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Phenotypic and evolutionary plasticity of organ masses in response to voluntary exercise in house mice¹

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Synopsis. We used a novel mouse model to study the effects of selective breeding for high locomotor activity (14 generations) on relative organ sizes, hematocrit (Hct), and blood hemoglobin (Hb) concentration. We also examined effects of exercise training and gene-by-environment interactions by housing animals for 8 weeks with wheels that were either free to rotate or locked. Mice from the four replicate High-Runner (HR) lines were smaller in total body mass but had larger body mass-adjusted kidneys relative to the four Control lines ($P < 0.05$). Control and HR lines did not differ significantly for mass-adjusted tail length or masses of the "triceps surae" hindlimb muscle group, heart (ventricle), spleen, liver, adrenal glands or gonads. Wheel access caused a reduction in body mass and an increase in relative heart mass. In females only, wheel access caused a reduction in relative spleen mass. Wheel access did not affect relative tail length or relative mass of the triceps surae, liver, adrenal gland or gonads. Significant interactions between selection history and wheel access were observed in females for spleen, liver, and gonad mass as well as Hct and Hb. Wheel access caused increases in both Hct and Hb, mainly in the HR lines. The mini-muscle phenotype, caused by a Mendelian recessive allele that halves hindlimb muscle mass, was significantly associated with several other body composition traits, including reduced body mass, increased tail length, increased heart mass, increased liver mass (females only), increased mean adrenal gland mass (females only), increased mean kidney mass (males only), and reduced Hct (wheel-access females only). Results are discussed in context of the beneficial acclimation hypothesis, genotype-by-environment interactions, and the potential for "nurture" to be self-reinforcing of "nature" in some complex behavioral-physiological phenotypes.

Keywords: adaptation, artificial selection, exercise physiology, experimental evolution, phenotypic plasticity, selective breeding, training

Introduction

Many organisms display the capacity to adjust or transform key anatomical, morphological or physiological characters in response to the environment. Such capacity for change is often referred to as phenotypic plasticity (Gordon, 1992; Via et al., 1995) or, when the changes are fully reversible, phenotypic flexibility (Piersma and Lindstrom, 1997). Generally beneficial responses to chronic exercise are well documented, and are prime examples of phenotypic flexibility. In rodents, such effects can be studied by use of either forced or voluntary exercise, with forced exercise protocols being more common in the literature. However, voluntary wheel running (Sherwin, 1998; Eikelboom, 1999) is also becoming widely used because many species of rodents will run voluntarily for many kilometers per day at relatively high intensity (Dewsbury, 1980; Rodnick et al., 1989; Girard et al., 2001).

Cardiac and skeletal muscle hypertrophy are commonly observed responses to chronic wheel access in both rats (e.g., Yano et al., 1997; Rodnick et al., 1989; Rodnick et al., 1992; Sexton, 1995) and mice (Allen et al., 2001; Harrison et al., 2002). Additionally, we and others have found that voluntary wheel running elicits a variety of physiological modifications in rodents, including decreases in body mass and body fat (Bell et al., 1997; Swallow et al., 1999; Swallow et al., 2001; Dumke et al., 2001), increases in maximal O₂ consumption (Lambert and Noakes, 1990; Yano et al., 1997; Swallow et al., 1998b), and metabolic alteration of cardiac and skeletal muscles (Rodnick et al., 1989; Zhan et al., 1999; Dumke et al., 2001; Ishihara et al., 1991; Houle-Leroy et al., 2000; Allen et al., 2001; Harrison et al., 2002).

But not all traits "train" in response to chronic exercise. For example, the acute relationship between physical activity and plasma corticosterone level in house mice does not change over several weeks of wheel access (Girard and Garland, 2001). Perhaps counter-intuitively, most human studies

show that hematocrit or blood hemoglobin concentration does not increase with prolonged exercise (Scheuer and Tipton, 1977; Sawka et al., 2000). This may be because training generally results in an overall increase in blood volume resulting from equivalent but nonsynchronous increases in both plasma volume and erythrocyte volume (Sawka et al., 2000). In rodent studies, hematocrit and hemoglobin content either showed no change (Yashiro and Kimura, 1979; Pfeil, 1988) or decreased (Spodaryk et al., 1985) after access to running wheels. Systematic studies of the relationship between locomotor activity and changes in blood volume are not available for rodents.

From the perspective of ecological and evolutionary physiology, the relationship between organ structures and correlated metabolic features is of interest because mounting evidence indicates that plastic changes in internal morphology in response to chronic energy challenges are correlated with improved functional capacity and metabolic function (Konarzewski and Diamond, 1995). For example, rodents exposed to cold exhibit cardiac muscle hypertrophy, and they also exhibit hypertrophy of many internal organs required to support nutrient extraction and utilization, including the gastrointestinal tract, liver, and kidney (Harri et al., 1984; Hammond et al., 1994; Konarzewski and Diamond, 1994; McDevitt and Speakman, 1994). Similar patterns are exhibited in response to increased energy demand as a result of lactation and exposure to high altitude (Hammond et al., 1999, 2001; Rezende et al., 2005).

Access to running wheels results in significant increases in energy expenditure, as indexed by increased food consumption, as compared to sedentary controls (Swallow et al., 2001), and therefore might result in similar changes in the internal organs required to support nutrient extraction and utilization. However, previous studies of rats do not show significant changes in kidney mass and are contradictory with regard to liver mass (Ring et al., 1970; Pitts, 1984). Unfortunately, these studies are difficult to

interpret because organ masses were reported only on a per gram body mass basis (e.g., see Hayes, 2001, and refs. therein) and because body mass and adipose composition can vary dramatically between active and sedentary individuals. No data are available for house mice.

A key factor in studies of the effects of voluntary exercise is how much the subjects actually exercise. All else being equal, one would expect that higher levels of physical activity would lead to greater training effects. However, too much exercise could be deleterious, as "over-training" effects are documented in a variety of organisms (e.g., Garland et al., 1987; Tyler et al., 1996; Budget, 1998).

We have developed a novel mouse model that can be used to test these propositions. Beginning in 1993, we have used selective breeding to create four replicate lines of house mice that exhibit high voluntary wheel running (High-Runner or HR lines) as compared with four random-bred Control lines (Swallow et al., 1998a; Swallow, 1998; Garland, 2003). After 14 generations of selection, HR mice ran more than twice the distance per day as compared with Control mice (Swallow et al., 1999). Because high levels of voluntary activity may require a high level of endurance, we hypothesized that selection for wheel running might be accompanied by a concomitant increase in whole-animal aerobic capacity (Swallow et al., 1998b). Previous studies on these lines after 10 generations of selection, when the activity difference between linetypes was 75% (Swallow et al., 1998a), showed that untrained mice from the HR lines exhibited a modest (6%) increase in maximum oxygen consumption, comparable to that elicited by 8 weeks of access to running wheels (Swallow et al., 1998b). However, this elevation of VO_2 max was not accompanied by significant increases in contractile performance, isotonic endurance or succinate dehydrogenase activity of the medial gastrocnemius muscle (Zhan et al., 1999). (Moreover, a statistically significant elevation of VO_2 max has not been found in three other studies at later generations [unpublished observations].) By generation 14,

though, untrained mice from the HR lines exhibited a trend towards higher muscle aerobic capacities, as indicated by mitochondrial and glycolytic enzyme activities, and lower anaerobic capacities (Houle-Leroy et al., 2000). As expected, training resulted in an increase in aerobic capacity in both the Control and HR lines. Moreover, two-way analysis of covariance revealed significant interactions between linetype and activity group: activities of several key enzymes showed greater training responses in the HR lines (Houle-Leroy et al., 2000). Furthermore, by generation 17, mice from the HR lines showed a higher insulin-stimulated glucose uptake in some muscles of HR vs. Control lines. In this case, however, neither linetype exhibited a training response after 8 weeks of wheel access, which differs from rats, which do show a positive training effect (Dumke et al., 2001).

In the present study, we sought to determine whether a series of organs hypothesized to be important for sustained, aerobic exercise would be altered constitutively in the High-Runner lines and/or would respond plastically after 8 weeks of access to running wheels. Because mice from the HR lines exhibit substantially elevated levels of wheel running, they may be more likely to exhibit a training response or may exhibit a greater training response, as compared with "normal" Control mice. Hence, power to detect effects of voluntary exercise should be increased by use of the HR lines. In addition, differences between HR and C lines in the magnitude of the plastic response might exist, given that plasticity itself is generally heritable and has been the subject of successful selection experiments in various organisms (Scheiner, 1993, 2002). A difference in plasticity would constitute a genotype-by-environment interaction. Thus, we also sought to compare the magnitude and direction of "phenotypic" adaptation to chronic wheel running with the magnitude and direction of any evolutionary or "genetic" adaptation, as indicated by the constitutive difference (if any) between HR and Control lines that exists when both are housed without access to functional wheels. (Some caution must be maintained here,

however, as it is possible the HR lines may exhibit elevated physical activity even when housed with locked wheels [see Rhodes et al., 2005].) Thus, in a two-way design, we studied the effects of genetic selection history (14 generations) and exercise history (8 weeks of access to running wheels) on cardiac and triceps surae muscle masses, as well as liver, spleen, kidney, adrenal gland, and gonad masses, tail length, hematocrit, and blood hemoglobin content.

Finally, we tested whether the "mighty mini-muscle" phenotype is associated with variation in any of the measured traits. As described previously, we discovered post hoc that the outbred population of mice from which the selection experiment was started contained a Mendelian recessive allele that halves hindlimb muscle mass (Garland et al., 2002). It has increased in frequency in two of the HR lines, and indeed is now (2004; unpublished results) fixed in one of them. The phenotype (representing the double-recessive genotype) has a variety of pleiotropic effects, including on organ masses, muscle enzyme activities, muscle glycogen concentration, and HSP72 expression (Houle-Leroy et al., 2003; Belter et al., 2004; Gomes et al., 2003, 2004). These or other pleiotropic effects may help to explain why the mini-muscle allele has been favored in the HR lines (Garland et al., 2002).

Materials and methods

Study Animals

Mice were sampled from the 14th generation of selective breeding for high voluntary wheel running. Full details of the selection experiment are provided elsewhere (Swallow, 1998; Swallow et al., 1998a), so only a brief description will be provided here. Body mass data for the same individuals used here were reported in a previous study (Swallow et al., 1999), which also give further information on housing and maintenance. Other papers that report data from these same individuals are Houle-Leroy et al. (2000, 2003) on exercise-relevant enzyme activities in hindlimb muscle, Thomson et al. (2002) on Sod-2 enzyme activity in liver, and Belter et al. (2004) on HSP72 expression in triceps surae (females only).

The original progenitors of the selection experiment were outbred, genetically variable Hsd:ICR house mice obtained from Harlan Sprague Dawley, Indianapolis, IN. Ten pairs (families) of mice were used to propagate each of the 8 closed lines, four selected for high activity and four randomly bred as controls. Each generation, when mice were 6-8 weeks old, they were housed individually with access to Wahman-type activity wheels for six days. Daily wheel running activity was monitored using an automated system. The selection criterion was the number of revolutions run on days 5 and 6 of access to the wheels. In the High-Runner lines, the highest running male and female from each family were selected for breeding. In the Control lines, a male and female from each family were chosen at random for breeding. By generation 10, the High-Runner lines had diverged significantly from the Controls with a 75% increase in total revolutions/day, caused primarily by High-Runner mice running faster rather than for more minutes each day (Swallow et al., 1998a).

Animals used in this study were from second litters; their siblings (first litters) were part of the

routine selection protocol. At 21 days of age, two male and two female pups from five families within each of the 8 lines were weaned, weighed, and toe clipped for identification. Mice were housed with three siblings until the following day, when they were housed individually and one sib from each sex and family was assigned to the Sedentary or Active group. Housing was in standard clear plastic cages (27x17x12.5 cm deep) with metal tops and wood shavings as bedding. Throughout the selection experiment and in the present study, food (Harlan Teklad Laboratory Rodent Diet (W) 8604) and water were available *ad libitum*. A constant 12:12 h photoperiod and ~22 °C ambient temperature were maintained.

Running wheels (1.12 m circumference) were attached to the cages via a 7.7-diameter tube. A photograph of the wheels can be found at:

<http://biology.ucr.edu/people/faculty/Garland/MusonWheel.jpg>

For the Sedentary group, wheels were prevented from rotating (locked) by use of a wire tie. For the Active group, wheel running was monitored daily from 22 days of age until the day prior to measurement of body (Swallow et al., 1999) and organ masses (mean age 78.9 days; range 75-82 days).

After 8 weeks of access to running wheels, mice were weighed to the nearest 0.1 g and a minimum of 4 blood samples (75 µl each) were collected from the peri-orbital sinus using heparinized micro-hematocrit tubes. Two of these tubes were centrifuged for 6.5 minutes in a Clay-Adams microfuge (Autocrit Ultra 3) to determine hematocrit (Hct). For measurement of hemoglobin concentration, 25-µl blood samples were drawn from an additional micro-hematocrit tube and transferred to 5 ml of Drabkin's reagent. Concentration of cyanmethemoglobin (Hb) was then determined at 540 nm (Brown, 1973) with a Beckman spectrophotometer using Sigma reagents.

Hemoglobin and hematocrit were determined in duplicate (or more) and means were analyzed.

After blood sampling, mice were killed by cervical dislocation. Following a mid-ventral incision, the heart was lifted with forceps and the ventricles were cut free from the atria and major blood vessels. Ventricles were blotted and any coagulated blood was removed before weighing. The gall bladder was removed before the liver was dissected and weighed. The spleen was removed and weighed. The right and left "triceps surae" (including soleus, plantaris, and both lateral and medial heads of the gastrocnemius) were removed by cutting the muscle from the lateral condyle of the tibia and medial condyle of the fibula, followed by cutting the Achilles' tendon midway between its origin and the muscles insertion. The foregoing organs were weighed to the nearest 0.1 mg. Tail length was measured to the nearest mm from the anogenital opening to the tip of the tail using a plastic ruler. Carcasses were then frozen. At a later date, they were defrosted and the kidneys, adrenal glands, and gonads (testes or ovaries) were dissected and weighed to the nearest 0.01 mg.

The MIXED procedure in SAS 6.12 was used to apply analysis of covariance (ANCOVA) models. Parameter estimation using the MIXED procedure uses maximum likelihood and, unlike the GLM procedure of SAS, provides the proper standard errors associated with least-squares means (see below) when the design is unbalanced (e.g., with respect to the mini-muscle phenotype) and contains random effects. A two-way ANCOVA model was used to test simultaneously the effects of linetype (High-Runner vs. Control) and activity group (Sedentary vs. Active). These two main grouping factors were fixed effects, whereas replicate line (N = 8 total) nested within linetype and family nested within line were random effects. As in previous analyses of these mice (Swallow et al., 1999; Houle-Leroy et al., 2000; Thomson et al., 2002), the significance of linetype was tested relative to line with 1 and 6 d.f. Both activity group and the activity-by-linetype interaction terms were tested relative to the line-by-

activity group interaction with 1 and 6 d.f. Following Belter et al. (2004), presence/absence of the mini-muscle phenotype (see Garland et al., 2002; Houle-Leroy et al., 2003) was used as an additional class variable (factor) in the model; its effect was tested over the error term with 1 and 26-28 d.f. (depending on sex and any missing values for particular traits). We did not attempt statistical comparisons of the sexes because they are known to differ in many respects; including amount, speed, and duration of wheel running, as well as body mass (e.g., see Swallow et al., 1998a, 1999). Thus, the sexes were analyzed separately. Sex comparisons will be the subject of future papers. Nevertheless, inspection of the least-squares means and standard errors (see below) presented in Table 3 allow one to see major sex differences, such as in body mass or mass-adjusted kidney mass (see also Fig. 2).

A P value of < 0.05 was used to judge statistical significance. For simplicity, all P values reported in the tables are 2-tailed. In several cases, we had directional predictions, so we sometimes mention the 1-tailed P value if the difference was in the predicted direction.

All P values are unadjusted for multiple comparisons. How best to correct for multiple comparisons is a complicated and controversial subject (e.g., see Curran-Everett, 2000). It is made more complicated by the fact that our analyses include a mixture of tests for which we had no directional hypothesis (e.g., effects of linetype on relative kidney mass), others for which we did have a directional hypothesis (e.g., effects of both linetype and wheel access on blood [Hb]), tests of interaction effects for which power may be relatively low (see Wahlsten, 1990; Houle-Leroy et al., 2000), and covariates (e.g., age) that constitute nuisance variables.

Body mass (\log_{10} transformed), age, time of sacrifice, and (z-transformed time of sacrifice)² were used as covariates in all analyses (the squared term for time allows for possible non-linear relationships). Body mass was log transformed because allometric first principles and many empirical

studies suggest that organ masses will often be linearly related to body mass on a log-log scale.

However, it is not clear that adjusting for body mass is biologically or statistically appropriate for all traits. For example, unlike most organ masses, blood hemoglobin content and hematocrit are not, *a priori*, expected to show a strong positive correlation with body mass. Empirically, we found that adrenal glands and gonads showed relatively weak correlations with body mass. Therefore, following analyses of enzyme activities by Houle-Leroy et al. (2000) and Thomson et al. (2002), for those traits we also performed analyses that did not include log body mass as a covariate.

Least-squares (adjusted) means and standard errors for each trait were also calculated in SAS PROC MIXED, using all factors and covariates in the model, regardless of statistical significance. Following Belter et al. (2004), potential outliers were examined using the dummy variable test described in Cook and Weisberg (1999). Based on this test, two individual data points were eliminated, one for testes mass and one for tail length (a female). Note that in some previous analyses of these mice (Swallow et al., 1999; Houle-Leroy et al., 2000), which employed SAS PROC GLM, missing values for some traits or removal of individuals as statistical outliers led us to drop the corresponding same-sex sibling from the analysis in order to maintain a balanced design. This was not necessary with SAS PROC MIXED, so sample sizes here are slightly larger than in those previous papers (usually one additional male, three additional females).

Results

Wheel running. As reported by Houle-Leroy et al. (2000) for animals used in this study, and consistent with all other studies, HR mice of both sexes ran significantly greater distances (rev/day) and at higher average velocities (RPM) as compared with mice from Control lines (Table 1). High-Runner mice also tended to spend more time running on the wheels (min/day; $P \sim 0.05$ in both sexes). Thus, the response to selection for increased wheel running has been accomplished primarily by HR mice running faster, not longer, in comparison with Controls (see also Koteja et al., 1999a; Koteja et al., 1999b; Swallow et al., 1998a; Swallow et al., 1998b; Swallow, 1998). In both the HR and Control lines, females ran more revolutions per day, ran for more minutes per day, and ran at higher velocities as compared to males (Table 1).

Body mass. Access to running wheels reduced body mass in both sexes and both linetypes (Table 2), as reported previously for these same mice (Swallow et al., 1999) and as found in other generational samples (e.g., Swallow et al., 2001). Also as reported previously (Koteja et al., 1999b; Swallow et al., 1999), mice from the HR lines were smaller than Controls, regardless of housing conditions (Tables 2 and 3). The effects of linetype and wheel access were additive, i.e., the interaction term was not statistically significant for either sex. Given the group differences in body mass, statistical analyses that adjust for those differences (i.e., analysis of covariance models) are generally appropriate, at least for traits that clearly must show some relationship to overall size of the animal, such as heart mass.

Organ masses, hematocrit, and hemoglobin. Table 2 presents significance levels and Table 3 presents least-squares means and standard errors. Mice with wheel access had larger hearts (Fig. 1: adjusted for differences in body mass) and elevated hematocrit and blood hemoglobin content in both

sexes. Wheel access also reduced relative spleen mass in females only. As compared with the Control lines, HR mice had relatively larger kidneys for both sexes (Fig. 2).

In our analysis, interactions between linetype and access to running wheels can be considered a genotype-by-environment interaction. For males, no traits measured in this study showed statistically significant interactions (Table 2). For females, however, spleen, liver, and ovary mass, as well as Hct and Hb, showed significant ($P < 0.05$) interactions (Table 2). Thus, for these traits in females, the effect of linetype was contingent on activity environment, and vice versa.

Inspection of the least-squares means and standard errors (Table 3) along with separate ANCOVAs of Sedentary and Active mice (not shown) indicated the following patterns. For Hct in males, the linetype effect was never significant. For Hb in males, the linetype effect was significant only for the wheel-access group (and only when body mass was not in the model). For both Hct and Hb in females, the linetype effect was significant only for the wheel-access group, and this was also true for the mini-muscle effect.

One obvious possible explanation for this pattern is that Control females simply do not run enough to elicit a training effect. To explore this, we considered only the wheel-access individuals and used ANCOVA with amount of running (total revolutions) during the final week as an additional covariate (see Fig. 4 for females). As shown in Table 4, results differed between the sexes. In males, wheel running was a highly significant positive predictor of individual variation in Hct and Hb, and the linetype effect remained non-significant (cf. Table 2). In females, however, wheel running was not a significant predictor, and in the models without body mass the linetype effect remained almost statistically significant (2-tailed $P = 0.065$ and 0.061 for Hct and Hb, respectively). These results suggest that HR females may be inherently more plastic for these two traits as compared with Control

females.

Effects of the mini-muscle phenotype. Mice with the mini-muscle phenotype were significantly smaller in both sexes. This factor was not analyzed in our previous paper on body masses of these same individual mice (Swallow et al., 1999), but a similar difference has been reported in other samples from these lines (Garland et al., 2002), although not in all samples (Houle-Leroy et al., 2003). Furthermore, the mini-muscle phenotype had wide-ranging effects on body composition (Table 2). By definition, the triceps surae was significantly reduced in mass in the mini-muscle individuals, resulting in a nearly halving of the mass of this skeletal muscle group (Table 3). However, the relative mass of cardiac muscle was increased in animals with this phenotype (see also Garland et al., 2002). Several additional mini-muscle effects were observed: increases in relative liver mass (females only), kidney mass (males only), mean adrenal gland mass (females only), and tail length (both sexes); decrease in Hct (females only). As described in the previous paragraph, we also performed separate analyses for the sedentary and wheel-access groups for Hct and Hb. These separate analyses (i.e., within sedentary or wheel-access groups) revealed that the negative effects of the mini-muscle phenotype occurred only in the mice housed with wheel access (Table 4). Spleen mass tended to be larger in animals of both sexes with the mini-muscle phenotype, but the P values were marginally non-significant (Table 2). However, because of the highly unbalanced design with regard to this factor, our power to detect an effect may be reduced.

Discussion

The term "complex trait" has various meanings (e.g., Fuller et al., 2005). From a phenotypic perspective, it generally refers to traits at higher levels of biological organization, such as behaviors or aspects of life history (e.g., growth rate, litter size), with the corollary that all lower-level traits are embedded within (components of) one or more complex phenotypes (Ghalambor et al., 2003; Sinervo and Calsbeek, 2003; Rezende et al., 2005). From a genetic perspective, it usually refers to traits that develop under effects from multiple loci, with the expectation that multiple environmental effects also act during ontogeny and may interact (i.e., have non-additive effects) with genetic effects (Pomp et al., 2004). Biomedically oriented research on the genetic and environmental bases of complex traits is accelerating at a rapid pace, especially with such model organisms as laboratory house mice (e.g., see <http://complextrait.org/>). The reason for this research effort is that many health issues and diseases are clearly complex traits in both the phenotypic and genetic senses (e.g., cancer, obesity, alcoholism; see also Koch and Britton, 2005; Wisløff et al., 2005). From the perspectives of ecology and evolutionary biology, complex traits are of interest because natural and sexual selection usually act most directly on higher-level traits, such as behavior and life history components, as expressed within the context of the overwhelming complexity of the natural world (Irschick and Garland, 2001). In addition, selection in the wild will generally occur simultaneously on multiple traits (Arnold, 2003; Ghalambor et al., 2003; Sinervo and Calsbeek, 2003).

Complex traits are made all the more complex by phenotypic plasticity and flexibility (Gordon, 1992; Scheiner, 1993; Via et al., 1995; Piersma and Lindstrom, 1997; Rezende et al., 2005). It is generally assumed that phenotypic changes in response to environmental cues or changes are conducive to Darwinian fitness, i.e., that they constitute adaptive plasticity (Schmitt et al., 2003). Acclimation is

one form of phenotypic plasticity, and although most biologists probably presume that the "beneficial acclimation hypothesis" is generally true, it should not be taken as a foregone conclusion for all traits or organisms (Scheiner, 1993; Leroi et al., 1994; Huey et al., 1999; Rezende et al., 2005).

Wheel-running activity is a genetically heritable trait, as indicated by a more than two-fold increase in activity levels after 14 generations of replicated selection for high voluntary wheel running in outbred laboratory house mice (Swallow et al., 1999). This selection protocol led to a concomitant, correlated reduction in body mass, an increase in relative kidney mass, and an increased frequency of the mini-muscle phenotype in two lines (see Garland et al., 2002; Houle-Leroy et al., 2003). In subsequent generations, we have found other statistically significant constitutive differences between the High-Runner and Control lines in suborganismal traits that may facilitate aerobic locomotor activity, including increased ventricle mass (Gomes et al., 2003), increased insulin-stimulated glucose uptake in some hindlimb muscles (Dumke et al., 2001), increased muscle glycogen concentration (Gomes et al., 2004), increased plasma corticosterone levels (Girard and Garland, 2002), and increased symmetry in the hindlimb long bones (T. Garland, Jr. and P. A. Freeman, unpublished). Still, as argued in Rhodes et al. (2005), the number and magnitude of these changes are less than might have been expected, and apparently less extensive than changes that have occurred in the brain (see also Bronikowski et al., 2004). These results highlight the complex genetic architecture of voluntary wheel running and its correlates with underlying morphological and physiological traits.

Access to running wheels for 8 weeks caused a significant decrease in body mass and an increase in relative heart mass in both sexes and linetypes. These effects are generally consistent with findings of previous studies of rodents (see Introduction). However, our finding that both hematocrit and hemoglobin content increased with wheel access, especially in the HR lines (Table 2), is actually

counter to the preponderance of human training studies (reviews in Scheuer and Tipton, 1977; Sawka et al., 2000) and the few rodent wheel-running studies that we found (Yashiro and Kimura, 1979; Spodaryk et al., 1985; Pfeil, 1988). Because our study used an 8-week training period, it seems unlikely that these mice had not yet reached a training equilibrium with respect to blood volume changes (Sawka et al., 2000). It is also important to note that the training effects were considerably greater in the HR lines than in the Control lines (see Table 3 and Fig. 3).

Intuitively, one might expect that voluntary exercise would lead to changes that are conducive to such exercise, e.g., cardiac hypertrophy or elevated blood hemoglobin levels. This can be thought of as a particular case of the beneficial acclimation hypothesis, one in which the organism has a major effect on its own "environment." Indeed, most exercise physiologists assume that changes induced by training will be in the "right" direction. However, a priori, it is not clear that this should be the case. All else being equal, changes in gene expression induced by an environmental change (including access to a running wheel) should have a 50:50 chance of being beneficial or deleterious. But organisms are products of long evolutionary histories, and it is reasonable to presume that past selection may have favored plastic responses that are generally in a beneficial direction. On the other hand, if the HR lines were constitutively higher in, say, hemoglobin levels, then they might actually exhibit a reduced ability to increase those levels in response to aerobic exercise training.

But it is also possible that too much exercise could be deleterious, as has been documented in some vertebrate training studies (e.g., Garland et al., 1987; Tyler et al., 1996; Budget, 1998). Although the HR lines run much more on wheels than do typical laboratory mice, and more than at least one population of wild house mice, they still run less than has been observed for some species of wild rodents (see Dewsbury, 1980; Garland, 2003). Hence, their high activity levels should not be

considered "abnormal." Nevertheless, we have reported previously that HR lines housed with access to running wheels may exhibit learning impairment (Rhodes et al., 2003). And, in the present study, HR females with wheel access exhibited size-corrected spleens and ovaries that were smaller than for any of the other three experimental groups. Although we have no data to test the proposition, it could be that these reductions are detrimental. A smaller spleen might indicate reduced immune function or erythrocyte storage capacity. A smaller ovary could reflect low ovarian stroma mass, reduced number or size of developing follicles, or reduced number or size of corpora lutea, any of which might be associated with reduced fertility. In any case, we clearly found that organs responded differently to chronic wheel access, and that the responses differed between the HR and Control lines.

The design of our experiment highlights the fact that phenotypes are the product of both their genotype and environment. Interestingly, the direction of "genetic" and "phenotypic" adaptation is in the same direction for the traits that exhibited both significant linetype and wheel-access effects. Thus, wheel running itself affects hemoglobin content in a way that should facilitate the ability to run. Although selection, by itself, did not lead to large number of correlated responses in "untrained" animals, mice from the High-Runner lines, when given access to running wheels, often experienced larger physiological responses to training. Thus, selection, by means of increased wheel-running behavior (which serves as an intermediate phenotype), interacted with the environment (access to running wheels) in a synergistic way that enhanced the realized expression of traits that influence running capacity. We have noted similar patterns with regard to hindlimb metabolic profile, measured by enzyme activities, in these same individual mice (Houle-Leroy et al., 2000). Mice in the HR lines tended toward higher muscle aerobic capacities, but did not reach levels of statistical significance when comparing animals without access to running wheels. However, HR mice with access to running wheels experienced a greater training

response, highlighting the potential for "nurture" to be self-reinforcing of "nature" in this study system.

We speculate that the phenomenon of a behavior influencing physiological (or morphological) traits that in turn enhance the ability to perform the behavior may be a common feature of complex, behavioral-physiological phenotypes, and one that has not been sufficiently appreciated in the evolutionary literature. For example, in a review of the relationships between behavior and evolution, Wcislo (1989) lists as two of five principle hypotheses the ideas that behavioral flexibility enables animals to compensate for their structural or physiological characteristics and that many behavioral traits develop later in ontogeny as compared with structural ones, such that environmental perturbations of structure can influence behavior. These ideas are related to but different from what we are suggesting, i.e., that some behaviors influence structure or physiology in ways that are in turn beneficial to expression of that very behavior (see also Houle-Leroy et al., 2000).

As noted above, on first principles, it should be equally probably that expression of a particular behavior would cause physiological changes that are *detrimental* to further expression of the behavior. For example, exhibiting high locomotor activity might possibly lead to muscle damage or atrophy, which would impede further activity. However, we hypothesize that selection on complex behavioral-physiological phenotypes should generally favor phenotypic plasticity in the "right" direction, i.e., physiological changes that will facilitate further expression of the behavior.

We are not aware of any review that has attempted to document its extent, but we speculate that the phenomenon of "self-induced adaptive plasticity" may be widespread. For example, in lines of rats selected for high and low treadmill running endurance capacity (see Koch and Britton, 2005), "training" responses with regard to body composition and organ masses paralleled responses found in our lines of mice. Rats from the high-endurance line ran significantly more revolutions per day on

activity wheels than did rats from the low-endurance line. Furthermore, 8 weeks of access to running wheels caused decreases in body mass and body fat, but increases in relative heart, liver, stomach, small intestine, and large intestine mass compared to sedentary animals in both lines (Swallow et al., unpublished).

Additional examples of self-induced adaptive plasticity may include a wide range of complex behavioral-physiological phenotypes. For example, animals that feed on particular foods may experience shifts in digestive enzymes that facilitate their ability to eat those foods (McWilliams and Karasov, 2001). Birds that engage in altitudinal migration might make "trial runs" that would induce cardiovascular changes that would improve their ability to function at high altitude (see also Rezende et al., 2005). In rats, maternal behavior is hormone-dependent in first-time mothers, but is less so in experienced mothers (Numan and Insel, 2003). Similarly, male-male agonistic interactions in vertebrates may result in the winners experiencing elevated testosterone levels, which could facilitate their subsequent performance in such interactions.

One interesting aspect of the genetic response to selection for increased wheel-running activity has been the increase in frequency of the mini-muscle allele, an apparent Mendelian recessive that causes an approximate 50% reduction in hindlimb muscle mass (Garland et al., 2002). The mini-muscle phenotype has been (unintentionally) favored by our selection protocol, and it exhibits several pleiotropic effects. The mini-muscle allele displays significant pleiotropic effects, for at least one sex, on many organs measured in this study. For example, body mass is reduced by approximately 13%, while heart, kidney, and liver mass are increased in the affected individuals. Reduced body mass (and limb mass) may contribute to lowering the energetic cost of locomotion.

Behavioral traits are inherently complex in that they involve the interaction of traits at many

lower-levels of organization (see also Overli et al., 2005). In the present study, selection for high wheel-running activity, a complex behavioral-physiological trait, has resulted in a variety of changes in lower-level physiological traits, including aspects of body composition. Some of these differences are present in "untrained" mice from the High-Runner lines and hence may be considered constitutive. Others occur only after mice have had the opportunity to self-train on running wheels for several weeks, which is an example of genotype-by-environment interaction. The present study also offers support for the beneficial acclimation hypothesis in that the observed training effects on relative heart size, Hct, and blood Hb concentration should improve the ability for sustained, aerobic locomotor activity. It also suggests that selection for high activity may have enhanced the phenotypic plasticity of some supportive traits in the HR lines points (in particular, Hct and Hb).

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Table 1. *Least-squares means and significance levels (P values; **bold** indicates $P < 0.05$, unadjusted for multiple comparisons) for wheel running during the final week of access to wheels.*

Trait	Control		High-Runner		HR/C		HR vs. C P value		Mini-muscle P value	
	M	F	M	F	M	F	M	F	M	F
Revolutions/Day	3,975	5,392	8,427	13,341	2.12	2.47	0.0196	0.0005	0.4857	0.2376
1-min Intervals/Day	309	391	406	493	1.31	1.26	0.0452	0.0535	0.9518	0.3988
Average RPM	13.0	14.4	20.9	27.7	1.61	1.92	0.0154	0.0002	0.3111	0.1883

Nested analysis of variance (no covariates) models were implemented in SAS PROC MIXED. Adjusted means for revolutions and for RPM are backtransformed from analyses of traits raised to the 0.2 power. N = 40 males and 40 females.

Table 2. Significance levels (*P* values; **bold** indicates $P < 0.05$, unadjusted for multiple comparisons) from nested analysis of covariance models implemented in SAS PROC MIXED.

Trait and transform	Sex	N	Selection	Wheel Access	Interaction	Mini-muscle	log Body Mass	Age	Time of Day	Time ²
d.f.			1,6	1,6	1,6	1,~26	1,~26	1,~26	1,~26	1,~26
log Body Mass	Male	79	0.0032 -	0.0108 -	0.4665	0.0371 -		0.6918	0.6056	0.8897
	Fem.	81	0.0313 -	0.0650 -	0.1333	0.0390 -		0.3565	0.8692	0.1507
log Triceps Surae	Male	78	0.3331 +	0.1269 +	0.7881	0.0001 -	0.0001	0.6069	0.0089	0.6018
	Fem.	80	0.9783 +	0.0829 +	0.9832	0.0001 -	0.0001	0.3040	0.1843	0.0676
log Ventricle	Male	79	0.0606 +	0.0372 +	0.4508	0.0080 +	0.0001	0.0260	0.0667	0.6166
	Fem.	81	0.2981 +	0.0430 +	0.4539	0.0303 +	0.0001	0.2681	0.0894	0.9514
log Spleen	Male	79	0.8334 +	0.1915 -	0.8329	0.0539+	0.0001	0.1364	0.8660	0.1660
	Fem.	81	0.3387 -	0.0301 -	0.0304	0.0608+	0.0001	0.2627	0.3301	0.3369
log Liver	Male	79	0.2287 +	0.6533 +	0.7484	0.0833+	0.0001	0.5033	0.0002	0.9623
	Fem.	81	0.5879 -	0.3263 +	0.0383	0.0001 +	0.0001	0.0251	0.9625	0.9896
log Mean Kidney	Male	79	0.0049 +	0.9959 +	0.5147	0.0037 +	0.0001	0.0038	0.5191	0.0311
	Fem.	80	0.0458	0.2236	0.7420	0.0810+	0.0001	0.2212	0.2043	0.6234

Table 2. ... continued ...

Trait and transform	Sex	N	Selection	Wheel Access	Interaction	Mini-muscle	log Body Mass	Age	Time of Day	Time ²
d.f.			1,6	1,6	1,6	1,~26	1,~26	1,~26	1,~26	1,~26
log Mean Testes	Male	78	0.2820 +	0.4757 +	0.6088	0.6518-	0.0660	0.5311	0.4318	0.6659
Ovary	Fem.	81	0.9088 -	0.4189 -	0.0360	0.2066+	0.0127	0.1268	0.9974	0.3069
log Tail	Male	79	0.4302 +	0.7088 -	0.8884	0.0238+	0.0004	0.5856	0.8621	0.9289
	Fem.	80	0.9362 +	0.6361 +	0.6667	0.0042+	0.0001	0.8067	0.7653	0.5553
Hematocrit	Male	79	0.4402 +	0.0352 +	0.3450	0.4004-	0.3903	0.2014	0.1300	0.0092
	Fem.	81	0.1756 +	0.0211 +	0.0465	0.0044-	0.0890	0.6350	0.9829	0.0848
no mass	Male	79	0.2434 +	0.0164 +	0.3124	0.5067-		0.1893	0.1232	0.0091
no mass	Fem.	81	0.0272 +	0.0139 +	0.0323	0.0123-		0.5101	0.9111	0.0695
Hemoglobin	Male	79	0.3225 +	0.0161 +	0.2374	0.7985-	0.1449	0.0633	0.8378	0.0015
	Fem.	81	0.0520 +	0.0078 +	0.0146	0.0892-	0.3439	0.1803	0.2624	0.0184
no mass	Male	79	0.0967 +	0.0065 +	0.2122	0.9417+		0.0583	0.8243	0.0019
no mass	Fem.	81	0.0105 +	0.0060 +	0.0119	0.1469-		0.1554	0.2412	0.0159

"Selection" refers to effect of linetype (High-Runner vs. Control lines), "Wheel Access" refers to whether mice were housed with access to wheels that were either free to rotate (Active group) or locked (Sedentary group), "Interaction" refers to statistical interaction between Selection and Wheel Access factors, "Mini-muscle" refers to effect of having the mini-muscle phenotype (see text). The four remaining columns indicate significance levels for covariates.

The sign following each P value indicates the direction of the effect (as can also be seen from the adjusted mean presented in Table 3). For Selection, + indicates that HR lines were higher than Control lines; for Wheel Access, + indicates that Active group were higher than Sedentary group; for Mini-muscle, + indicates that individuals with the small-muscle phenotype (Garland et al., 2002) had higher values. When log body mass had a significant effect ($P < 0.05$), it was always positive, as would be expected for organ sizes. An empty body mass column indicates it was not used as a covariate for that analysis.

Table 3. *Least-squares (adjusted) means and standard errors from SAS PROC MIXED, corresponding to tests presented in Table 2.*

Trait	Se	Control Lines				High-Runner Lines				Mini-muscle			
		Sedentary		Active		Sedentary		Active		Normal		Mini	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
log Body Mass	M	1.539	0.0120	1.521	0.0119	1.489	0.0098	1.461	0.0105	1.520	0.0055	1.485	0.0154
(g)	F	1.433	0.0160	1.430	0.0161	1.392	0.0150	1.367	0.0146	1.424	0.0091	1.387	0.0182
log Triceps Surae	M	2.074	0.0130	2.085	0.0128	2.092	0.0116	2.100	0.0128	2.217	0.0073	1.958	0.0146
(mg)	F	1.975	0.0153	1.986	0.0155	1.976	0.0150	1.987	0.0153	2.109	0.0096	1.853	0.0155
log Ventricle	M	2.121	0.0126	2.133	0.0122	2.150	0.0108	2.171	0.0125	2.121	0.0064	2.166	0.0153
(mg)	F	2.043	0.0138	2.060	0.0139	2.051	0.0130	2.084	0.0137	2.039	0.0061	2.080	0.0173
log Spleen	M	1.956	0.0243	1.930	0.0233	1.958	0.0204	1.939	0.0246	1.913	0.0105	1.978	0.0307
(mg)	F	1.953	0.0237	1.952	0.0240	1.974	0.0217	1.883	0.0233	1.908	0.0095	1.974	0.0321
log Liver	M	2.955	0.0116	2.956	0.0110	2.969	0.0097	2.975	0.0119	2.950	0.0046	2.978	0.0147
(mg)	F	2.854	0.0098	2.880	0.0099	2.867	0.0091	2.856	0.0097	2.831	0.0038	2.897	0.0132
log Mean Kidney	M	2.395	0.0137	2.389	0.0132	2.450	0.0117	2.456	0.0137	2.394	0.0060	2.450	0.0168
(mg)	F	2.218	0.0139	2.234	0.0140	2.259	0.0132	2.268	0.0138	2.230	0.0069	2.260	0.0163
log Mean Adrenal	M	0.317	0.0323	0.320	0.0305	0.260	0.0261	0.265	0.0321	0.273	0.0131	0.308	0.0381
(mg)	F	0.589	0.0314	0.596	0.0315	0.558	0.0293	0.582	0.0306	0.544	0.0166	0.620	0.0370
no mass	M	0.311	0.0315	0.320	0.0306	0.264	0.0253	0.274	0.0284	0.272	0.0133	0.312	0.0374
no mass	F	0.602	0.0304	0.607	0.0307	0.541	0.0275	0.546	0.0266	0.545	0.0152	0.603	0.0373
log Left Adrenal	M	0.337	0.0272	0.349	0.0259	0.291	0.0224	0.310	0.0271	0.312	0.0119	0.331	0.0347
(mg)	F	0.615	0.0316	0.614	0.0318	0.582	0.0291	0.618	0.0308	0.580	0.0158	0.634	0.0403
no mass	M	0.328	0.0276	0.346	0.0268	0.297	0.0222	0.324	0.0238	0.311	0.0127	0.336	0.0348
no mass	F	0.628	0.0317	0.624	0.0321	0.564	0.0285	0.581	0.0274	0.582	0.0154	0.617	0.0406

log Mean Testes	M	2.003	0.0235	2.015	0.0230	2.041	0.0209	2.044	0.0231	2.032	0.0130	2.020	0.0273
Ovary (mg)	F	0.745	0.0466	0.786	0.0469	0.799	0.0437	0.718	0.0459	0.724	0.0240	0.800	0.0587
log Tail	M	1.990	0.0063	1.988	0.0062	1.995	0.0056	1.994	0.0063	1.983	0.0031	2.000	0.0073
(mm)	F	1.975	0.0058	1.975	0.0058	1.979	0.0054	1.980	0.0057	1.968	0.0026	1.987	0.0074
Hematocrit	M	49.06	0.750	49.74	0.728	49.39	0.646	50.80	0.745	50.14	0.377	49.35	0.909
(%)	F	48.51	0.695	48.76	0.701	48.66	0.653	50.90	0.689	50.63	0.334	47.78	0.900
no mass	M	48.94	0.732	49.72	0.723	49.54	0.618	51.10	0.656	50.12	0.372	49.52	0.882
no mass	F	48.44	0.642	48.70	0.650	49.01	0.580	51.51	0.556	50.58	0.284	48.25	0.840
Hemoglobin	M	16.88	0.236	17.15	0.229	17.01	0.203	17.59	0.235	17.20	0.117	17.12	0.284
(g/100 ml)	F	16.80	0.204	16.87	0.206	16.85	0.190	17.81	0.202	17.33	0.088	16.83	0.273
no mass	M	16.81	0.224	17.14	0.221	17.08	0.189	17.75	0.200	17.19	0.109	17.21	0.272
no mass	F	16.79	0.191	16.87	0.193	16.91	0.172	17.92	0.164	17.32	0.077	16.93	0.254

Table 4. Significance levels (*P* values; **bold** indicates $P < 0.05$, unadjusted for multiple comparisons) from nested analysis of covariance models implemented in SAS PROC MIXED for wheel-access (Active) mice only.

Trait and transform	Sex	Selection	Revolutions in Final Week	Mini-muscle	log Body Mass	Age	Time of Day	Time ²
d.f.		1,6	1,~26	1,~26	1,~26	1,~26	1,~26	1,~26
Hematocrit	Male	0.6382 +	0.0152 +	0.3046-	0.8701	0.3029	0.2037	0.0308
	Fem.	0.1559 +	0.6449 +	0.0044-	0.3292	0.5047	0.2762	0.5009
no mass	Male	0.6476 +	0.0067 +	0.2598-		0.2787	0.1863	0.0285
no mass	Fem.	0.0653 +	0.5588 +	0.0065-		0.4806	0.3163	0.5104
Hemoglobin	Male	0.4753 +	0.0058 +	0.2942-	0.8087	0.1481	0.9007	0.0130
	Fem.	0.1375 +	0.5450 +	0.0154-	0.2898	0.3491	0.2955	0.2821
no mass	Male	0.4003 +	0.0010 +	0.2975-		0.1475	0.9153	0.0121
no mass	Fem.	0.0613 +	0.4154 +	0.0255-		0.3363	0.3716	0.2478

The sign following each P value indicates the direction of the effect. For Selection, + indicates that HR lines were higher than Control lines; for Running in Final Week, + indicates a positive effect of amount of running; for Mini-muscle, + indicates that individuals with the small-muscle phenotype (Garland et al., 2002) had higher values. An empty body mass column indicates it was not used as a covariate for that analysis. N = 40 males and 40 females.

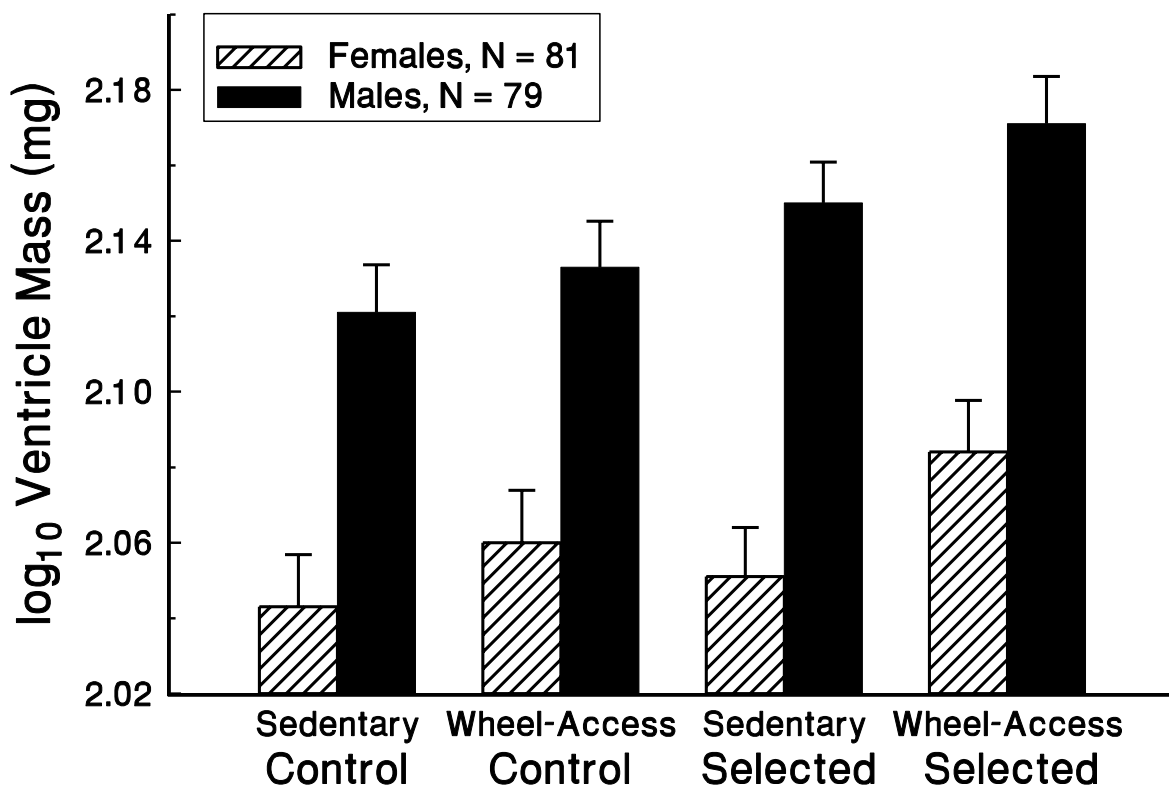
Figure Legends

Fig. 1. Bar graph illustrating group differences for mass-adjusted \log_{10} ventricle mass (least-squares means and standard errors from Table 3, corresponding to statistical tests shown in Table 2: covariates included log body mass, age, time of day of sacrifice, and (Z-transformed time of day)²). Wheel access had a significant positive effect in both sexes (see Table 2 for P values). However, linetypes did not differ significantly for either sex (although for males the 2-tailed P value was 0.0606), and interactions between the main effects were not significant. Mice with the mini-muscle phenotype had significantly larger ventricles in both sexes (Tables 2 and 3).

Fig. 2. Bar graph illustrating group differences for mass-adjusted \log_{10} kidney mass (see Fig. 1 legend for further details). In both sexes, mice from the High-Runner lines had significantly larger kidneys. Mice with the mini-muscle phenotype also had larger kidneys, although the difference was not statistically significant in females (Tables 2 and 3).

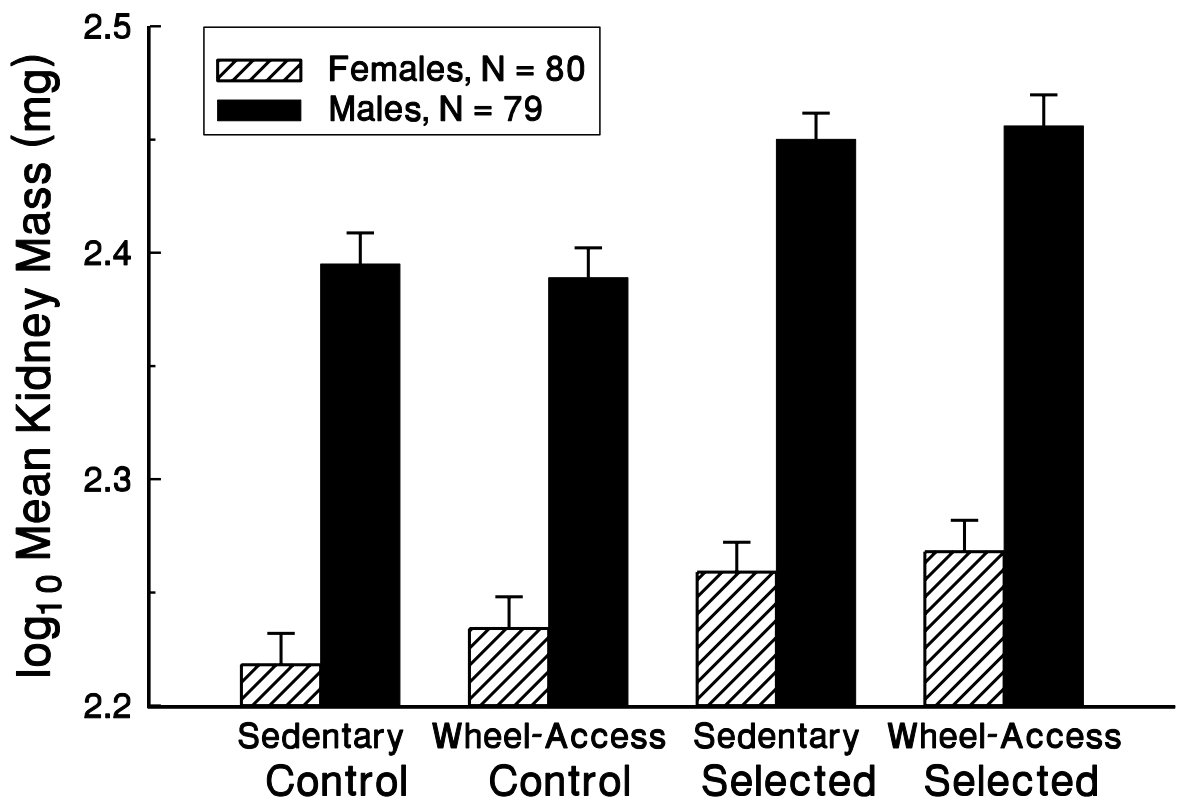
Fig. 3. Bar graph illustrating group differences for blood [hemoglobin] (not mass-adjusted; least-squares means and standard errors from Table 3; statistical tests presented in Table 2). In both sexes, mice from the High-Runner lines had more Hb than Control lines when housed with wheel access (see text).

Fig. 4. Relation between blood [Hb] and amount of wheel running during final week for female mice. As shown in Table 4, ANCOVA suggests that the difference between High-Runner and Control lines is not simply a function of higher running by the former.



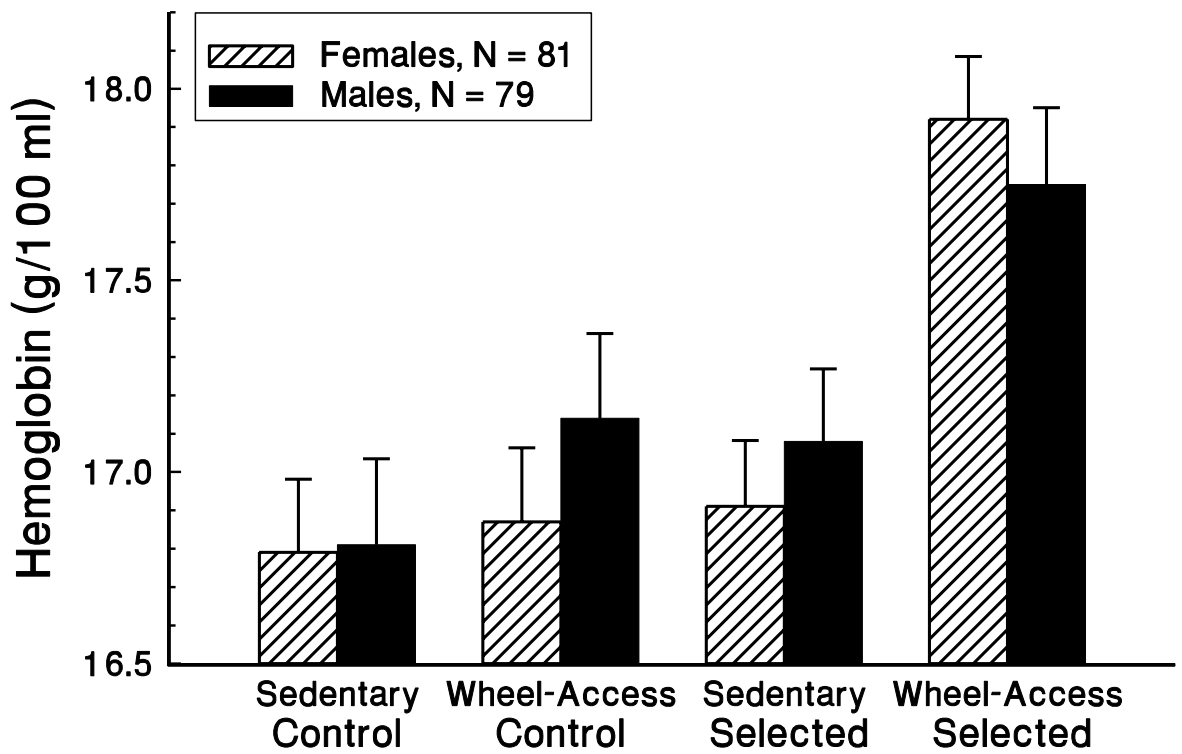
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Fig. 1



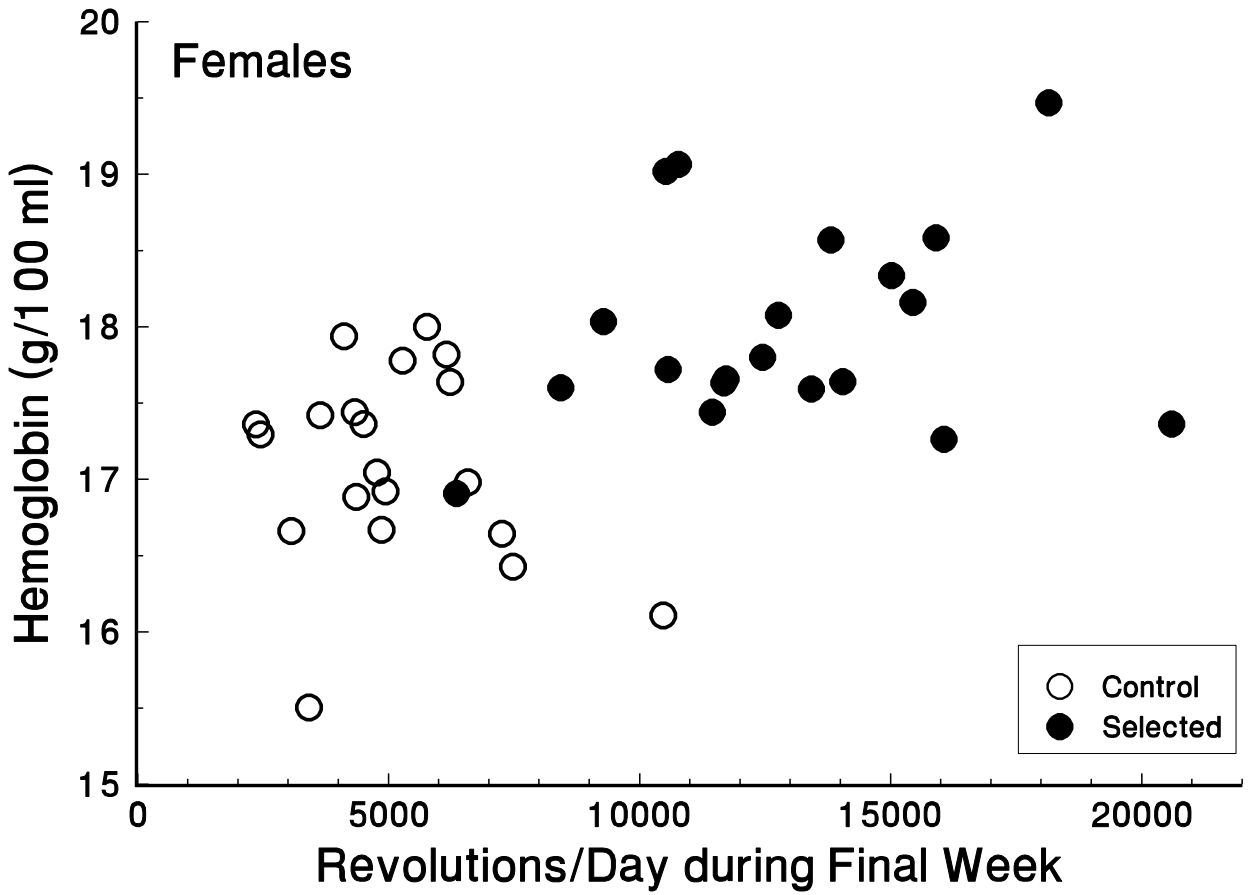
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Fig. 2



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Fig. 3



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Fig. 4