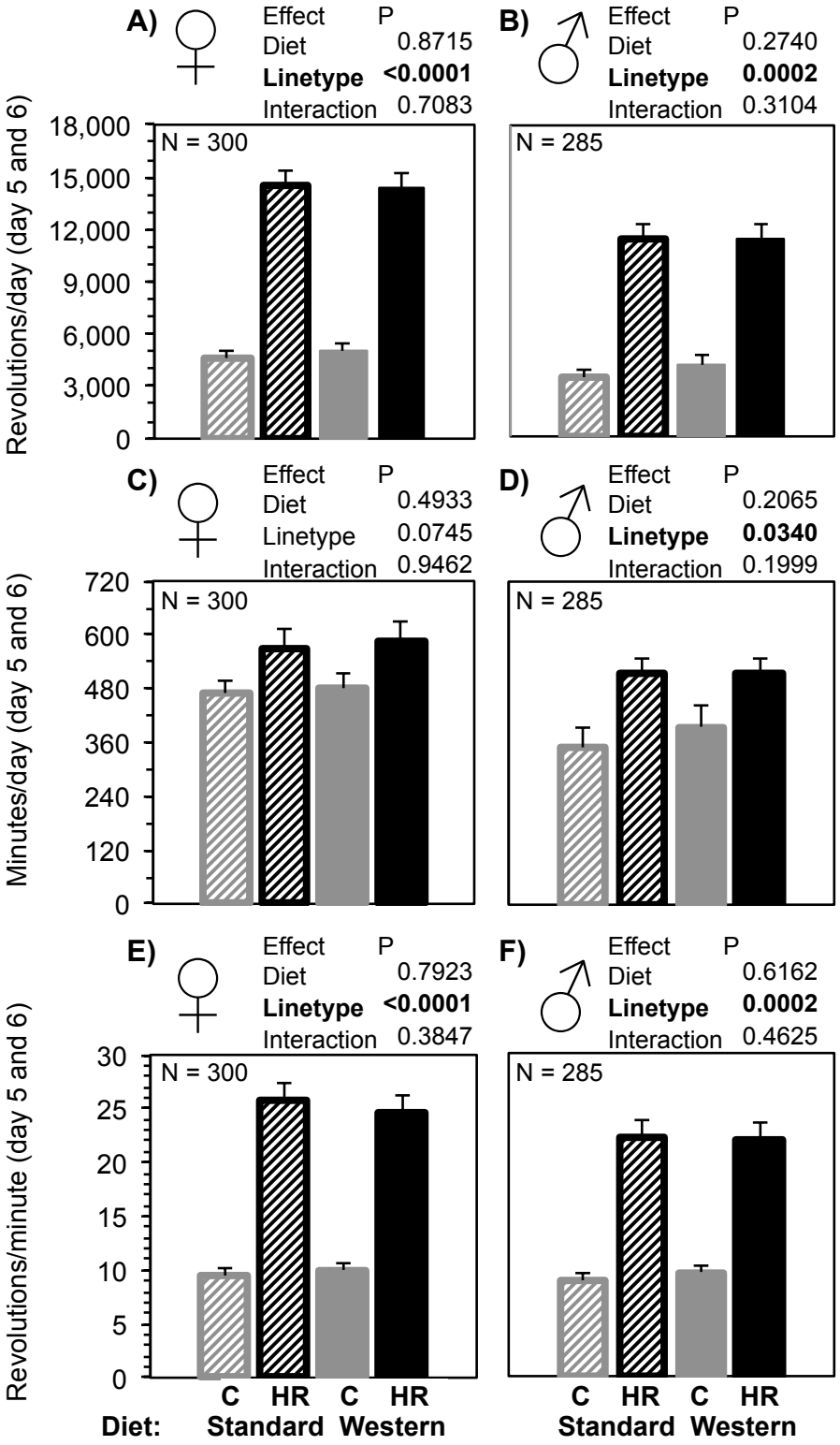


Fig. 4.8. Wheel running revolutions by replicate lines.

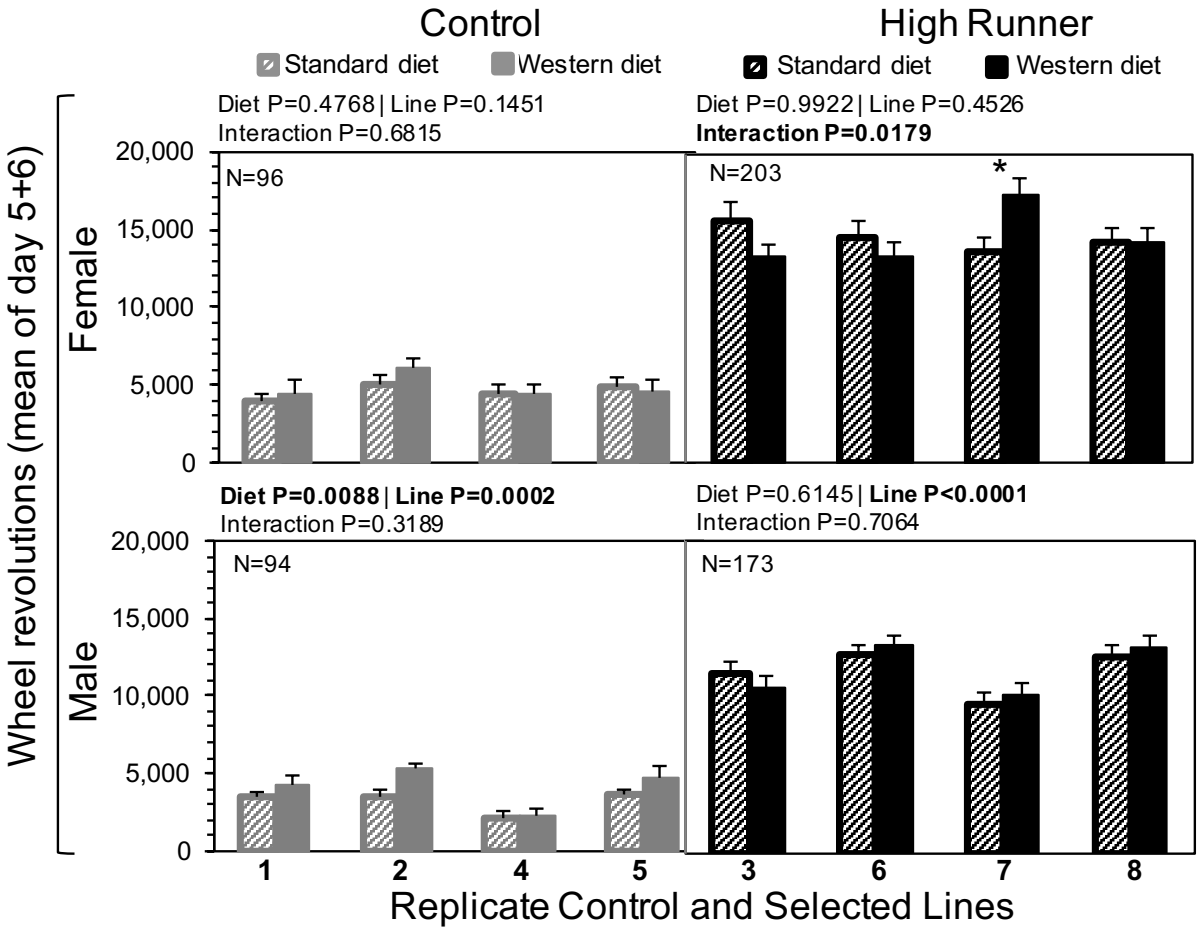


Fig. 4.9. Running duration by replicate lines.

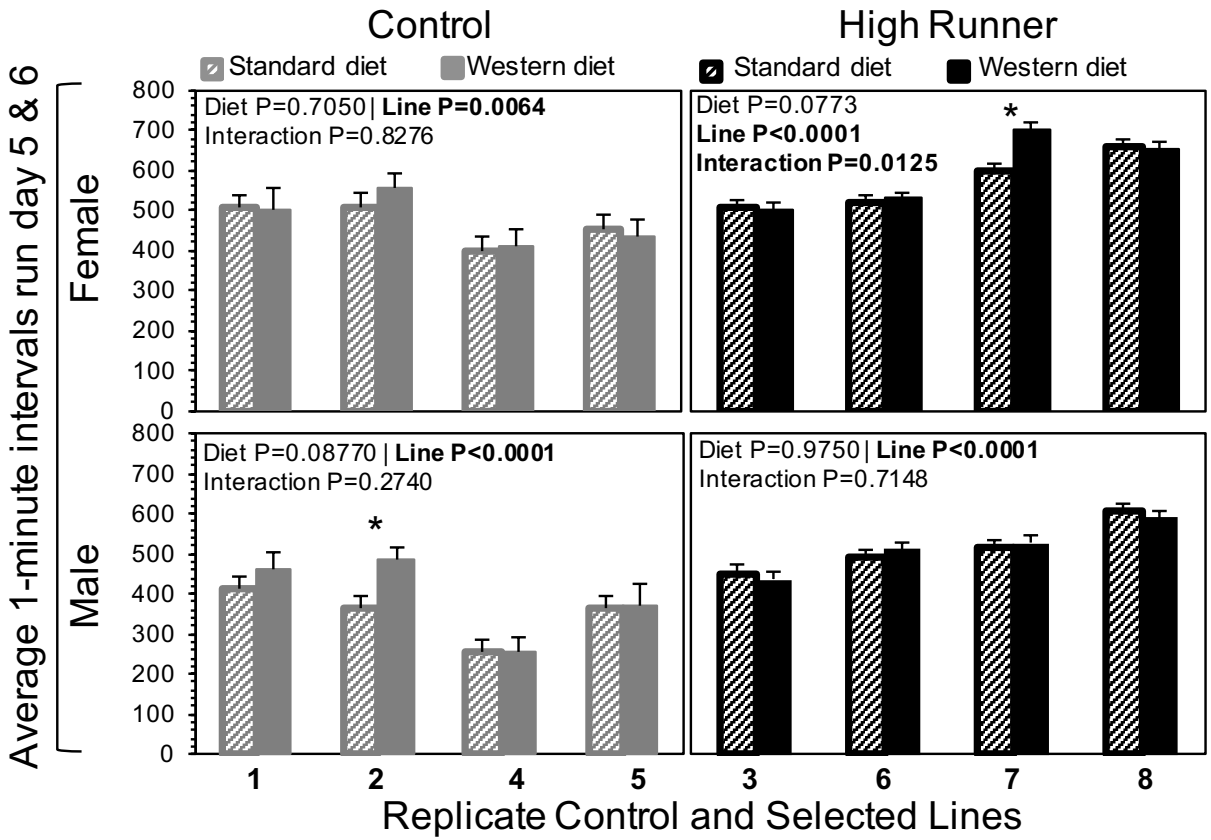


Fig. 4.10. Average running speed by replicate lines.

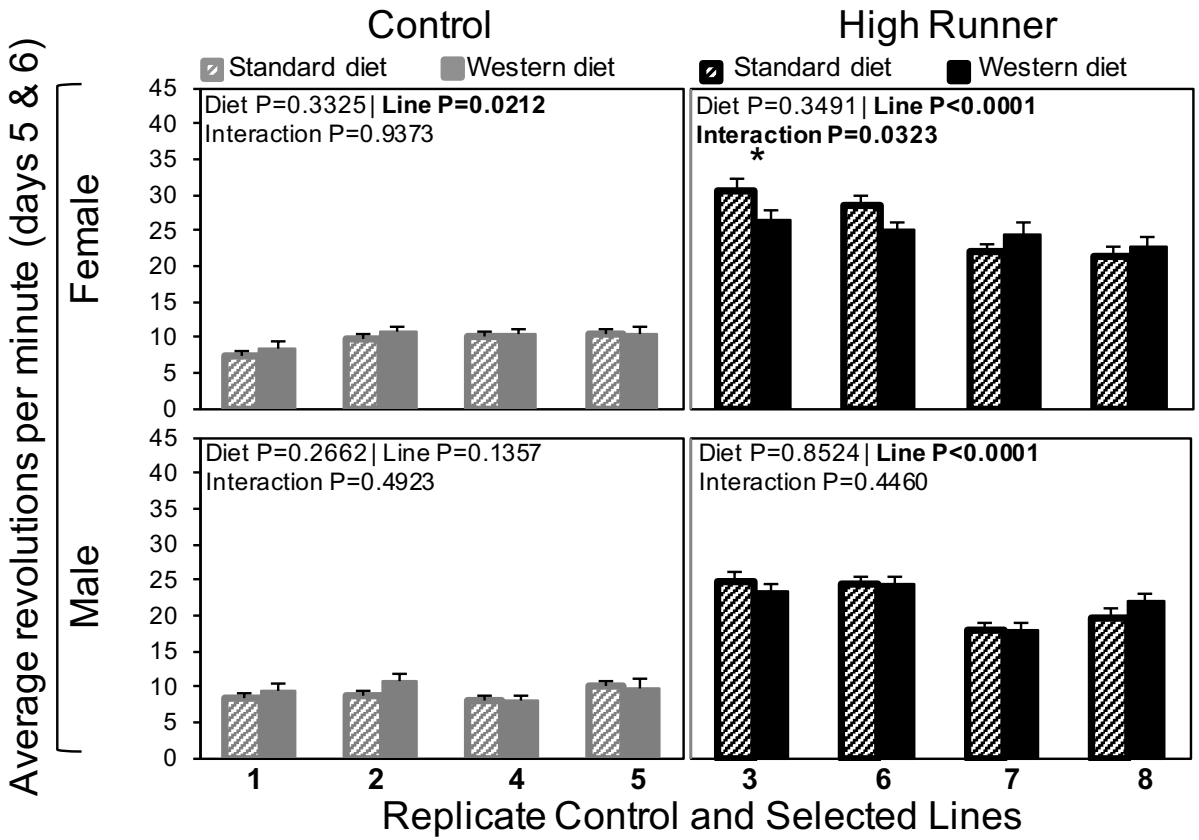


Fig. 4.11. Maximal running speed by replicate lines.

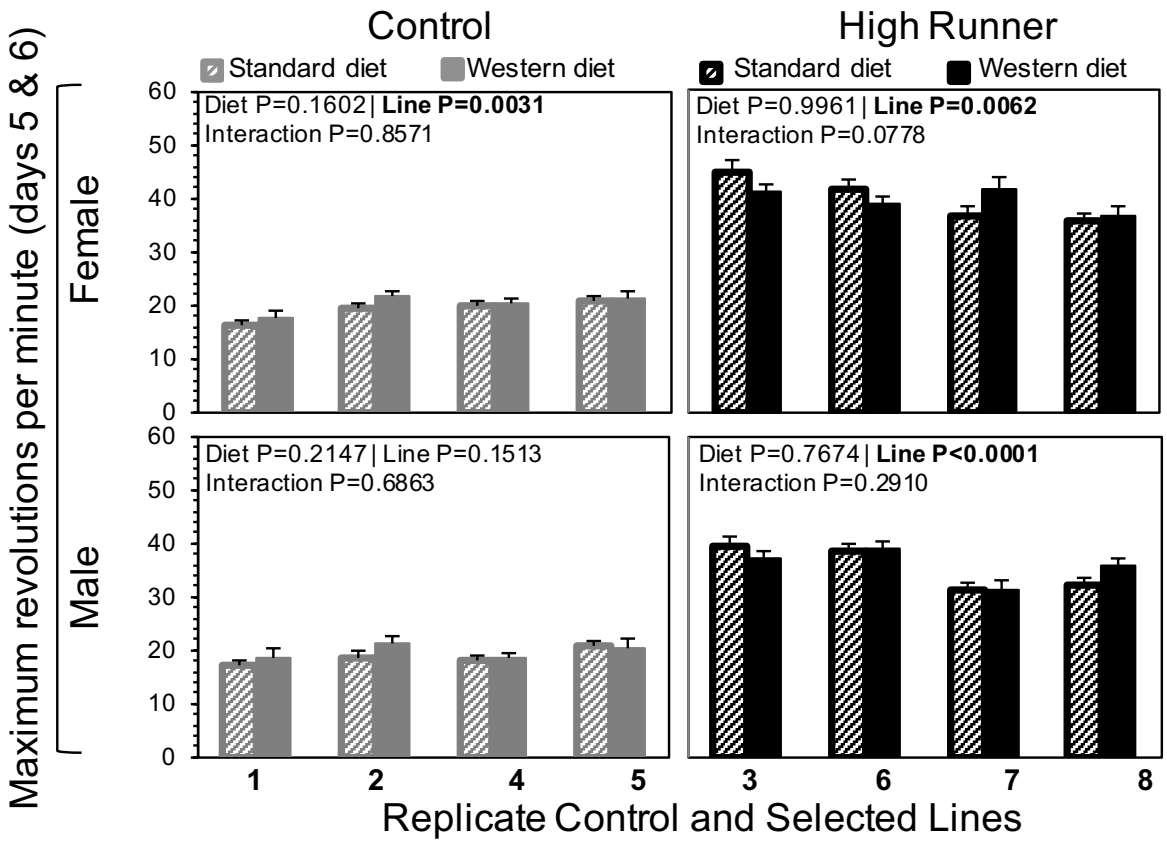


Fig. 4.12. Adult body mass by replicate lines.

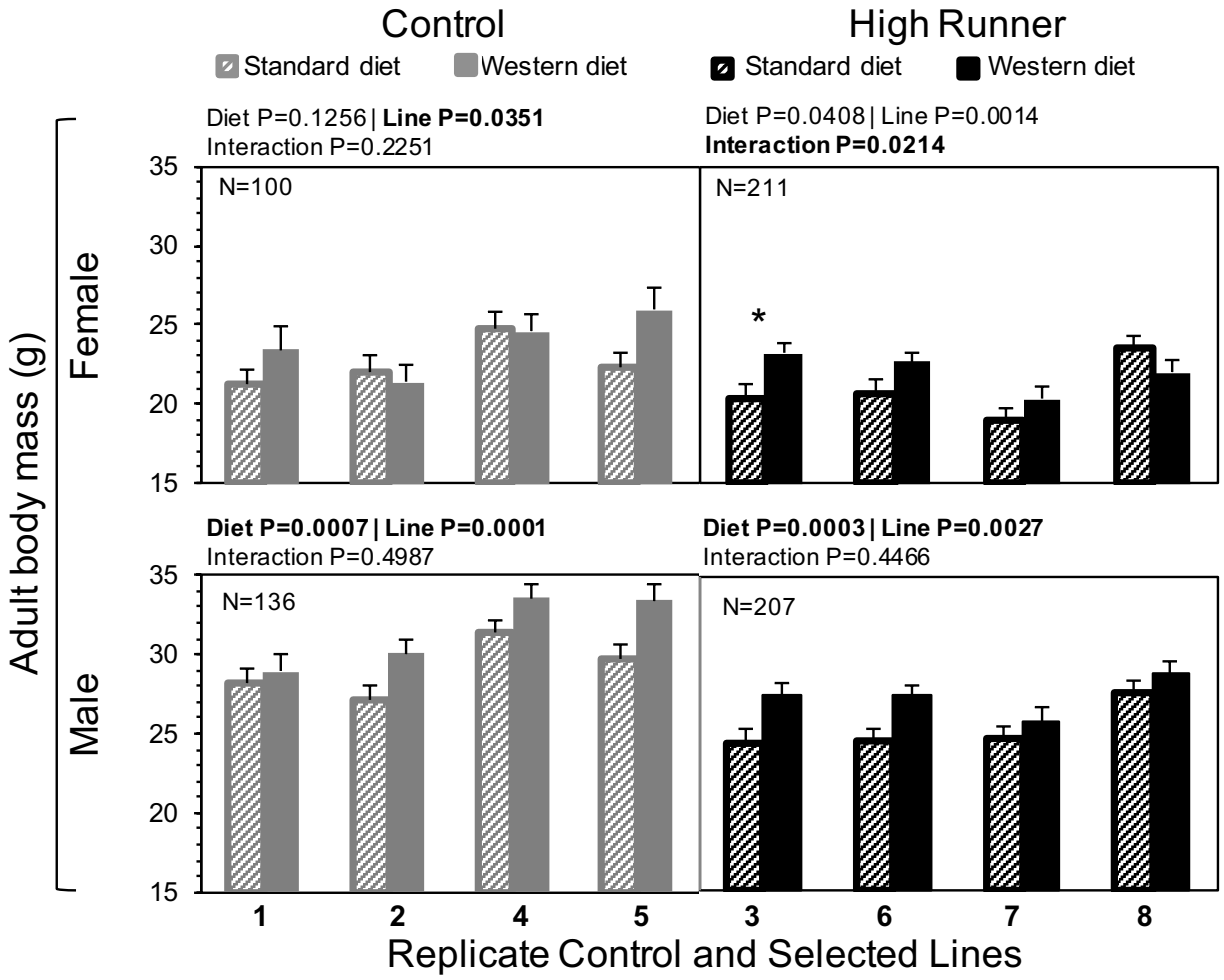


Fig. 4.13. Grand-offspring wheel revolutions by replicate lines.

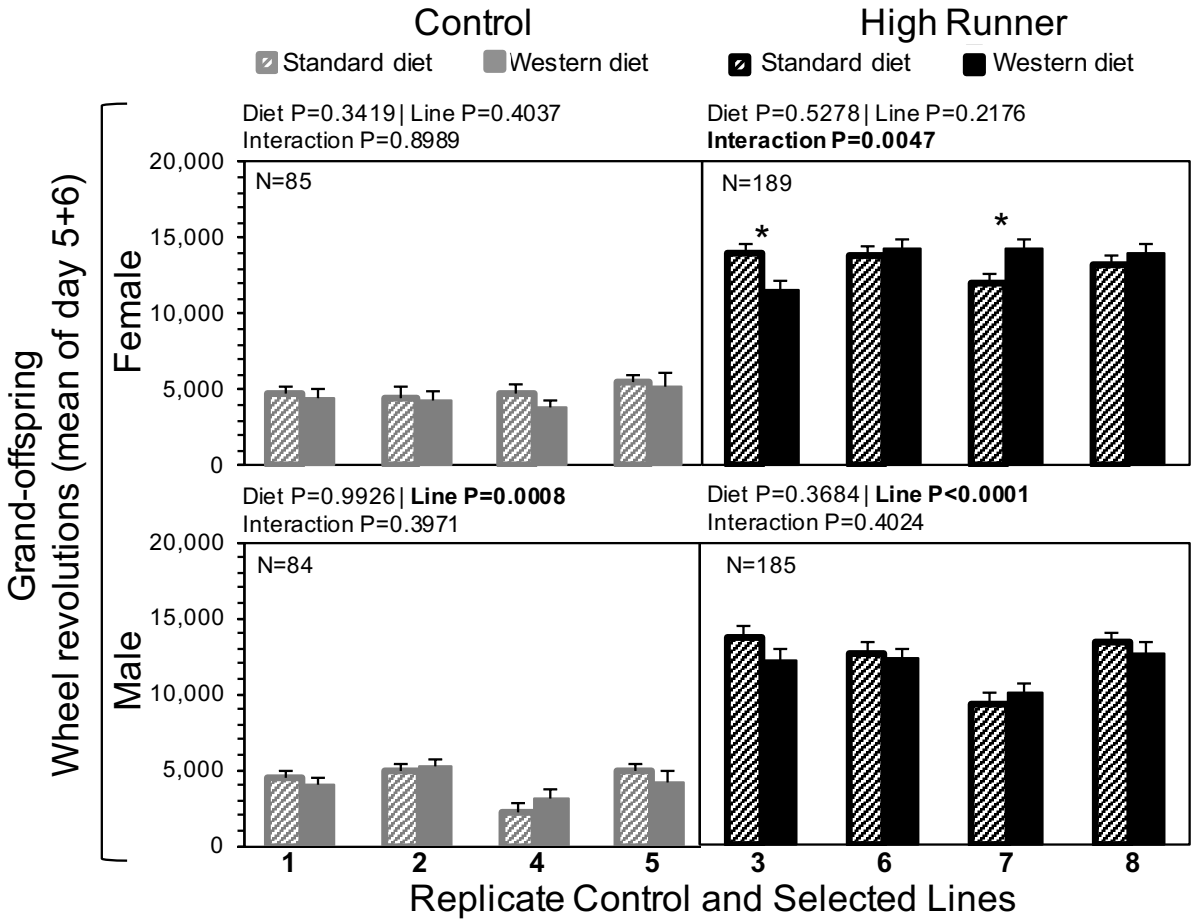
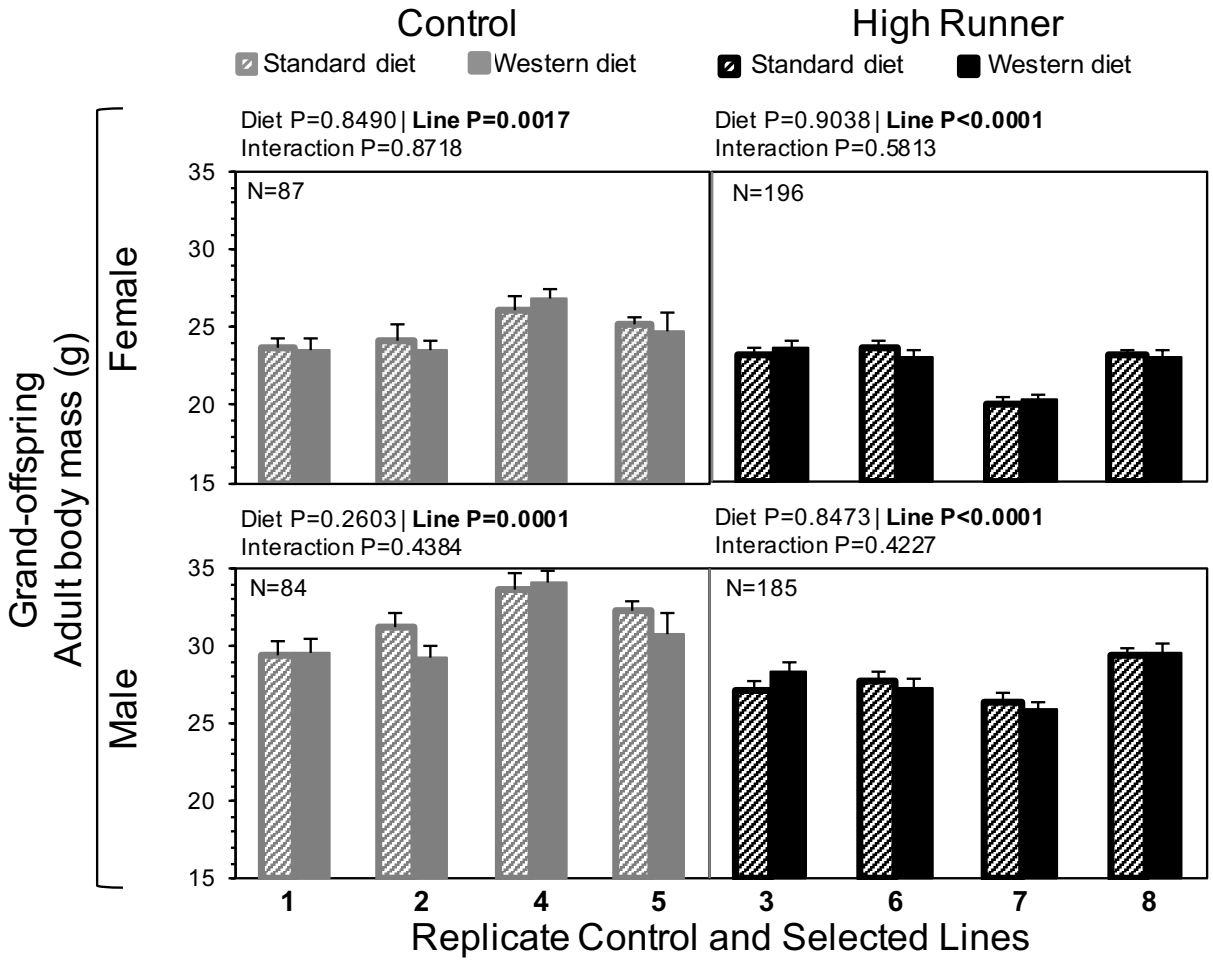


Fig. 4.14. Grand-offspring body mass by replicate lines.



Conclusions

Artificial selection for increased wheel-running behavior in mice produced 4 replicate High-Runner (HR) lines that run 2.5 to 3 times more revolutions on wheels on days 5 and 6 of a 6-day test compared with 4 replicate control (C) lines. Despite continued selection, the HR lines have been at a selection limit since generations 17-25 (differing by replicate line and sex) (Careau et al. 2013). Contrary to expectations from quantitative genetic theory, additive genetic variance (V_A) had not been depleted, selection differentials and realized selection differentials had not gone to zero, and no clear evidence of counterpoising natural selection was observed at their selection limits (Careau et al. 2013). This dissertation attempted to uncovered other potential mechanisms of the selection limits observed in the HR lines, and in so doing contributed more broadly to the understanding of complex behavioral traits and their evolution.

Chapter 1 demonstrated that “phenotypic epistasis" (non-additive interactions among the hierarchical component traits of a complex behavior) alone apparently does not allow the maintenance of additive genetic variance at a selection limit. However, antagonistic pleiotropy at the genetic level (with alleles segregating at some loci having opposite effects on motivation for speed vs. motivation for duration of running, and another set of alleles jointly affecting ability for both speed and duration), can lead to maintenance of V_A at a selection limit for running distance. Future studies should examine the evolution of the genetic correlations between and among the component traits through further simulations, attempt to identify genes that have these sorts of effects

in mice, and then determine whether their frequencies have changed in ways that could explain the selection limits in the HR lines.

Chapter 2 explored one of the possible genetic constraints on the evolution of wheel running by use of a hybrid cross with continued selection on it and the parental HR lines. Hybrid F_1 offspring showed heterosis for running distance, but this was lost in subsequent generations and the hybrid line did not break the limit. Both male and female hybrids ran faster than the parental lines for most generations, but running duration was intermediate or reduced, indicating different genetic architecture for these traits. The hybrid line had increased heritability for running speed and duration, but not for total distance, compared with the parental lines. The genetic correlation between running duration and speed evolved from positive in the starting (base) population to negative in the parental lines, and remained so in the hybrid line. This result, which represents a type of genetic constraint, could be caused by antagonistic pleiotropy, as suggested by the simulation results from Chapter 1. Together, Chapters 1 and 2 advance our understanding of the potential genetic mechanisms of selection limits.

Chapter 3 studied body composition (i.e., lean and fat mass) of mice before and after 6 days of wheel access, and its relationship to food consumption, wheel running, and home-cage activity. Before wheel testing, HR mice weighed less than C mice, primarily due to reduced lean mass, and females were lighter than males, entirely due to lower lean mass. Over 6 days of wheel access, all groups tended to gain small amounts of lean mass, but lose fat mass, resulting in overall loss of total body mass and altered body composition. HR mice lost less fat compared with C mice, apparently caused by

higher food consumption, in spite of the fact that they were much more active on wheels (and slightly more active in home-cages). All 4 groups by sex and linetype converged to a fat mass of ~1.7 g, suggesting an optimal body fat for these mice. If this optimal amount of body fat is required to sustain high levels of physical activity near the end of the 6 days of wheel access, then HR mice may be at a limit for wheel running related to their initial low body fat. That is, despite some amount of compensatory eating, HR mice still lose fat, so they may be unable to increase their activity beyond current levels, despite continued directional selection. This limit related to energy balance could be a general explanation for the selection limits experienced in HR lines. Analyses of individual variation within groups suggested that the complex relationships between body mass, activity levels, food consumption, and body composition differ between the sexes and between HR and C lines.

Chapter 4 examined energetic perturbations imposed in early-life. Dams were given high-fat, high-sugar "Western" diet (WD) or standard chow from 2 weeks prior to pairing until pups were 14 days of age, when all mice were switched to standard chow. From weaning to adulthood, offspring received physiological and behavioral tests. Maternal WD increased juvenile home-cage activity for both HR and C mice (only males tested). Maternal WD also increased fat and lean masses of offspring from weaning to adulthood. Maternal WD did not increase wheel running of adult offspring, indicating that fat availability itself does not increase wheel running. Thus, the previously observed increase in wheel running for WD-fed mice (from weaning to adulthood; Meek et al. 2010) may be due to effects of WD other than increased fat storage, such as changes in

the reward system (Acosta et al. 2017). The selection limit for wheel running may be more closely associated with limits to motivation for running compared with limits to physical ability. On average, all groups lost fat mass after 6 days of voluntary wheel running, but the absolute amount lost was greater for mice with maternal WD, resulting in a convergence of all mice to ~2 g of body fat, similar to the result in Chapter 3. Together, Chapters 3 and 4 suggest that the selection limits observed in the HR lines could be related to physiological constraints, specifically involving body fat.

Overall, these studies elucidate mechanisms underlying the evolution of complex behavioral traits.

References

Acosta, W., T. H. Meek, H. Schutz, E. M. Dlugosz, and T. Garland Jr. 2017. Preference for Western diet coadapts in High Runner mice and affects voluntary exercise and spontaneous physical activity in a genotype-dependent manner. Behav. Processes 135:56–65.

Careau, V., M. E. Wolak, P. A. Carter, and T. Garland, Jr. 2013. Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection. Evolution 67:3102–3119.

Falconer, D. S., and T. F. C. MacKay. 1996. Introduction to Quantitative Genetics. 4th ed. Benjamin Cummings.

Garland, Jr., T., and M. R. Rose (eds). 2009. Experimental evolution: concepts, methods, and applications of selection experiments. University of California Press, Berkeley.

Meek, T. H., J. C. Eisenmann, and T. Garland, Jr. 2010. Western diet increases wheel running in mice selectively bred for high voluntary wheel running. Int. J. Obes. 34:960–969.

Appendix

Genetic Model Code

Instructions for using the genetic model simulation code in R.

1. Open R (I used version 3.4.1)
2. File > New Document
3. Copy + Paste the entire code below
4. Edit > Execute to run the entire code
    a. Or, right click "Run line or selection" [Windows]
    b. Or, press Command + return [Mac]
    c. Alternatively, copy + paste into the R Console
5. Answer questions/prompts to set the working directory and parameters of the model
6. When finished, several output CSV files will be in your working directory

```
##################### CODE BEGINS HERE ####################
# file: "Dropbox/1 genetic model/model_5.R

# If want to re-create a run, uncomment the next line
# and select the random seed CSV file
# .Random.seed <-read.csv(file.choose())

# Remove current memory in R
rm(list=ls())
# To install packages, uncomment the next line
# install.packages("MASS");install.packages("moments")
# Call packages to generate random numbers
library(MASS);library(moments)

# This opening bracket ensures that the prompts work.
{
##################### PROMPTS ####################
  readline("Press enter to set the working directory. Select any
file within the working folder.")
  setwd(dirname(file.choose()))
  cat(paste("Files will be saved to ",getwd()))

  # Ask for replication number, default to 1
  RUN <- as.integer(readline("What number replication are you
running? (default:1)"))
  if (is.na(RUN)) {RUN <- 1}
  maxrun <- as.integer(readline("How many replicates do you want
to run? (default:1) "))
  if(is.na(maxrun)) {maxrun <- 1}
```

```
  # Ask for number of generations, default to 100
  Maxgen <- as.integer(readline("Enter the number of generations
(default: 100):"))
  if (is.na(Maxgen)) {Maxgen <- 100}

  # Ask for selection, default to yes
  sel <- readline("Do you want selection? y/n (default: y)")
  if(sel == "n"){sel <- "cont"} else {sel <- "sel"}

  # Ask for number of breeding pairs, default to 20
  pairs <- as.integer(readline("Enter the number of breeder pairs
(default: 20):"))
  if(is.na(pairs)){pairs <- 20}

  # Ask for litter size, defalut to 5
  litter <- as.integer(readline("Enter the litter size (per
family) (default: 5):"))
  if(is.na(litter)){litter <- 5}

  # Ask for allelic effects, default to leptokurtic
  biallele <- readline("Do you want a biallelic, leptokurtic, or
gaussian design? b/l/g (default: l)")
  if(biallele == "b"){biallele <- "y"} else if(biallele ==
"g"){biallele <- "g"} else {biallele <- "l"}

  # Ask for number of loci for each trait, default to 10
  total.loci <- as.integer(readline("How many loci are there for
each of the the lowest traits? (default: 10)"))
  if(is.na(total.loci)){total.loci <- 10}

  # Ask for overlap between loci, default to no
  # Note, if there are loci in common, they are: common for
speed, common for duration, common for ability, common for
motivation
  common.loci <- readline("Is there overlap between loci
(pleitropy)? y/n (default: n)")
    # If loci do overlap, ask for number of overlapping loci
    # If this option is left blank, default to zero
    if (common.loci == "y"){
    common.loci <- as.integer(readline("How many loci are there
in overlap (will be subtracted from total)?"))
    if (is.na(common.loci)){common.loci<-0}
    # If loci do overlap, ask for antagonistic pleiotropy
    # Note: antagonistic pleiotropy will result in
    # ability loci are + in speed and - in duration,
    # motivation loci are + in duration and - in speed.
    common.loci.neg <- readline("Are the effects of pleiotropic
loci opposite in speed vs duration? y/n (default:n)")
      } else {common.loci <- 0}
```

```
  # Ask for varying allele frequencies, default no
  # Note, currently they can only vary for biallelic models
  # with 10 total and 3 common loci models.
  if (biallele=="y" & total.loci==10 & common.loci==3){
    allele.freq <- readline("Do the allele frequencies vary? y/n
(default:n)")
    if(allele.freq=="y"){allele.freq <- "y"
    }else{allele.freq <- "n"}
  } else {allele.freq <- "n"}

  # Ask for within-family selection, dafult yes
  # Note, within-family selection changes number of pairs
  within.fam <- readline("Is there within-family selection? y/n
(default: y)\nNOTE: if y, pairs and litter = 10")
  if(within.fam == ""){within.fam <- "y"}
  if(within.fam == "y"){pairs <- 10; litter <- 10}

  # Ask for genetic dominance, default to no
  dom <- readline("Are there dominance effects at the lowest
level loci? y/n (default: n)")
    # If dominanc, ask for on which loci
    if (dom == "y") {dom <- 1
    } else if (dom == "y" &common.loci==3 & total.loci==10){
    domy <- readline("On all loci? y/n (default: y)")
    if(domy=="y") {dom <- 1} else {dom <- 2}
  } else {dom <- 0}

  # Ask to measure breeding values, dafult no
  bv <- readline("Do you want to measure breeding values? y/n
(default: n)")
  if (is.na(bv)) {bv <- "n"} else if (bv == "y") {bv <- "y"} else
{bv <- "n"}

  # Population size = number of pairs x litter size
  N.Pop <- pairs*litter
  cat(c("Your population size is",N.Pop,"."))
  # Number of pairs = number of parents x 2
  np <- pairs*2
  # Number of unique loci = total minus 2xcommon loci
  unique.loci <- total.loci - 2*common.loci

##################### FUNCTIONS ####################
# Function to assign loci based on allelic effects
# P = vector of initial allele frequencies of +'s
ASSIGN <- function(G.loci, N.Pop,P,biallele)
  # For allelic effects of +1 and -1
    {if(biallele == "y"){
    Total.loci <- N.Pop*2*G.loci # total number of alleles
```

```
    Alleles <- runif(Total.loci) # generate random numbers
    # Create + or - alleles based on P vector
    temp <- matrix(0,2*N.Pop,length(P))
    for(p in 1:length(P))
      temp[,p] <- sapply(((p-1)*2*N.Pop+1):((p-1)*2*N.Pop +
2*N.Pop), FUN=function(x) {if (Alleles[x]<P[p]){1} else {-1}})
    Alleles <- as.vector(temp)
    return(matrix(Alleles, N.Pop, 2*G.loci))
  # For allelic effects from a normal distribution
    } else if(biallele == "g") {
   return(matrix(rnorm(N.Pop*2*G.loci,0,1),N.Pop,2*G.loci))
  # For allelic effects from a leptokurtic distribution
    } else if(biallele == "l") {
    return(matrix(sample(c(rnorm(1320,0,0.26),
      rnorm(500,0,1.39),rnorm(150,0,2),rnorm(30,0,3)),
      replace=T,size=N.Pop*2*G.loci),N.Pop,2*G.loci))
  }}

# Function to allow mutations (change sign of effect)
# based on poisson distribution, with lambda = Pmut
MUTATION <- function(MS, Pmut, loci, N)
  {T.genes <- N*2*loci
  lambda <- Pmut*T.genes
  N.mutations <- rpois(1,lambda)
  Row  <- ceiling(runif(N.mutations, min=0, max=T.genes))
  Temp  <- matrix(MS)
  Temp[Row] <- ((-1)*Temp[Row])
  Temp <- c(N.mutations,Temp)
  return(Temp)}

# Function for dominance
# Affects the first half of the number of loci (rounded up)
dom.plus <-function(MS.loci,MSmatrix)
  {sapply(1:length(MSmatrix[,1]),function(y)
  {gms<-sum(sapply(seq(1,MS.loci*2,2),FUN=function(x)
  {if(x<MS.loci) {2*max(MSmatrix[y,x],MSmatrix[y,(x+1)])
    } else {sum(MSmatrix[y,x],MSmatrix[y,(x+1)])}}))})}
dom.minus <-function(MS.loci,MSmatrix)
  {sapply(1:length(MSmatrix[,1]),function(y)
  {gms<-sum(sapply(seq(1,MS.loci*2,2),FUN=function(x)
  {if(x<MS.loci) {2*min(MSmatrix[y,x],MSmatrix[y,(x+1)])
    } else {sum(MSmatrix[y,x],MSmatrix[y,(x+1)])}}))})}

# Function to create offspring's genetic matrices
offspring <- function(G.matrix,loci,i,parent,P1,P2)
  { for(j in 1:litter){
    G.matrix[((i-1)*litter+j),
      seq(1,loci*2,2)] <-sapply(seq(1,loci*2,2),
      FUN=function(x){sample(parent[P1,x:(x+1)],1)})
```

```
    G.matrix[((i-1)*litter+j),
       seq(2,loci*2,2)] <- sapply(seq(1,loci*2,2),
       FUN=function(x){sample(parent[P2,x:(x+1)],1)})}
  return(G.matrix)}

# Function to create all possible matings
# used for calculating breeding values
MATE <- function(offspring,N,loci,matrix,p1,p2)
  {offspring <- matrix(0,N,loci*2)
   for(i in 1:N){
     offspring[i,seq(1,loci*2,2)]<- sapply(seq(1,loci*2,2),
     FUN=function(x){sample(matrix[p1,x:(x+1)],1)})
     offspring[i,seq(2,loci*2,2)]<- sapply(seq(1,loci*2,2),
     FUN=function(x){sample(matrix[p2,x:(x+1)],1)})}
   return(offspring)}

# Function to calculate phenotypes
RUNNING <-function(MSmatrix,ASmatrix,MDmatrix,ADmatrix,
  CSmatrix,CDmatrix,CAmatrix,CMmatrix)
{
### Genotype to phenotype - Motivation for Speed ###
# Add up alleles to get a genotypic score,
# depending on pleiotropy and genetic dominance
if(common.loci > 0){
 if(common.loci.neg == "y"){
   if(dom == 0){
     G.MS <- 16+rowSums(MSmatrix)+
      rowSums(CSmatrix)-rowSums(CMmatrix)
   }else if(dom == 1){
     G.MS <- 16+dom.plus(MS.loci,MSmatrix)+
      dom.plus(CS.loci,CSmatrix)-dom.plus(CM.loci,CMmatrix)
   }else if(dom == 2) {
     G.MS <-16+rowSums(MSmatrix[,c(1:2,7:8)])+
      rowSums(CSmatrix[,5:6])-rowSums(CMmatrix[,1:2])+
      dom.plus(1,MSmatrix[,5:6])+
      dom.plus(1,CSmatrix[,1:2])-
      dom.plus(1,CMmatrix[,5:6])+
       dom.minus(1,MSmatrix[,3:4])+
       dom.minus(1,CSmatrix[,3:4])-
      dom.minus(1,CMmatrix[,3:4])}
  }else{
   if(dom == 0){
    G.MS <- 16+rowSums(MSmatrix)+
     rowSums(CSmatrix)+ rowSums(CMmatrix)
   }else if(dom == 1){
    G.MS <- 16+dom.plus(MS.loci,MSmatrix)+
      dom.plus(CS.loci,CSmatrix)+dom.plus(CM.loci,CMmatrix)
   }else if(dom == 2){
    G.MS <- 16 + rowSums(MSmatrix[,c(1:2,7:8)]) +
```

```
       rowSums(CSmatrix[,5:6])+rowSums(CMmatrix[,1:2])+
       dom.plus(1,MSmatrix[,5:6]) +
       dom.plus(1,CSmatrix[,1:2]) +
       dom.plus(1,CMmatrix[,5:6]) +
       dom.minus(1,MSmatrix[,3:4]) +
       dom.minus(1,CSmatrix[,3:4]) +
       dom.minus(1,CMmatrix[,3:4])}
 }} else {
   if(dom == 0){
    G.MS <- 16+ rowSums(MSmatrix)
   }else if (dom == 1){
    G.MS <- 16 +dom.plus(MS.loci,MSmatrix)}
}
# Truncate motivation speed genotype, min 1 and max 39
G.MS  <- sapply(seq(1,length(G.MS)), FUN = function(x)
  {if(G.MS[x]<1){1} else if(G.MS[x]>39){39
  }else{G.MS[x]}})
# Environmental effects with a mean of zero
Env <- mvrnorm(n=length(G.MS), mu=c(0,0), Sigma=diag(2))
# Add genotype + enviroment, with arbitrary scaling
P.MS  <- 0.7*G.MS + 3*Env[,1]
# Truncate motivation speed phenotype, min 1 max 39
P.MS  <- sapply(seq(1,length(P.MS)), FUN = function(x)
  {if(P.MS[x]<1){1} else if(P.MS[x]>39){39
  }else{P.MS[x]}})

### Genotype to phenotype - Ability for Speed ###
# Add up alleles to get a genotypic score,
# depending on pleiotropy and genetic dominance
if(common.loci>0){
 if(common.loci.neg == "y"){
  if(dom == 0){
    G.AS <- 16 + rowSums(ASmatrix) +
     rowSums(CSmatrix) + rowSums(CAmatrix)
  }else if(dom == 1){
    G.AS <- 16 + dom.plus(AS.loci,ASmatrix) +
     dom.plus(CS.loci,CSmatrix) + dom.plus(CA.loci,CAmatrix)
  }else if(dom == 2){
    G.AS <- 16+ rowSums(ASmatrix[,c(1:2,3:4)]) +
     rowSums(CSmatrix[,5:6]) + rowSums(CAmatrix[,1:2]) +
     dom.plus(1,ASmatrix[,7:8])+
     dom.plus(1,CSmatrix[,1:2])+
     dom.plus(1,CAmatrix[,3:4])+
     dom.minus(1,ASmatrix[,5:6])+
     dom.minus(1,CSmatrix[,3:4]) +
     dom.minus(1,CAmatrix[,5:6])}
  }else{
   if(dom == 0){
    G.AS <- 16 + rowSums(ASmatrix)+
```

```
      rowSums(CSmatrix) + rowSums(CAmatrix)
    }else if(dom == 1){
     G.AS <- 16 + dom.plus(AS.loci,ASmatrix) +
      dom.plus(CS.loci,CSmatrix)+dom.plus(CA.loci,CAmatrix)
    }else if(dom == 2){
     G.AS <- 16+ rowSums(ASmatrix[,c(1:2,3:4)]) +
      rowSums(CSmatrix[,5:6]) + rowSums(CAmatrix[,1:2]) +
      dom.plus(1,ASmatrix[,7:8]) +
      dom.plus(1,CSmatrix[,1:2]) +
      dom.plus(1,CAmatrix[,3:4]) +
      dom.minus(1,ASmatrix[,5:6]) +
      dom.minus(1,CSmatrix[,3:4]) +
      dom.minus(1,CAmatrix[,5:6])}
 }} else {
    if(dom == 0){
     G.AS <- 16+ rowSums(ASmatrix)
    }else if(dom == 1){
     G.AS <- 16 + dom.plus(AS.loci,ASmatrix)}
}
# Truncate ability speed genotype, min 1 and max 39
G.AS  <- sapply(seq(1,length(G.AS)), FUN = function(x)
  {if(G.AS[x]<1){1} else if(G.AS[x]>39){39
  }else{G.AS[x]}})
# Add genotype + enviroment, with arbitrary scaling
P.AS  <- 0.7*G.AS + 3*Env[,2]
# Truncate ability speed phenotype, min 1 and max 39
P.AS  <- sapply(seq(1,length(P.AS)), FUN = function(x)
  {if(P.AS[x]<1){1} else if(P.AS[x]>39){39
  }else{P.AS[x]}})

# Save variances for motivation and ability for speed
VeMS <- var(Env[,1]) # Environmental variance of motivation
VgMS <- var(G.MS)    # Genotypic variance of motivation
VeAS <- var(Env[,2]) # Environmental variance of ability
VgAS <- var(G.AS)    # Genotypic variance of ability

# Speed phenotype = lower of motivation or ability
P.S <- sapply(seq(1, length(P.MS)), FUN = function(x)
  ifelse(P.MS[x] <= P.AS[x], P.MS[x], P.AS[x]))


### Genotype to phenotype - Motivation for Duration ####
if(common.loci>0){
 if(common.loci.neg == "y"){
   if(dom == 0){
    G.MD <- 500 + 20*(rowSums(MDmatrix) +
     rowSums(CDmatrix) + rowSums(CMmatrix))
   }else if(dom == 1){
    G.MD <- 500 + 20*(dom.plus(MD.loci,MDmatrix) +
```

```
      dom.plus(CD.loci,CDmatrix)+dom.plus(CM.loci,CMmatrix))
    }else if(dom == 2){
     G.MD <- 500+ 20*(rowSums(MDmatrix[,c(1:2,7:8)]) +
      rowSums(CDmatrix[,5:6]) + rowSums(CMmatrix[,1:2]) +
     dom.plus(1,MDmatrix[,5:6])+
     dom.plus(1,CDmatrix[,1:2]) +
     dom.plus(1,CMmatrix[,5:6]) +
     dom.minus(1,MDmatrix[,3:4]) +
     dom.minus(1,CDmatrix[,3:4]) +
     dom.minus(1,CMmatrix[,3:4]))}
 }else{
    if(dom == 0){
     G.MD <- 500 + 20*(rowSums(MDmatrix) +
      rowSums(CDmatrix) + rowSums(CMmatrix))
    }else if(dom == 1){
     G.MD <- 500 + 20*(dom.plus(MD.loci,MDmatrix) +
      dom.plus(CD.loci,CDmatrix)+dom.plus(CM.loci,CMmatrix))
    }else if(dom == 2){
     G.MD <- 500+ 20*(rowSums(MDmatrix[,c(1:2,7:8)]) +
      rowSums(CDmatrix[,5:6]) + rowSums(CMmatrix[,1:2]) +
      dom.plus(1,MDmatrix[,5:6]) +
      dom.plus(1,CDmatrix[,1:2]) +
      dom.plus(1,CMmatrix[,5:6]) +
      dom.minus(1,MDmatrix[,3:4]) +
      dom.minus(1,CDmatrix[,3:4]) +
      dom.minus(1,CMmatrix[,3:4]))}
 }} else {
    if(dom == 0){
     G.MD <- 500 + 20*rowSums(MDmatrix)
    }else if(dom == 1){
     G.MD <- 500 + 20*dom.plus(MD.loci,MDmatrix)}
}
# Truncate motivation duration genotype, min 10 and max 960
G.MD  <- sapply(seq(1,length(G.MD)), FUN = function(x)
  {if(G.MD[x]<10){10} else if(G.MD[x]>960){960
  }else {G.MD[x]}})
# Environmental effects with a mean of zero
EnvD <- mvrnorm(n=length(G.MS), mu=c(0,0), Sigma=diag(2))
# Add genotype + enviroment, with arbitrary scaling
P.MD  <- 0.7*G.MD + 25*EnvD[,1]
# Truncate motivation duration phenotype, min 10 max 960
P.MD  <- sapply(seq(1,length(P.MD)), FUN = function(x)
  {if(P.MD[x]<10){10} else if(P.MD[x]>960){960
  }else{P.MD[x]}})

### Genotype to phenotype - Ability for Duration ####
if(common.loci>0){
 if(common.loci.neg == "y"){
    if(dom == 0){
```

```
     G.AD <- 500 + 20*(rowSums(ADmatrix) +
      rowSums(CDmatrix) - rowSums(CAmatrix))
    }else if(dom == 1){
     G.AD <- 500 + 20*(dom.plus(AD.loci,ADmatrix) +
      dom.plus(CD.loci,CDmatrix)-dom.plus(CA.loci,CAmatrix))
    }else if(dom == 2){
     G.AD <- 500+ 20*(rowSums(ADmatrix[,c(1:2,3:4)]) +
      rowSums(CDmatrix[,5:6]) - rowSums(CAmatrix[,1:2]) +
      dom.plus(1,ADmatrix[,5:6]) +
      dom.plus(1,CDmatrix[,1:2]) -
      dom.plus(1,CAmatrix[,3:4]) +
      dom.minus(1,ADmatrix[,7:8]) +
      dom.minus(1,CDmatrix[,3:4]) -
      dom.minus(1,CAmatrix[,5:6]))}
 }else{
    if(dom == 0){
     G.AD <- 500 + 20*(rowSums(ADmatrix) +
      rowSums(CDmatrix) + rowSums(CAmatrix))
    }else if(dom == 1){
     G.AD <- 500 + 20*(dom.plus(AD.loci,ADmatrix) +
      dom.plus(CD.loci,CDmatrix)+dom.plus(CA.loci,CAmatrix))
    }else if(dom == 2){
     G.AD <- 500+ 20*(rowSums(ADmatrix[,c(1:2,3:4)]) +
      rowSums(CDmatrix[,5:6]) + rowSums(CAmatrix[,1:2]) +
      dom.plus(1,ADmatrix[,5:6]) +
      dom.plus(1,CDmatrix[,1:2]) +
      dom.plus(1,CAmatrix[,3:4]) +
      dom.minus(1,ADmatrix[,7:8]) +
      dom.minus(1,CDmatrix[,3:4]) +
      dom.minus(1,CAmatrix[,5:6]))}
}}else{
    if(dom == 0){
     G.AD <- 500 + 20* rowSums(ADmatrix)
    }else if(dom == 1){
     G.AD <- 500 + 20*dom.plus(AD.loci,ADmatrix)}
}
# Truncate ability duration genotype, min 10 and max 960
G.AD  <- sapply(seq(1,length(G.AD)), FUN = function(x)
  {if(G.AD[x]<10){10} else if(G.AD[x]>960){960
  }else{G.AD[x]}})
# Add genotype + enviroment, with arbitrary scaling
P.AD  <- 0.7*G.AD + 25*EnvD[,2]
# Truncate ability duration phenotype, min 10 and max 960
P.AD  <- sapply(seq(1,length(P.AD)), FUN = function(x)
  {if(P.AD[x]<10){10} else if(P.AD[x]>960){960
  }else{P.AD[x]}})

# Save variances for motivation and ability for duration
VeMD <- var(EnvD[,1])# Environmental variance of motivation
```

```
VgMD <- var(G.MD)    # Genotypic variance of motivation
VeAD <- var(EnvD[,2])# Environmental variance of ability
VgAD <- var(G.AD)    # Genotypic variance of ability

# Duration phenotype = lower of motivation or ability
P.D <- sapply(seq(1, length(P.MD)), FUN = function(x)
  ifelse(P.MD[x] <= P.AD[x], P.MD[x], P.AD[x]))

# Running phenotype = mulitply speed and duration
P.R <- P.S*P.D

return(c(P.S,P.D,P.R,P.MS,P.AS,P.MD,P.AD,VeMS,VgMS,VeAS,VgAS,VeMD
,VgMD,VeAD,VgAD))
}


##################### ACTION ####################
for(RUN in RUN:(RUN+maxrun-1)){
runif(1)
randomseed <- .Random.seed

# Number of loci per each lowest-level trait
MS.loci <- unique.loci # Motivation for speed
AS.loci <- unique.loci # Ability for speed
CS.loci <- common.loci # Common for ability and motivation for
speed
MD.loci <- unique.loci # Motivation for duration
AD.loci <- unique.loci # Ability for duration
CD.loci <- common.loci # Common for ability and motivation for
duration
CM.loci <- common.loci # Common for speed motivation and duration
motivation
CA.loci <- common.loci # Common for speed ability and duration
ability

# Create genetic matrices for each trait
MSmatrix<-ASSIGN(MS.loci,N.Pop,c(rep(.5,MS.loci)),biallele)
ASmatrix<-ASSIGN(AS.loci,N.Pop,c(rep(.5,AS.loci)),biallele)
MDmatrix<-ASSIGN(MD.loci,N.Pop,c(rep(.5,MD.loci)),biallele)
ADmatrix<-ASSIGN(AD.loci,N.Pop,c(rep(.5,AD.loci)),biallele)
# Create genetic matrices for each trait, loci in common
if(common.loci >0){
(CSmatrix<-ASSIGN(CS.loci,N.Pop,c(rep(.5,CS.loci)),biallele))&
(CDmatrix<-ASSIGN(CD.loci,N.Pop,c(rep(.5,CD.loci)),biallele))&
(CAmatrix<-ASSIGN(CA.loci,N.Pop,c(rep(.5,CA.loci)),biallele))&
(CMmatrix<-ASSIGN(CM.loci,N.Pop,c(rep(.5,CM.loci)),biallele))}
# Create genetic matrices for each trait, vary allele frequency
if(allele.freq == "y" & common.loci == 3 & unique.loci == 4){
MSmatrix<-ASSIGN(MS.loci, N.Pop ,c(.65,.55,.45,.35),biallele)
```

```
ASmatrix<-ASSIGN(AS.loci, N.Pop, c(.35,.95,.85,.75),biallele)
MDmatrix<-ASSIGN(MD.loci, N.Pop, c(.65,.55,.45,.35),biallele)
ADmatrix<-ASSIGN(AD.loci, N.Pop, c(.95,.85,.75,.35),biallele)
CSmatrix<-ASSIGN(CS.loci, N.Pop, c(.25,.15,.05),biallele)
CDmatrix<-ASSIGN(CD.loci, N.Pop, c(.25,.15,.05),biallele)
CMmatrix<-ASSIGN(CM.loci, N.Pop, c(.95,.85,.75),biallele)
CAmatrix<-ASSIGN(CA.loci, N.Pop, c(.65,.55,.45),biallele)}


# Set up data tables which will be filled up later
P <- matrix(0,(np/2),2)
all <-matrix(0,N.Pop*Maxgen,11,
  dimnames=list(1:(N.Pop*Maxgen),rbind("ID","SireID","DamID",
  "Gen","running","speed","duration","ms","as","md","ad")))
Output.phenotype <- matrix(0, Maxgen,8,dimnames=list(1:Maxgen,
  rbind("Igen","P.MS","P.AS","P.MD","P.AD","P.S","P.D","P.R")))
Output.h2 <- matrix(0, Maxgen, 35, dimnames=list(1:Maxgen,
  rbind("Igen","VpR","VpS","VpD","VpMS","VeMS","VgMS","h2_MS",
  "VpAS","VeAS","VgAS","h2_AS","VpMD","VeMD","VgMD","h2_MD",
  "VpAD","VeAD","VgAD","h2_AD","pcorRS","pcorRD","pcorSD",
  "pcorRMS","pcorRAS","pcorRMD","pcorRAD","pcorSMS","pcorSAS",
  "pcorDMD","pcorDAD","pcorMSAS","pcorMDAD","pcorMSMD",
  "pcorASAD")))
Output.slopes<-matrix(0,Maxgen,2,dimnames=list(1:Maxgen,
  rbind("slope","std error")))
Output.mutations <- matrix(0,Maxgen,9,dimnames=list(1:Maxgen,
  rbind("Igen","MS","AS","MD","AD","CS","CD","CA","CM")))
if (bv == "y") {Output.breedingvalue <- matrix(0,Maxgen,3,
  dimnames=list(1:Maxgen,rbind("breeding.value.S",
  "breeding.value.D","breeding.value.R")))}
dev.new()

###### Iterate over generations ######
# Generation 1 is the base population
for (Igen in 1:Maxgen)
{

# Caluclate breeding values
if(bv == "y" & Igen > 1){
  offspring.means.S<-matrix(0,N.Pop,N.Pop)
  offspring.means.D<-matrix(0,N.Pop,N.Pop)
  offspring.means.R<-matrix(0,N.Pop,N.Pop)
  N.bv <- 5
  for(i in 1:(N.Pop-1)){
    for(j in (i+1):N.Pop){
     offspringMS <- MATE(offspringMS,N.bv,MS.loci,MSmatrix,i,j)
     offspringAS <- MATE(offspringAS,N.bv,AS.loci,ASmatrix,i,j)
     offspringMD <- MATE(offspringMD,N.bv,MD.loci,MDmatrix,i,j)
     offspringAD <- MATE(offspringAD,N.bv,AD.loci,ADmatrix,i,j)
```

```
  if(common.loci >0){
 (offspringCS <- MATE(offspringCS,N.bv,CS.loci,CSmatrix,i,j)) &
 (offspringCD <- MATE(offspringCD,N.bv,CD.loci,CDmatrix,i,j)) &
 (offspringCM <- MATE(offspringCM,N.bv,CM.loci,CMmatrix,i,j)) &
 (offspringCA <- MATE(offspringCA,N.bv,CA.loci,CAmatrix,i,j))}
 running <- RUNNING(offspringMS,offspringAS,offspringMD,
   offspringAD,offspringCS,offspringCD,offspringCA,offspringCM)
  offspring.means.S[i,j]<- mean(running[1:N.bv])
  offspring.means.S[j,i]<- mean(running[1:N.bv])
  offspring.means.D[i,j]<- mean(running[(N.bv+1):(2*N.bv)])
  offspring.means.D[j,i]<- mean(running[(N.bv+1):(2*N.bv)])
  offspring.means.R[i,j]<- mean(running[(2*N.bv+1):(3*N.bv)])
  offspring.means.R[j,i]<- mean(running[(2*N.bv+1):(3*N.bv)])}}
breeding.value.S <- 1:N.Pop
pop.mean.S<-mean(offspring.means.S[upper.tri(offspring.means.S)])
for(b in 1:N.Pop){breeding.value.S[b]<-2*(pop.mean.S-
  mean(offspring.means.S[b,-b]))}
Output.breedingvalue[Igen,1] <- var(breeding.value.S)
breeding.value.D <- 1:N.Pop
pop.mean.D<-mean(offspring.means.D[upper.tri(offspring.means.D)])
for(b in 1:N.Pop) {breeding.value.D[b] <- 2*(pop.mean.D-
  mean(offspring.means.D[b,-b]))}
Output.breedingvalue[Igen,2] <- var(breeding.value.D)
breeding.value.R <- 1:N.Pop
pop.mean.R<-mean(offspring.means.R[upper.tri(offspring.means.R)])
for(b in 1:N.Pop) {breeding.value.R[b] <- 2*(pop.mean.R-
  mean(offspring.means.R[b,-b]))}
Output.breedingvalue[Igen,3] <- var(breeding.value.R)
}


# Calculate running phenotype
running <- RUNNING(MSmatrix,ASmatrix,MDmatrix,ADmatrix,
  CSmatrix,CDmatrix,CAmatrix,CMmatrix)

# Extract phenotypes from running matrix
P.S <- running[1:N.Pop]
P.D <- running[(N.Pop+1):(2*N.Pop)]
P.R <- running[(2*N.Pop+1):(3*N.Pop)]
P.MS <- running[(3*N.Pop+1):(4*N.Pop)]
P.AS <- running[(4*N.Pop+1):(5*N.Pop)]
P.MD <- running[(5*N.Pop+1):(6*N.Pop)]
P.AD <- running[(6*N.Pop+1):(7*N.Pop)]
# Calculate phenotypic variances
VpS <- var(P.S)
VpD <- var(P.D)
VpR<- var(P.R)
VpMS <- var(P.MS)
VpAS <- var(P.AS)
```

```
VpMD <- var(P.MD)
VpAD <- var(P.AD)
# Extract genetic and environmental variances from running matrix
VeMS <- running[(7*N.Pop+1)]
VgMS <- running[(7*N.Pop+2)]
VeAS <- running[(7*N.Pop+3)]
VgAS <- running[(7*N.Pop+4)]
VeMD <- running[(7*N.Pop+5)]
VgMD <- running[(7*N.Pop+6)]
VeAD <- running[(7*N.Pop+7)]
VgAD <- running[(7*N.Pop+8)]
# Caluclate heritabilities for lowest-level traits
h2_MS <- VgMS/VpMS
h2_AS <- VgAS/VpAS
h2_MD <- VgMD/VpMD
h2_AD <- VgAD/VpAD

# Calculate phenotypic correlations
pcorRS <- cor(P.R,P.S)  # running and speed
pcorRD <- cor(P.R,P.D)  # running and duration
pcorSD <- cor(P.S,P.D)  # speed and duration

pcorRMS <- cor(P.R,P.MS) # running and motivation for speed
pcorRAS <- cor(P.R,P.AS) # running and ability for speed
pcorRMD <- cor(P.R,P.MD) # running and motivation for duration
pcorRAD <- cor(P.R,P.AD) # running and ability for duration

pcorSMS <- cor(P.S,P.MS) # speed and motivation for speed
pcorSAS <- cor(P.S,P.AS) # speed and ability for speed
pcorDMD <- cor(P.D,P.MD) # duration and motivation for duration
pcorDAD <- cor(P.D,P.AD) # duration and ability for duration

pcorMSAS <- cor(P.MS,P.AS) # motivation and ability for speed
pcorMDAD <- cor(P.MD,P.AD) # motivation and ability for duration
pcorMSMD <- cor(P.MS,P.MD) # motivation for speed and duration
pcorASAD <- cor(P.AS,P.AD) # ability for speed and duration

# Save phenotypes, variances, and correlations
if(np>2){
all[(Igen*N.Pop-(N.Pop-1)):(Igen*N.Pop),1:11] <-
  cbind(seq((Igen*N.Pop-(N.Pop-1)),Igen*N.Pop),
  rep(P[,1],each=litter),rep(P[,2],each=litter),
  Igen,P.R,P.S,P.D,P.MS,P.AS,P.MD,P.AD)
}else{
all[(Igen*N.Pop-(N.Pop-1)):(Igen*N.Pop),1:11] <-
  cbind(seq((Igen*N.Pop-(N.Pop-1)),Igen*N.Pop),
  rep(P[1],each=litter),rep(P[2],each=litter),
  Igen,P.R,P.S,P.D,P.MS,P.AS,P.MD,P.AD)}
```

```
# Mid-parent-on-offspring regressions
if (Igen > 1){
  reg <- matrix(rep(Parent,each=(litter)),N.Pop,2)
  mean <- sapply(seq(1,np,2),FUN=function(x){
     (ParentP.R[x]+ParentP.R[x+1])/2})
  reg <- cbind(reg,rep(mean,each=litter),P.R)
  plot(reg[,3],reg[,4],xlab='avgparents',ylab='offspring',
     main="offspring-on-parent regression",
     xlim=c(0,20000),ylim=c(0,20000))
  legend("topleft",legend=Igen)
  res=lm(reg[,4]~reg[,3])
  abline(res)
Output.slopes[Igen,]<-summary(res)$coefficients[2,1:2]}

# Store results
Output.phenotype[Igen,1:8] <- c(Igen,mean(P.MS),mean(P.AS),
  mean(P.MD),mean(P.AD),mean(P.S),mean(P.D),mean(P.R))
Output.h2[Igen,1:35] <- c(Igen,VpR,VpS,VpD,VpMS,VeMS,VgMS,
  h2_MS,VpAS,VeAS,VgAS,h2_AS,VpMD,VeMD,VgMD,
  h2_MD,VpAD,VeAD,VgAD,h2_AD,pcorRS,pcorRD,pcorSD,
  pcorRMS,pcorRAS,pcorRMD,pcorRAD,pcorSMS,
  pcorSAS,pcorDMD,pcorDAD,pcorMSAS,pcorMDAD,pcorMSMD,pcorASAD)


# Mutations
Pmut <- .0001
MStemp <- MUTATION(MSmatrix, Pmut, MS.loci, N.Pop)
MSmatrix <- matrix(MStemp[2:length(MStemp)],N.Pop,2*MS.loci)
AStemp <- MUTATION(ASmatrix, Pmut, AS.loci, N.Pop)
ASmatrix <- matrix(AStemp[2:length(AStemp)],N.Pop,2*AS.loci)
MDtemp <- MUTATION(MDmatrix, Pmut, MD.loci, N.Pop)
MDmatrix <- matrix(MDtemp[2:length(MDtemp)],N.Pop,2*MD.loci)
ADtemp <- MUTATION(ADmatrix, Pmut, AD.loci, N.Pop)
ADmatrix <- matrix(ADtemp[2:length(ADtemp)],N.Pop,2*AD.loci)
if(common.loci>0){
  (CStemp <- MUTATION(CSmatrix, Pmut, CS.loci, N.Pop))&
  (CDtemp <- MUTATION(CDmatrix, Pmut, CD.loci, N.Pop))&
  (CAtemp <- MUTATION(CAmatrix, Pmut, CA.loci, N.Pop))&
  (CMtemp <- MUTATION(CMmatrix, Pmut, CM.loci, N.Pop))&
  (CSmatrix <- matrix(CStemp[2:length(CStemp)],N.Pop,2*CS.loci))&
  (CDmatrix <- matrix(CDtemp[2:length(CDtemp)],N.Pop,2*CD.loci))&
  (CAmatrix <- matrix(CAtemp[2:length(CAtemp)],N.Pop,2*CA.loci))&
  (CMmatrix <- matrix(CMtemp[2:length(CMtemp)],N.Pop,2*CM.loci))
}else{(CStemp<-CDtemp<-CAtemp<-CMtemp<-0)}
# Save number of mutations per trait per generation
Output.mutations[Igen,1:9] <- c(Igen,MStemp[1],AStemp[1],
  MDtemp[1],ADtemp[1],CStemp[1],CDtemp[1],CAtemp[1],CMtemp[1])
```

```
# Make breeding pairs
if(within.fam == "y"){
 if(sel == "sel") {
  # Select highest running individuals within families
  preParent <- c(sapply(seq(1,N.Pop,litter),
    FUN = function(i) {order(P.R[i:(i+litter-1)],
      decreasing=TRUE)[1:2]+(i-1)}))
  } else {
  # Randomly select breeders within families
  preParent <- c(sapply(seq(1,N.Pop,litter),
    FUN = function(i) {sample(i:(i+litter-1),2,replace=F)}))}
} else {
 if(sel == "sel"){
  # Select highest running individuals, mass selection
  preParent <- order(P.R,decreasing=TRUE)
 } else {
  # Randomly select individuals
  preParent <- sample(N.Pop,N.Pop,replace=FALSE)}
}
# Create matrices of parental alleles
ParentMS <- MSmatrix
ParentAS <- ASmatrix
ParentMD <- MDmatrix
ParentAD <- ADmatrix
if(common.loci >0){
  (ParentCS <- CSmatrix) &
  (ParentCM <- CDmatrix) &
  (ParentCA <- CAmatrix) &
  (ParentCD <- CDmatrix)}
# Make preliminary pairings, but these are all sib-pairs
P <- matrix(preParent,np/2,2, byrow=TRUE)
# Randomize 2nd column so that pairings are now random
P <- cbind(P,sample(P[,2],np/2,replace=F))
# Check if any pairs are still sib-pairs
same <- which((P[,2] - P[,3])== 0)
if(length(same)>0){
 if(length(same)==1){
   # If there is one sib-pair
   # switch partners with the first non-sib-pair
   switcheroo <- which((P[,2] - P[,3]) != 0)[1]
   P[c(same,switcheroo),3] <- P[c(switcheroo,same),3]
 }else{
   # If there is more than one sib-pair
   # Take only the sib-pairs and rotate them
   tmp2 <- c(same[length(same)],same[-length(same)])
   P[same,3] <- P[tmp2,3]}}
# Now we have the non-sib pairs IDs
P <- P[,c(1,3)]
# Parents as in P, by column
```

```
Parent <- c(P)
# Phenotypes of parents in order of offspring IDs
if(np > 2) {
  ParentP.R <- c(sapply(1:(np/2),
    FUN=function(x){c(P.R[P[x,1]],P.R[P[x,2]])}))
}else{
  ParentP.R <- c(sapply(1:(np/2),
    FUN=function(x){c(P.R[P[1]],P.R[P[2]])}))}

# Make matrices of offspring genotypes
for(i in 1:(N.Pop/litter)) {
if(np > 2){
  MSmatrix<-offspring(MSmatrix,MS.loci,i,ParentMS,P[i,1],P[i,2])
  ASmatrix<-offspring(ASmatrix,AS.loci,i,ParentAS,P[i,1],P[i,2])
  MDmatrix<-offspring(MDmatrix,MD.loci,i,ParentMD,P[i,1],P[i,2])
  ADmatrix<-offspring(ADmatrix,AD.loci,i,ParentAD,P[i,1],P[i,2])
if(common.loci >0){
(CSmatrix<-offspring(CSmatrix,CS.loci,i,ParentCS,P[i,1],P[i,2]))&
(CDmatrix<-offspring(CDmatrix,CD.loci,i,ParentCD,P[i,1],P[i,2]))&
(CMmatrix<-offspring(CMmatrix,CM.loci,i,ParentCM,P[i,1],P[i,2]))&
(CAmatrix<-offspring(CAmatrix,CA.loci,i,ParentCA,P[i,1],P[i,2]))}
}else{
  MSmatrix <- offspring(MSmatrix,MS.loci,i,ParentMS,P[1],P[2])
  ASmatrix <- offspring(ASmatrix,AS.loci,i,ParentAS,P[1],P[2])
  MDmatrix <- offspring(MDmatrix,MD.loci,i,ParentMD,P[1],P[2])
  ADmatrix <- offspring(ADmatrix,AD.loci,i,ParentAD,P[1],P[2])
  if(common.loci >0){
   (CSmatrix<-offspring(CSmatrix,CS.loci,i,ParentCS,P[1],P[2]))&
   (CDmatrix<-offspring(CDmatrix,CD.loci,i,ParentCD,P[1],P[2]))&
   (CMmatrix<-offspring(CMmatrix,CM.loci,i,ParentCM,P[1],P[2]))&
   (CAmatrix<-offspring(CAmatrix,CA.loci,i,ParentCA,P[1],P[2]))
}}}

print(paste("Finished generation",Igen,"of",Maxgen,"of RUN",RUN))
# The following bracket is the end of each generation
}


#################### GRAPHS ####################

##### Parent-on-offspring regression #####
dev.new()
par(mfrow=c(1,1))
plot(1:Maxgen,Output.slopes[,1],type='l', xlab="Generation",
  ylab="midparent-on-offspring regression slope",
  main = "Midparent-on-Offspring Regression Slopes",
  ylim=c(-1,1))
abline(h = 0, lty=3, col="light gray")
```

```
##### Phenotypes panel-running, speed, duration, MS,AS,MD,AD)
dev.new()
par(mfrow=c(2,3), oma=c(0,0,2,0), mar=c(1.5,1.5,2,0.5))
# Running
plot(Output.phenotype[,"Igen"],Output.phenotype[,"P.R"],
  xlab='Generation', ylab="revolutions per day",
  type="l",main="Revolutions")
abline(v=seq(0,Maxgen,10),lty=3,col="light gray")
# Speed
plot(Output.phenotype[,"Igen"],Output.phenotype[,"P.S"],
  xlab='Generation', ylab="Speed (revolutions per minute)",
  type="l",main="Mean Speed") #plot speed phenotype
abline(v=seq(0,Maxgen,10),lty=3,col="light gray")
# Duration
plot(Output.phenotype[,"Igen"],Output.phenotype[,"P.D"],
  xlab='Generation', ylab="Duration (minutes per day)",
  type="l",main="Duration")
abline(v=seq(0,Maxgen,10),lty=3,col="light gray")
# Blank space in panel
frame()
# Motivation and ability for speed
plot(Output.phenotype[,"Igen"],Output.phenotype[,"P.AS"],
  xlab="Generation", ylab="speed (revolutions per minute)",
  main="Mean MS and AS", type="l", ylim = c(5,40))
points(Output.phenotype[,"Igen"],
  Output.phenotype[,"P.AS"], pch=0)  # Open square = AS
lines(Output.phenotype[,"Igen"],Output.phenotype[,"P.MS"])
points(Output.phenotype[,"Igen"],
  Output.phenotype[,"P.MS"], pch=1)  # Open circle = MS
legend("bottomright",legend=c("ability","motivation"),pch=c(0,1),
title="legend",  bty="n",cex=.9)
abline(v=seq(0,Maxgen,10),lty=3,col="light gray")
# Motivation and ability for duration
plot(Output.phenotype[,"Igen"],Output.phenotype[,"P.AD"],
  xlab="Generation", main="mean MD and AD",
  ylab="duration (minutes per day)", type="l", ylim=c(300,700))
points(Output.phenotype[,"Igen"],
  Output.phenotype[,"P.AD"],pch=15)  # Closed square = AD
lines(Output.phenotype[,"Igen"],Output.phenotype[,"P.MD"])
points(Output.phenotype[,"Igen"],
  Output.phenotype[,"P.MD"], pch=16) # Closed circle = MD
legend("bottomright",legend=c("ability","motivation"),
  pch = c(15,16), title="legend",bty="n", cex=.9)
abline(v=seq(0,Maxgen,10),lty=3,col="light gray")
title(main = list("Phenotypes", cex =2), outer = TRUE)

##### breeding value ######
if (bv == "y"){
h2_R<-Output.breedingvalue[,"breeding.value.R"]/Output.h2[,"VpR"]
```

```
h2_S<-Output.breedingvalue[,"breeding.value.S"]/Output.h2[,"VpS"]
h2_D<-Output.breedingvalue[,"breeding.value.D"]/Output.h2[,"VpD"]

dev.new()
par(mfrow=c(3,4), oma=c(0,0,2,0)) #plot in a 3x3
# Running
plot(Output.breedingvalue[,"breeding.value.R"],
  xlab="Generation", ylab = "Va - Running",
  type = "l", main = "Va")
plot(Output.h2[,"Igen"],Output.h2[,"VpR"],xlab="Generation",
  ylab="Vp - Running",type="l", main="Vp")
plot(Output.h2[,"Igen"],
  Output.breedingvalue[,"breeding.value.R"],
  xlab='Generation', main="Vg",ylab="Vg - Running",type="l")
plot(Output.h2[,"Igen"],h2_R,xlab='Generation',
  main="H2",ylab="H2 - Running",type="l")
abline(v=seq(0,Maxgen,10),lty=3,col="light gray")
# Speed
plot(Output.breedingvalue[,"breeding.value.S"],
  xlab="Generation", ylab = "Va - Speed", type = "l")
plot(Output.h2[,"Igen"],Output.h2[,"VpS"],xlab="Generation",
  ylab="Vp - Speed",type="l")
plot(Output.h2[,"Igen"],
  Output.breedingvalue[,"breeding.value.S"],xlab='Generation',
  ylab="Vg - Speed",type="l")
plot(Output.h2[,"Igen"],h2_S,xlab='Generation',
  ylab="H2 - Speed",type="l")
abline(v=seq(0,Maxgen,10),lty=3,col="light gray")
# Duration
plot(Output.breedingvalue[,"breeding.value.D"],
  xlab="Generation", ylab = "Va - Duration", type = "l")
plot(Output.h2[,"Igen"],Output.h2[,"VpD"],xlab="Generation",
  ylab="Vp - Duration",type="l")
plot(Output.h2[,"Igen"],
  Output.breedingvalue[,"breeding.value.D"],
  xlab='Generation',ylab="Vg - Duration",type="l")
plot(Output.h2[,"Igen"],h2_D,xlab='Generation',
  ylab="H2 - Duration",type="l")
abline(v=seq(0,Maxgen,10),lty=3,col="light gray")
title(main = list("heritabilities of composite traits (from
breeding values)", cex =2), outer = TRUE)
}

##### Vg, Ve, Vp, and h2 for MS, AS, MD, AD #####
dev.new()
par(mfrow=c(4,4), oma=c(0,0,2,0)) #plot in a 4x4
#MS
plot(Output.h2[,"Igen"],Output.h2[,"VpMS"],xlab="Generation",
  ylab="Vp - motivation for speed",type="l", main="Vp")
```

```
plot(Output.h2[,"Igen"],Output.h2[,"VeMS"],xlab='Generation',
  main="Ve", ylab="Ve - MS",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"VgMS"],xlab='Generation',
  main="Va",ylab="Va - MS",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"h2_MS"],xlab='Generation',
  main="h2", ylab="h2 - MS",type="l")
#AS
plot(Output.h2[,"Igen"],Output.h2[,"VpAS"],xlab="Generation",
  ylab="Vp - ability for speed",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"VeAS"],xlab='Generation',
  ylab="Ve - AS",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"VgAS"],xlab='Generation',
  ylab="Va - AS",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"h2_AS"],xlab='Generation',
  ylab="h2 - AS",type="l")
#MD
plot(Output.h2[,"Igen"],Output.h2[,"VpMD"],xlab="Generation",
  ylab="Vp - motivation for duration",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"VeMD"],xlab='Generation',
  ylab="Ve - MD",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"VgMD"],xlab='Generation',
  ylab="Va - MD",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"h2_MD"],xlab='Generation',
  ylab="h2 - MD",type="l")
#AD
plot(Output.h2[,"Igen"],Output.h2[,"VpAD"],xlab="Generation",
  ylab="Vp - ability for duration",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"VeAD"],xlab='Generation',
  ylab="Ve - AD",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"VgAD"],xlab='Generation',
  ylab="Va - AD",type="l")
plot(Output.h2[,"Igen"],Output.h2[,"h2_AD"],xlab='Generation',
  ylab="h2 - AD",type="l")
title(main = list("heritabilities of lowest level traits from
direct allelic calculations", cex =2), sub = "warning: the
heritabilities do not make sense because of the way they interact
/ epistasis", outer = TRUE)


##################### SAVE CSV FILES ####################

# Name files according to parameters used
file <- paste("model3_",sel,"_",biallele,"biallele_",
  common.loci,"common_",allele.freq,"freq_",
  within.fam,"wfam_",dom,"dom_",N.Pop,"pop_run",RUN,sep="")

# Save file of variance estimates from breeding values
if (bv == "y"){
write.csv(Output.breedingvalue,
```

```
  file=paste(file,"_Output.breedingvalue.csv",sep=""),
    row.names = F)}

# Save file of variances
write.csv(Output.h2,file=paste(file,"_Output.h2.csv",sep=""),
  row.names = F)

# Save file of phenotypes
write.csv(Output.phenotype,
  file=paste(file,"_Output.phenotype.csv",sep=""),row.names = F)

# Save file of parent-offspring regression slopes
write.csv(Output.slopes,file=paste(file,"_","Output.slopes.csv",
  sep=""),row.names = F)

# Save file of pedigree + phenotypes of every individual
all[(N.Pop+1):(N.Pop*Maxgen),"SireID"] <-
  sapply((N.Pop+1):(N.Pop*Maxgen),
   FUN=function(x){(all[x,"Gen"]-2)*N.Pop+all[x,"SireID"]})
all[(N.Pop+1):(N.Pop*Maxgen),"DamID"] <-
  sapply((N.Pop+1):(N.Pop*Maxgen),
   FUN=function(x){(all[x,"Gen"]-2)*N.Pop+all[x,"DamID"]})
write.csv(all,file=paste(file,"_all.csv",sep=""),row.names = F)

# Save file of random seed
sampleseed <- sample(1:10,1)
attr(sampleseed,"seed") <- randomseed
write.csv(attr(sampleseed,"seed"),
  file=paste(file,"_RandomSeed.csv",sep=""),row.names=F)

# These two brackets sign the end of the code.
# The enable the prompts at the beginning to work.
# Do not leave any lines after the bracket.
}}
```