© 2017. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2017) 220, 1038-1047 doi:10.1242/jeb.148759

RESEARCH ARTICLE

# Effects of activity, genetic selection and their interaction on muscle metabolic capacities and organ masses in mice

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## ABSTRACT

Chronic voluntary exercise elevates total daily energy expenditure and food consumption, potentially resulting in organ compensation supporting nutrient extraction/utilization. Additionally, species with naturally higher daily energy expenditure often have larger processing organs, which may represent genetic differences and/or phenotypic plasticity. We tested for possible adaptive changes in organ masses of four replicate lines of house mice selected (37 generations) for high running (HR) compared with four non-selected control (C) lines. Females were housed with or without wheel access for 13–14 weeks beginning at 53–60 days of age. In addition to organ compensation, chronic activity may also require an elevated aerobic capacity. Therefore, we also measured hematocrit and both citrate synthase activity and myoglobin concentration in heart and gastrocnemius. Both selection (HR versus C) and activity (wheels versus no wheels) significantly affected morphological and biochemical traits. For example, with body mass as a covariate, mice from HR lines had significantly higher hematocrit and larger ventricles, with more myoglobin. Wheel access lengthened the small intestine, increased relative ventricle and kidney size, and increased skeletal muscle citrate synthase activity and myoglobin concentration. As compared with C lines, HR mice had greater training effects for ventricle mass, hematocrit, large intestine length and gastrocnemius citrate synthase activity. For ventricle and gastrocnemius citrate synthase activity, the greater training was quantitatively explainable as a result of greater wheel running (i.e. 'more pain, more gain'). For hematocrit and large intestine length, differences were not related to amount of wheel running and instead indicate inherently greater adaptive plasticity in HR lines.

**KEY WORDS: Adaptive plasticity, Artificial selection, Exercise, Muscle metabolic capacities, Phenotypic plasticity, Organ masses**

## INTRODUCTION

A multitude of studies have documented 'training responses' (phenotypic plasticity) resulting from chronic wheel access or forced treadmill exercise in both mice and rats, including: cardiac and skeletal muscle hypertrophy (e.g. Swallow et al., 2005); decreases in body mass and body fat (e.g. Dumke et al., 2001); increases in maximal $O_2$ consumption (e.g. Swallow et al., 1998b); and metabolic alteration of cardiac and skeletal muscles (e.g. Saltin and Golnick, 1983; Harrison et al., 2002). However, not all traits change in response to chronic exercise (for bone architecture examples, see Kelly et al., 2006; for organ mass examples, see Swallow et al., 2005).

Genetic factors are also known to mediate the proximate effects of locomotion on physiological systems. For example, phenotypic traits (organ masses and muscle metabolic capacities in the present study) have a genetic basis that may support innate or intrinsic exercise abilities. In addition, the propensity to engage in exercise has a genetic basis (Kelly et al., 2010; Kelly and Pomp, 2013), and this variation in activity may or may not facilitate a training response in the given phenotype (which also has a genetic basis). Therefore, there is the potential for a complex interaction between the genetic or intrinsic 'value' of a phenotypic trait and the exercise-induced changes (Swallow et al., 2010). One or both of these values of the phenotype may be essential to further support locomotion above baseline levels (see fig. 2 in Middleton et al., 2008a). For example, in humans, habitual activity levels (Perusse et al., 1989), health-related physical fitness traits (Bouchard and Perusse, 1994) and aerobic performance (Bouchard et al., 2000) are all affected by genetic components, but the genetic link, or correlation, between these traits was not examined in these studies. Here, we examine the effects of physical activity (voluntary exercise on wheels), genetic selection and their interaction on muscle metabolic capacities and organ masses in lines of mice selectively bred for high levels of voluntary wheel running. We examined organs and biochemical properties that are either directly or indirectly related to nutrient processing and $O_2$ transport.

Beginning in 1993, selective breeding was utilized to create four replicate lines of house mice (*Mus domesticus*; Hsd:ICR strain) that exhibited high voluntary wheel running (HR lines), while also maintaining four non-selected lines to serve as controls (C lines; Garland et al., 2011b; Swallow et al., 1998a). Mice in the HR lines diverged rapidly from controls and reached apparent selection limits 2.5- to 3-fold above those of controls at generations 16–28, depending on sex and line (Careau et al., 2013). HR mice primarily run faster rather than for more minutes per day, although in males some increase in amount of time spent running is also observed (Swallow et al., 1998a; Garland et al., 2011a). In several experiments, mice from both the HR and C lines have been housed with or without wheel access for several days, weeks (as in the present study) or months to test for differences in plasticity (training effects) in a variety of morphological (e.g. Middleton et al., 2008b) and physiological (Kelly et al., 2014) traits. For example, Swallow et al. (2001) found that HR mice with wheel access consumed 24.5% more food than their sedentary counterparts, and C mice with wheel access consumed 19.5% more food than sedentary individuals. Among the group with wheel access (HR and C lines), genetic selection led to HR lines consuming 9–13% more

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**Received 24 August 2016; Accepted 26 December 2016**



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food per day, but the elevated food consumption could be statistically explained by the higher wheel running of HR lines (Swallow et al., 2001). Conversely, when both groups were housed with locked wheels (unable to rotate), food consumption differed by 8.4%, with HR lines consuming more food per day (Swallow et al., 2001). Koteja et al. (1999) demonstrated that when housed with access to wheels, HR lines had slightly higher (2–5% increase in females) daily energy expenditures than their C line counterparts (see table 2 in Koteja et al., 1999), although that study was conducted before the HR lines had reached selection limits and so likely underestimates the differences that would exist for mice used in the present study (Rezende et al., 2009). Therefore, the energetic demands of high levels of locomotion coupled with increases in energy assimilation rate may be facilitated partially by adjustments in the physiological systems responsible for nutrient extraction (i.e. the gastrointestinal system) and utilization.

Genetic factors and training effects of phenotypic traits that may facilitate voluntary physical activity have been studied extensively. However, rarely have these effects been studied in concert on the same set of animals. An exception is work in rats selected for low and high intrinsic treadmill endurance exercise capacity (e.g. Koch et al., 2012). Here, we utilize a mouse model to simultaneously address the genetically mediated, exercise-related effects on organ morphology and muscle metabolic capacities, using mice that had been selectively bred for high levels of voluntary wheel running. We hypothesized that the energetic demands associated with high levels of voluntary exercise would result in alterations of organs and biochemical processes relevant in nutrient processing and $O_2$ transport. Specifically, we hypothesized that both selection history (HR versus C) and exercise training would alter relative organ sizes and muscle metabolic capacities, and that genotype-by-environment interactions might be indicative of the relative importance of particular traits.

## MATERIALS AND METHODS

### Study animals and experimental protocol

All procedures in this study were approved by and are in accordance with guidelines set forth by the Institutional Animal Care and Use Committee at the University of California, Riverside. Mice were sampled from the 37th generation of selective breeding for high voluntary wheel running. A full description of the selection experiment is detailed elsewhere (Swallow et al., 1998a). Animals utilized in the present study were also the focus of a previous study on ventilatory responses (Kelly et al., 2014). Full details of the methods can be found in Kelly et al. (2014) and only pertinent features are presented here. At 74–81 days of age (mean=79 days), 12 female mice from each of the eight lines were weighed and housed individually for 13–14 weeks in standard cages, half of which were attached to a running wheel (1.12 m circumference, Wahman-type; Lafayette Instruments, Lafayette, IN, USA). Cages with and without wheels were placed alternately on racks (Fig. S1). Four groups were compared: mice from C lines housed without wheels (sedentary, $N$=24); mice from C lines housed with wheels (active, $N$=24); mice from HR lines housed without wheels (sedentary, $N$=24); and mice from HR lines housed with wheels (active, $N$=24). Female mice were chosen as they run greater distances at higher speeds (Swallow et al., 1998a), and thus any phenotypic responses to exercise may be more pronounced than in male mice. As was done in a large majority of previous studies using this mouse model (e.g. Swallow et al., 1998a), wheel-running activity was recorded in 1-min intervals daily for ≥23 h. From this information, the following daily traits were calculated: total daily revolutions, time spent running (i.e. cumulative 1-min intervals in which at least one revolution was recorded), average speed (total revolutions/time spent running) and maximum speed (highest number of revolutions in any 1-min interval within a 24 h period). Water and food [Harlan Teklad Laboratory Rodent diet (W) 8604] were provided *ad libitum*, photoperiod was maintained at constant 12 h:12 h light:dark (lights on at 0700 h) and room temperature was controlled (~22°C).

After 13–14 weeks of wheel access (or not for the sedentary group), mice were euthanized ($CO_2$ inhalation) in batches to allow for harvesting of organs. The 'active' group had wheel access up to and including the day of euthanasia. Mean (±s.e.m.) age at euthanization was 175.6±1.76 days (range=171–179 days). Prior to euthanasia, mice were weighed, anesthetized by methoxy-flurane, and four blood samples (75 µl each) were collected from the peri-orbital sinus using heparinized micro-hematocrit tubes (Hoff, 2000). Tubes were centrifuged for 5 min in a Clay–Adams microfuge (Autocrit Ultra 3) to determine hematocrit (Hct) or the percent volume of red blood cells. After blood sampling, mice were euthanized and body length was measured to the nearest millimeter from the rostrum to the anus. Following a mid-ventral incision, the peritoneal fat pad was removed and weighed. The heart was then lifted with forceps and the ventricles were cut free from the atria and connecting blood vessels. Ventricles were blotted and any coagulated blood was removed before weighing. The right and left soleus, plantaris and gastrocnemius were removed by separating the complex from the tibia–fibula, cutting the Achilles' tendon midway between its origin and the muscles' insertion, separating the muscles with forceps, and cutting the individual muscles from the lateral condyle of the tibia and medial condyle of the fibula. The spleen, liver, lungs and kidneys were removed and weighed. The gastro-intestinal tract (stomach, small intestine, cecum, large intestine) was removed, separated, flushed with saline and weighed. Length measurements (to the nearest cm) were also obtained for the small and large intestine. The lungs, stomach, small intestine, cecum and large intestine were dried at 60°C for 7 days and reweighed. All organs were weighed to the nearest 0.0001 g and stored at −80°C for further analysis (with the exception of the dried organs).

Utilizing spectrophotometry (Spectra*Max* Plus; Molecular Devices, Sunnyvale, CA, USA), citrate synthase (CS) activity and myoglobin concentration were measured in both the gastrocnemius and ventricle. CS activity was estimated by measuring the rates of transfer of sulfhydryl groups to 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm (described previously by Rezende et al., 2006a). Total dilution of gastrocnemius and heart tissues for CS assays was 1:20,000 (w/v). CS was measured in duplicate for each tissue type. Following Reynafarje (1963), myoglobin concentration was calculated from the difference between absorbance obtained at 538 and 568 nm. Myoglobin, an oxygen-binding protein found in muscle, concentration was measured in quadruplicate for each individual and for each tissue type. Exceptions included individuals with the mini-muscle phenotype, where some measurements were performed in triplicate because of limited gastrocnemius tissue. The mini-muscle phenotype is a Mendelian recessive that halves hind limb muscle mass and exhibits numerous pleiotropic effects, including effects on organ masses and muscle enzyme activities (Garland et al., 2002; Kelly et al., 2013). For this study, individuals with the mini-muscle phenotype were identified by examining the mass of the triceps surae muscle in relation to body mass (e.g. see fig. 2 in Garland et al., 2002).

### Statistical analysis

The Mixed procedure in SAS (SAS Institute, Cary, NC, USA) was used to apply nested analysis of covariance (ANCOVA) models

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with restricted maximum likelihood (REML) estimation and Type 3 tests of fixed effects (e.g. see Kelly et al., 2014). A cross-nested, two-way ANCOVA was used to simultaneously test the effects of line type (HR versus C lines) and activity (wheels versus no wheels). Replicate line ($N$=8 total), nested within line type, was always considered a random effect, and the significance of line type was tested relative to line with 1 and 6 d.f. The two main grouping factors, line type and activity, were considered fixed effects. Effects of activity and the activity-by-line type interaction were tested relative to the activity-by-line interaction (d.f.=1, 6). Presence/absence of the mini-muscle phenotype (see Garland et al., 2002; Kelly et al., 2013) was used as an additional factor in the model and was tested relative to the error term with 1 and ~70 d.f. (depending on any missing values for particular traits). Body mass ($\log_{10}$ transformed), age, time of euthanasia and ($z$-transformed time of euthanasia)$^2$ were included as covariates, also tested with 1 and ~70 d.f. The squared term for time of euthanasia was included in the analysis as it allows for possible non-linear relationships between time of day and the dependent variable of interest. When analyzing body mass, multiple models were employed utilizing models including body length and age as covariates.

Given that we have performed multiple statistical tests on the same set of individuals, it is appropriate to adjust individual $P$-values (significance levels) in order to control the 'experiment-wise' Type I error rate. The total number of hypotheses resulting in $P$-values is 306 ($P$-values from Tables 1–3). See Fig. S2 for a distribution of $P$-values in relation to the $\pi_0$ statistic (the overall proportion of true null hypotheses; Storey and Tibshirani, 2003; Storey, 2003). To control for the multiple comparisons, we employed the positive false discovery rate (pFDR) procedure developed by Storey (2002) utilizing the *qvalue* software package ('bootstrap' option) in R. We determined a $P$-value cut-off that would yield a global, experiment-wise FDR of 5%. Results of the pFDR analysis of the 306 $P$-values indicated that those of 0.0044 or smaller could be considered significant, as opposed to the typical alpha level of 0.05. In the subsequent text we discuss unadjusted two-tailed $P$-values (unless specifically noted for traits for which we had *a priori* predictions about the direction of main effect or

**Table 1. Tests of high runner versus control lines, effects of activity (wheel access or not), genotype-by-environment interactions, and effects of the mini-muscle phenotype on organ masses and muscle metabolic capacities**

| Trait | $N$ | $P_{selection}$ | $P_{activity}$ | $P_{interaction}$ | $P_{mini}$ | $P_{mass}$ | $P_{age}$ | $P_{time}$ | $P_{time^2}$ |
|---|---|---|---|---|---|---|---|---|---|
| log Body mass | 94 | **0.0270**– | **0.0495**– | 0.2571 | 0.7641+ | | 0.9463– | **0.0039**– | 0.5670– |
| log Body mass[a] | 94 | 0.0825– | 0.0694– | 0.7754 | 0.4787– | **<0.0001+** | 0.3107– | **0.0005**– | 0.1121– |
| log Snout–rump | 94 | **0.0368**– | 0.2479– | **0.0772** | 0.1030+ | | 0.2662+ | 0.9642– | 0.3054+ |
| BMI (kg m$^{-2}$) | 92 | **0.0458**– | 0.0535– | 0.5258 | 0.4891– | | 0.6997– | **0.0003**– | 0.2002– |
| log Ventricle | 94 | **0.0227+** | **0.0028+** | **0.0648** | **0.0242+** | **<0.0001+** | 0.9859– | **0.0483**– | 0.9117+ |
| Hematocrit | 91 | **0.0415+** | 0.1158+ | 0.2003 | 0.9010– | 0.3658– | **0.0156**– | 0.7640+ | 0.1165– |
| Hematocrit no mass | 91 | **0.0125+** | 0.0726+ | 0.2342 | 0.8449– | | **0.0181**– | 0.6044+ | 0.1247– |
| log Soleus | 93 | 0.7809+ | 0.9191– | 0.5537 | **<0.0001+** | **<0.0001+** | 0.6031– | 0.1167– | 0.2600– |
| log Plantaris | 94 | 0.3979– | 0.4109+ | 0.9524 | **0.0236**– | **<0.0001+** | 0.9360– | 0.3614– | 0.6573– |
| log Gastrocnemius | 94 | 0.3301– | 0.6535+ | 0.9952 | **<0.0001**– | **<0.0001+** | 0.0865– | 0.6410+ | 0.6907– |
| log Peritoneal fat | 94 | 0.4454+ | 0.1271– | 0.9520 | 0.1932+ | **<0.0001+** | **0.0112+** | 0.1030+ | 0.4600+ |
| log Spleen | 90 | 0.2270– | 0.4586– | 0.5417 | **0.0244+** | **<0.0001+** | 0.1916– | 0.6013– | 0.0555– |
| log Liver | 94 | 0.6463– | 0.7208+ | 0.1136 | **0.0131+** | **<0.0001+** | **0.0285**– | **<0.0001**– | 0.5646– |
| log Kidney | 94 | 0.9811+ | **0.0261+** | 0.4711 | **0.0160+** | **<0.0001+** | 0.4599– | 0.2763– | 0.5832+ |
| log Lung wet | 92 | 0.1679+ | 0.1407+ | 0.5834 | **0.0286+** | **<0.0001+** | 0.4348+ | 0.9257+ | 0.9339+ |
| log Lung dry | 92 | 0.0952+ | 0.4085+ | 0.9018 | **0.0414+** | **<0.0001+** | 0.8039+ | 0.7392+ | 0.5346+ |
| log Stomach wet | 93 | 0.2413– | 0.4443– | 0.7924 | 0.0665+ | **<0.0001+** | 0.0868– | **0.0065**– | 0.5802+ |
| log Stomach dry | 93 | 0.2434– | 0.9040+ | 0.7780 | **0.0466+** | **<0.0001+** | **0.0313**– | 0.0623– | 0.1635+ |
| log Small intestine wet | 94 | 0.4536– | 0.1556+ | 0.8088 | 0.3710+ | **<0.0001+** | 0.9893+ | 0.6443– | 0.1194– |
| log Small intestine dry | 92 | 0.7402– | 0.0553+ | 0.7316 | 0.3919+ | **<0.0001+** | **0.0012**– | **0.0149+** | 0.0553– |
| log Small intestine length | 93 | 0.7630+ | **0.0331+** | 0.4189 | 0.0935+ | **0.0003+** | 0.9127+ | 0.3201– | 0.3267+ |
| log Cecum wet | 94 | 0.2521– | 0.2165– | 0.1172 | 0.1314+ | **0.0170+** | 0.3845– | **0.0010**– | 0.8029+ |
| log Cecum dry | 94 | 0.8211+ | 0.7044– | 0.1467 | 0.0919+ | **0.0003+** | 0.4400– | 0.0856– | 0.6602+ |
| log Large intestine wet | 92 | 0.1533+ | 0.0871+ | 0.3566 | 0.3608+ | **<0.0001+** | 0.2136– | 0.0785+ | 0.8496– |
| log Large intestine dry | 92 | 0.1745+ | 0.4531+ | 0.4027 | 0.1851+ | **<0.0001+** | 0.0682– | 0.0722+ | 0.7309– |
| log Large intestine length | 94 | 0.0642+ | 0.6017+ | 0.1666 | 0.6563– | **0.0085+** | **0.0022+** | 0.4435+ | 0.9386+ |
| Ventricle CS | 90 | 0.1089+ | 0.1882+ | 0.7097 | 0.2971– | 0.1055 | **0.0074+** | **0.0038+** | 0.8822– |
| Ventricle CS no mass | 90 | 0.2965+ | 0.3209+ | 0.9595 | 0.4000– | | **0.0078+** | **0.0088+** | 0.8398– |
| Ventricle Mb | 92 | **0.0332+** | 0.9637+ | 0.6705 | 0.0774– | 0.0582+ | **0.0137+** | **0.0167+** | 0.0614+ |
| Ventricle Mb no mass | 92 | 0.0983+ | 0.6488– | 0.8963 | 0.1136– | | **0.0165+** | 0.0550+ | 0.0887+ |
| Gastrocnemius CS | 90 | 0.0657+ | **0.0469+** | 0.1933 | **<0.0001+** | 0.1281+ | 0.6970+ | 0.4657– | 0.5200– |
| Gastrocnemius CS no mass | 90 | 0.1498+ | 0.0625+ | 0.1292 | **<0.0001+** | | 0.7463+ | 0.1965– | 0.4357– |
| Gastrocnemius Mb | 90 | 0.0855+ | **0.0122+** | 0.1470 | **<0.0001+** | 0.4740+ | 0.8721– | 0.2373– | 0.1172– |
| Gastrocnemius Mb no mass | 90 | 0.0975+ | **0.0128+** | 0.1175 | **<0.0001+** | | 0.8501– | 0.1785– | 0.1075– |

Values represent nested ANCOVAs comparing all female mice [HR versus C lines housed with (active) versus without wheel access (sedentary)]. Values are for two-tailed tests (unadjusted for multiple comparisons), and those in bold indicate significant effects ($P$<0.05; with the exception of the interaction, where $P$<0.1 are in bold, see Materials and methods). Selection: HR versus C mice; Activity: mice with versus without wheels; Mini: effect of having the mini-muscle phenotype (see Materials and methods). log Body mass (which was included as cofactor unless noted otherwise by 'no mass'), age, time of day and (time of day)$^2$ represent covariates in the analyses. The $z$-transformed squared term for time of euthanasia (Time$^2$) was included in the analysis as it allows for possible non-linear relationships between time of day and the variable of interest. Snout–rump: a measure of body length (cm), distance from the tip of the nose to the anus; BMI: body mass index (kg m$^{-2}$); CS: citrate synthase activity; Mb: myoglobin concentration.

Signs following $P$-values indicate direction of effect based on the partial regression from the mixed model: $P_{selection}$, + indicates HR>C; $P_{activity}$, + indicates mice housed with wheels>those housed without; $P_{mini}$, + indicates mini-muscle mice>normal-muscle mice.

[a]Body length as the covariate for analysis of body mass.



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**Table 2. Tests of HR versus C, effects of replicate line, and effects of the mini-muscle phenotype on organ masses and muscle metabolic capacities in female sedentary mice only (no wheel access)**

| Trait | *N* | $P_{selection}$ | $P_{mini}$ | $P_{log\ mass}$ | $P_{age}$ | $P_{time}$ | $P_{time}^2$ | −2×ln REML first iteration | −2×ln REML final iteration | Line likelihood ratio test | $P_{line}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| log Body mass | 47 | **0.0333**− | 0.5787+ | | 0.9063− | 0.2547− | 0.3745− | −102.1 | −107.6 | 5.53 | **0.0187** |
| log Body mass[a] | 47 | 0.2272− | 0.5152− | **<0.0001+** | 0.9438− | **0.0355**− | 0.2220− | −128.0 | −132.3 | 4.30 | **0.0382** |
| log Snout–rump | 47 | **0.0037**− | **0.0249+** | | 0.7819− | 0.1086+ | 0.8810+ | −223.7 | −223.7 | 0.00 | 1.0000 |
| BMI | 46 | 0.1030− | 0.5341− | | 0.5961+ | **0.0168**− | 0.2853− | 27.7 | 18.5 | 9.18 | **0.0025** |
| log Ventricle | 47 | 0.2911+ | 0.1253+ | **<0.0001+** | 0.7942+ | 0.0773− | 0.5625− | −144.7 | −144.7 | 0.00 | 1.0000 |
| Hematocrit | 46 | 0.2914+ | 0.5041− | 0.5806− | **0.0094**− | 0.5206+ | 0.9622+ | −168.9 | −168.9 | 0.02 | 0.8832 |
| Hematocrit no mass | 46 | 0.1195+ | 0.4351− | | **0.0095**− | 0.4973+ | 0.9406+ | −172.1 | −172.1 | 0.02 | 0.9022 |
| log Soleus | 46 | 0.5344− | **<0.0001+** | **0.0017+** | 0.3904− | 0.1604− | 0.0758− | −98.8 | −106.7 | 7.87 | **0.0050** |
| log Plantaris | 47 | 0.4370− | 0.1103− | **<0.0001+** | 0.1771− | 0.5799− | 0.9024+ | −104.5 | −108.8 | 4.33 | **0.0375** |
| log Gastrocnemius | 47 | 0.2510− | **<0.0001**− | **0.0005+** | 0.8370− | 0.7024− | 0.1639− | −125.3 | −127.6 | 2.26 | 0.1331 |
| log Peritoneal fat | 47 | 0.3786+ | 0.1035+ | **<0.0001+** | 0.1154+ | 0.2248+ | 0.7463+ | −8.1 | −10.2 | 2.13 | 0.1446 |
| log Spleen | 46 | 0.2417− | **0.0186+** | **0.0016+** | 0.2303− | 0.7999− | 0.0984− | −72.8 | −79.1 | 6.37 | **0.0116** |
| log Liver | 47 | 0.0675− | **0.0208+** | **<0.0001+** | **0.0170**− | **<0.0001**− | 0.5634− | −130.6 | −131.8 | 1.28 | 0.2578 |
| log Kidney | 47 | 0.5135− | **0.0102+** | **<0.0001+** | 0.2857− | 0.4378− | 0.8427− | −113.5 | −113.6 | 0.01 | 0.9124 |
| log Lung wet | 47 | 0.3578+ | 0.0917+ | **<0.0001+** | 0.6628+ | 0.2423+ | 0.4660− | −120.9 | −125.7 | 4.76 | **0.0291** |
| log Lung dry | 47 | 0.3715+ | 0.1371+ | **<0.0001+** | 0.8594− | 0.4776+ | 0.6301− | −123.2 | −126.2 | 3.05 | 0.0807 |
| log Stomach wet | 46 | 0.2056− | 0.1918+ | **0.0198+** | 0.4534− | 0.1877− | 0.3341− | −107.6 | −107.6 | 0.00 | 1.0000 |
| log Stomach dry | 46 | 0.1301− | **0.0204+** | **0.0058+** | 0.2860− | 0.3430− | 0.7676+ | −113.1 | −113.1 | 0.00 | 1.0000 |
| log Small intestine wet | 47 | 0.1414− | 0.0788+ | **0.0090+** | 0.4493− | 0.3948− | 0.1635− | −109.2 | −111.2 | 1.91 | 0.1665 |
| log Small intestine dry | 46 | 0.3714− | 0.2671+ | **<0.0001+** | **0.0048**− | 0.0758+ | **0.0147**− | −123.9 | −123.9 | 0.00 | 1.0000 |
| log Small intestine length | 47 | 0.4115+ | **0.0326+** | 0.1337+ | 0.6541− | 0.8472− | 0.2181+ | −132.2 | −133.7 | 1.48 | 0.2234 |
| log Cecum wet | 47 | 0.5914− | 0.5747+ | 0.4297+ | **0.0176**− | 0.0633− | 0.7513− | −79.3 | −79.9 | 0.65 | 0.4209 |
| log Cecum dry | 47 | 0.5033+ | 0.2822+ | **0.0081+** | 0.0950− | 0.2603− | 0.3191− | −59.3 | −59.9 | 0.66 | 1.0000 |
| log Large intestine wet | 45 | 0.8477+ | 0.1771+ | **<0.0001+** | **0.0226**− | 0.1828+ | 0.1814− | −93.4 | −93.4 | 0.00 | 1.0000 |
| log Large intestine dry | 45 | 0.4937+ | 0.1523+ | **<0.0001+** | **0.0119**− | 0.1169+ | 0.2607− | −77.1 | −77.1 | 0.00 | 1.0000 |
| log Large intestine length | 47 | 0.7580+ | 0.8391+ | 0.0511+ | 0.4499+ | 0.4842+ | 0.9844+ | −104.6 | −104.6 | 0.00 | 1.0000 |
| Ventricle CS | 46 | 0.5978+ | 0.8955+ | 0.3906+ | **0.0227+** | **0.0048+** | 0.6668− | 435.4 | 435.4 | 0.00 | 1.0000 |
| Ventricle CS no mass | 46 | 0.9761+ | 0.7564+ | | **0.0221+** | **0.0045+** | 0.6295− | 448.0 | 448.0 | 0.00 | 1.0000 |
| Ventricle Mb | 45 | 0.2597+ | 0.7118− | 0.2351+ | 0.4291+ | **0.0079+** | 0.0966+ | 70.1 | 70.1 | 0.00 | 0.9774 |
| Ventricle Mb no mass | 45 | 0.5018+ | 0.7464− | | 0.4544+ | 0.0111+ | 0.1363+ | 74.0 | 74.0 | 0.07 | 0.7921 |
| Gastrocnemius CS | 44 | 0.5734+ | **<0.0001+** | 0.7843− | 0.3314− | 0.9433− | 0.8675+ | 367.9 | 367.9 | 0.00 | 1.0000 |
| Gastrocnemius CS no mass | 44 | 0.4389+ | **0.0002+** | | 0.3670− | 0.9591− | 0.8408+ | 345.2 | 345.2 | 0.01 | 0.9117 |
| Gastrocnemius Mb | 44 | 0.3990+ | **<0.0001+** | 0.7070+ | 0.4078− | 0.5416− | 0.8806+ | 34.9 | 34.9 | 0.00 | 1.0000 |
| Gastrocnemius Mb no mass | 44 | 0.4285 | **<0.0001+** | | 0.3902− | 0.5465− | 0.9001+ | 36.6 | 36.6 | 0.00 | 1.0000 |

Significance levels (bold indicates $P<0.05$, two-tailed, unadjusted for multiple comparisons) for the effects of both line type and line. Selection: HR versus C mice. Mini: effect of having the mini-muscle phenotype (see text). log Body mass (which was included as cofactor unless noted otherwise by 'no mass'), age, time of day and (time of day)$^2$ represent covariates in the analyses.

The *z*-transformed squared term for time of euthanasia (Time$^2$) was included in the analysis as it allows for possible non-linear relationships between time of day and the variable of interest.

Signs following *P*-values indicate direction of effect based on the partial regression from the mixed model: $P_{selection}$, + indicates HR>C; $P_{activity}$, mice housed with wheels>those housed without; $P_{mini}$, mini-muscle mice>normal-muscle mice.

In each one-way ANCOVA, to determine whether significant variation among replicate lines was present, the −2 REMLs of the first and last iteration evaluations within each ANCOVA were examined. The difference in −2 REMLs can be compared with a $\chi^2$ distribution with 1 d.f., for which the critical value for $P=0.05$ is 3.841.

[a]Snout–rump length as the covariate for analysis of body mass.

covariate) while emphasizing general patterns in the Results and Discussion that do not depend on details of how *P*-values may be adjusted for multiple comparisons.

As discussed elsewhere (Swallow et al., 2005; Garland and Kelly, 2006), chronic wheel exposure has the potential to positively (or negatively, in some cases) affect suborganismal traits that are hypothesized to promote the continuation or increase in wheel-running activity. Moreover, the effects (positive or negative) of the presence or absence of wheel access (environment) may depend on the genotype (HR versus C). Thus, for a given trait, wheel access may increase the value in HR lines relatively more (or less) than the effect in C lines. The greater effect of wheel access may be a function of the greater wheel running exhibited by HR lines ('more pain, more gain'), or the differences may reflect inherently greater plasticity in the HR lines (i.e. for a given amount of stimulus, such as wheel running per day, individuals in the HR lines show a greater response as compared with individuals in the C lines). The basis for this hypothesis and the statistical approach to test it are discussed in detail elsewhere (Garland and Kelly, 2006).

The general procedure to test the above hypothesis follows the subsequent example. For log ventricle mass, Table 3 shows that the ln maximum likelihood (not REML) of the nested ANCOVA model with wheel running (95.4) is larger than for the model without wheel running (91.8). As the former model contains one additional parameter (estimating the effect of wheel running), twice the difference in ln maximum likelihoods (7.16, in this case) can be compared with a $\chi^2$ distribution with 1 d.f., for which the critical value for $P=0.05$ is 3.841. Therefore, the model with wheel running as an additional covariate yields a significantly better fit to the data, and we conclude that the difference in log ventricle mass between HR and C mice when housed with wheel access can be explained as a simple function of the greater running by HR mice. In other words, HR mice show a greater training response not because they are inherently more plastic but because they 'train

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**Table 3. Tests of HR versus C and effects of replicate line on organ masses and muscle metabolic capacities in female active mice only (with wheel access)**

| Trait | *N* | ln max. likelihood (larger is better) | −2×ln max. likelihood | Likelihood ratio test | $P_{selection}$ | $P_{rev}$ | $P_{log\ mass}$ | −2×ln REML first interation | −2×ln REML final iteration | Line likelihood ratio test | $P_{line}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| log Body mass | 47 | 89.7 | −179.4 | 0.94 | 0.2932− | 0.3379− | | −123.2 | −124.0 | 0.75 | 0.3865 |
| | 47 | 89.2 | −178.5 | | **0.0201**− | | | −134.1 | −134.7 | 0.56 | 0.4543 |
| log Body mass[a] | 47 | 97.1 | −194.2 | 1.40 | 0.4429− | 0.2369− | **0.0003+** | −134.8 | −137.8 | 3.02 | 0.0822 |
| | 47 | 96.4 | −192.8 | | **0.0482**− | | **0.0003+** | −146.1 | −148.4 | 2.29 | 0.1302 |
| log Snout–rump | 47 | 151.1 | −302.2 | 0.17 | 0.7316− | 0.7006− | | −230.5 | −230.5 | 0.00 | 1.0000 |
| | 47 | 151.0 | −302.1 | | 0.2463− | | | −244.6 | −244.6 | 0.00 | 1.0000 |
| BMI | 46 | 14.0 | −28.0 | 0.08 | 0.1568− | 0.7212− | | 10.3 | 7.4 | 2.85 | 0.0914 |
| | 46 | 14.0 | −27.9 | | **0.0283**− | | | 2.0 | −0.8 | 2.82 | 0.0931 |
| log Ventricle | 47 | 95.4 | −190.7 | **7.16*** | 0.3046+ | **0.0163** | **<0.0001+** | −130.9 | −131.8 | 0.96 | 0.3272 |
| | 47 | 91.8 | −183.6 | | **0.0104+** | | **<0.0001+** | −135.7 | −137.8 | 2.10 | 0.1473 |
| Hematocrit | 45 | 125.3 | −250.5 | 3.39 | 0.4240+ | 0.0944+ | 0.6931− | −180.8 | −180.8 | 0.00 | 1.0000 |
| | 45 | 123.6 | −247.2 | | **0.0122+** | | 0.5676− | −191.2 | −191.2 | 0.00 | 1.0000 |
| Hematocrit no mass | 45 | 125.2 | −250.4 | 3.58 | 0.3675+ | 0.0813+ | | −184.2 | −184.2 | 0.00 | 1.0000 |
| | 45 | 123.4 | −246.8 | | **0.0044+** | | | −194.4 | −194.4 | 0.00 | 1.0000 |
| log Soleus | 47 | 81.8 | −163.6 | 0.00 | 0.1562+ | 0.9666− | **<0.0001+** | −89.2 | −110.7 | 21.46 | **0.0001** |
| | 47 | 81.8 | −163.6 | | 0.1243+ | | **<0.0001+** | −100.0 | −122.2 | 22.16 | **0.0001** |
| log Plantaris | 47 | 82.9 | −165.7 | 3.01 | 0.1011− | 0.1161+ | **<0.0001+** | −108.3 | −111.0 | 2.71 | 0.0997 |
| | 47 | 81.3 | −162.7 | | 0.3058− | | **<0.0001+** | −116.7 | −119.8 | 3.12 | 0.0773 |
| log Gastrocnemius | 47 | 84.4 | −168.7 | 0.00 | 0.3677− | 0.9476− | **<0.0001+** | −25.5 | −117.1 | 91.56 | **0.0001** |
| | 47 | 84.4 | −168.7 | | 0.3587− | | **<0.0001+** | −34.7 | −129.1 | 94.44 | **0.0001** |
| log Peritoneal fat | 47 | 21.2 | −42.4 | 0.00 | 0.6596+ | 0.9313− | **<0.0001+** | 3.4 | −6.8 | 10.23 | **0.0014** |
| | 47 | 21.2 | −42.4 | | 0.6348+ | | **<0.0001+** | −5.1 | −15.6 | 10.57 | **0.0011** |
| log Spleen | 44 | 58.3 | −116.7 | 1.23 | 0.8067− | 0.2819− | **0.0011+** | −55.2 | −69.7 | 14.46 | **0.0001** |
| | 44 | 57.7 | −115.4 | | 0.4140− | | **0.0006+** | −65.3 | −79.1 | 13.85 | **0.0002** |
| log Liver | 47 | 90.1 | −180.1 | 1.16 | 0.0897+ | 0.3613− | **<0.0001+** | −121.2 | −123.2 | 1.94 | 0.1637 |
| | 47 | 89.5 | −179.0 | | 0.1191+ | | **<0.0001+** | −130.8 | −134.0 | 3.25 | 0.0714 |
| log Kidney | 47 | 87.9 | −175.8 | 0.25 | 0.8037+ | 0.6562+ | **<0.0001+** | −110.3 | −120.4 | 10.09 | **0.0015** |
| | 47 | 87.8 | −175.5 | | 0.5712+ | | **<0.0001+** | −121.3 | −131.9 | 10.56 | **0.0012** |
| log Lung wet | 45 | 94.1 | −188.3 | 0.20 | 0.2772+ | 0.7221+ | **<0.0001+** | −122.2 | −129.9 | 7.77 | **0.0053** |
| | 45 | 94.0 | −188.1 | | 0.1484+ | | **<0.0001+** | −132.6 | −141.9 | 9.32 | **0.0023** |
| log Lung dry | 45 | 88.2 | −176.3 | 0.06 | 0.1204+ | 0.8828+ | **<0.0001+** | −116.4 | −119.2 | 2.77 | 0.0960 |
| | 45 | 88.1 | −176.2 | | **0.0451+** | | **<0.0001+** | −127.7 | −130.9 | 3.22 | 0.0727 |
| log Stomach wet | 47 | 88.4 | −176.9 | 0.76 | 0.6538+ | 0.4264− | **0.0064+** | −119.4 | −119.4 | 0.00 | 1.0000 |
| | 47 | 88.1 | −176.1 | | 0.7949− | | **0.0043+** | −130.3 | −130.3 | 0.00 | 1.0000 |
| log Stomach dry | 47 | 82.7 | −165.4 | 1.64 | 0.3112− | 0.2420+ | **0.0003+** | −109.6 | −109.6 | 0.00 | 1.0000 |
| | 47 | 81.9 | −163.8 | | 0.7994− | | **0.0004+** | −119.5 | −119.5 | 0.00 | 1.0000 |
| log Small intestine wet | 47 | 83.6 | −167.1 | 0.96 | 0.6682− | 0.4470+ | **<0.0001+** | −111.1 | −111.4 | 0.31 | 0.5777 |
| | 47 | 83.1 | −166.2 | | 0.8598+ | | **<0.0001+** | −121.4 | −122.2 | 0.84 | 0.3594 |
| log Small intestine dry | 46 | 96.3 | −192.6 | 0.48 | 0.6884− | 0.5824+ | **<0.0001+** | −131.2 | −133.4 | 2.18 | 0.1398 |
| | 46 | 96.1 | −192.1 | | 0.9605− | | **<0.0001+** | −141.9 | −145.1 | 3.21 | 0.0732 |
| log Small intestine length | 46 | 112.1 | −224.2 | 0.47 | 0.3770+ | 0.5390+ | **0.0002+** | −158.2 | −160.2 | 2.03 | 0.1542 |
| | 46 | 111.8 | −223.7 | | 0.1135+ | | **0.0002+** | −170.3 | −172.5 | 2.17 | 0.1407 |
| log Cecum wet | 47 | 51.7 | −103.4 | 0.10 | 0.6064− | 0.8541− | **0.0230+** | −56.8 | −57.2 | 0.34 | 0.5598 |
| | 47 | 51.7 | −103.3 | | 0.3427− | | **0.0184+** | −66.7 | −67.1 | 0.43 | 0.5120 |
| log Cecum dry | 47 | 37.3 | −74.6 | 0.01 | 0.8899− | 0.8929+ | **0.0336+** | −32.3 | −32.5 | 0.14 | 0.7083 |
| | 47 | 37.3 | −74.6 | | 0.9495− | | **0.0315+** | −41.7 | −41.8 | 0.13 | 0.7184 |
| log Large intestine wet | 47 | 68.4 | −136.8 | 0.49 | 0.1425+ | 0.5216− | **0.0137+** | −85.3 | −85.3 | 0.00 | 1.0000 |
| | 47 | 68.2 | −136.3 | | 0.1011+ | | **0.0099+** | −95.5 | −95.5 | 0.00 | 1.0000 |
| log Large intestine dry | 47 | 57.7 | −115.4 | 2.03 | 0.1019+ | 0.1915− | **0.0348+** | −67.1 | −67.1 | 0.00 | 1.0000 |
| | 47 | 56.7 | −113.4 | | 0.2007+ | | **0.0227+** | −75.6 | −75.6 | 0.00 | 1.0000 |
| log Large intestine length | 47 | 85.4 | −170.7 | 0.45 | 0.0676+ | 0.54100− | 0.2043+ | −114.2 | −114.2 | 0.00 | 1.0000 |
| | 47 | 85.2 | −170.3 | | **0.0286+** | | 0.1709+ | −125.2 | −125.2 | 0.00 | 1.0000 |
| Ventricle CS | 44 | −219.2 | 438.4 | 0.93 | 0.3205+ | 0.3916− | 0.8199+ | 399.4 | 399.3 | 0.12 | 0.7290 |
| | 44 | −219.7 | 439.4 | | 0.5500+ | | 0.7597+ | 402.6 | 402.4 | 0.16 | 0.6892 |
| Ventricle CS no mass | 44 | −219.3 | 438.6 | 1.01 | 0.3285+ | 0.3722− | | 411.4 | 411.3 | 0.16 | 0.6892 |
| | 44 | −219.8 | 439.6 | | 0.6047+ | | | 414.7 | 414.4 | 0.22 | 0.6390 |
| Ventricle Mb | 47 | −26.7 | 53.4 | 3.36 | 0.6688− | 0.0765+ | 0.1951+ | 77.2 | 75.8 | 1.37 | 0.2418 |
| | 47 | −28.4 | 56.7 | | 0.2572+ | | 0.3210+ | 73.0 | 72.3 | 0.70 | 0.4028 |
| Ventricle Mb no mass | 47 | −27.6 | 55.2 | 2.59 | 0.5244− | 0.1167+ | | 81.6 | 80.6 | 1.02 | 0.3125 |
| | 47 | −28.9 | 57.8 | | 0.3922+ | | | 77.1 | 76.5 | 0.59 | 0.4424 |
| Gastrocnemius CS | 46 | −209.8 | 419.5 | **4.76*** | 0.3128+ | **0.0429+** | **0.0114+** | 398.8 | 384.2 | 14.60 | **0.0001** |
| | 46 | −212.2 | 424.3 | | **0.0487+** | | **0.0307+** | 402.7 | 389.7 | 13.05 | **0.0001** |

Continued



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**Table 3. Continued**

| Trait | *N* | ln max. likelihood (larger is better) | −2×ln max. likelihood | Likelihood ratio test | $P_{selection}$ | $P_{rev}$ | $P_{log\ mass}$ | −2×ln REML first interation | −2×ln REML final iteration | Line likelihood ratio test | $P_{line}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Gastrocnemius CS no mass | 46 | −213.7 | 427.3 | 2.55 | 0.4762+ | 0.1315+ | | 416.6 | 402.0 | 14.60 | **0.0001** |
| | 46 | −214.9 | 429.9 | | 0.1148+ | | | 419.6 | 405.7 | 13.92 | **0.0001** |
| Gastrocnemius Mb | 46 | −7.7 | 15.3 | 1.08 | 0.2455+ | 0.3335+ | 0.3471+ | 71.2 | 40.9 | 30.35 | **0.0001** |
| | 46 | −8.2 | 16.4 | | 0.1238+ | | 0.4354+ | 64.6 | 34.1 | 30.53 | **0.0001** |
| Gastrocnemius Mb no mass | 46 | −8.2 | 16.4 | 0.74 | 0.2705+ | 0.4158+ | | 74.6 | 44.0 | 30.63 | **0.0001** |
| | 46 | −8.6 | 17.1 | | 0.1437+ | | | 67.9 | 36.9 | 31.04 | **0.0001** |

Significance levels (bold indicates $P<0.05$, two-tailed, unadjusted for multiple comparisons) for the effects of both line type and line. Selection: HR versus C mice. Rev: revolutions run during the final week of wheel access. log mass: represents one covariate in the analyses. Age, time of day and (time of day)$^2$ were also included as covariates (results not shown). Signs following *P*-values indicate direction of effect based on the partial regression from the mixed model: $P_{selection}$, + indicates HR>C.

Twice the difference in log maximum likelihood is distributed as a $\chi^2$ with 1 d.f., i.e. 3.841 for $P=0.05$. Values larger than this indicate that the model including amount of running during final week as a covariate (full model) fits the data significantly better than a model that does not include this covariate (reduced model). In each one-way ANCOVA, to determine whether significant variation among replicate lines was present, the −2 log REMLs of the first and last iteration evaluations within each ANCOVA were examined. The difference in −2 log REMLs can be compared with a $\chi^2$ distribution with 1 d.f., for which the critical value for $P=0.05$ is 3.841. There were no mini-muscle individuals from line 6 (lab designation) housed with wheel access. However, the mini-muscle phenotype is fixed (100% of individuals express it) in one HR line (lab designation is line 3). Therefore, to prevent the confounding of line and mini-muscle, mini-muscle was not used as a factor in the statistical model.

[a]Snout–rump length as the covariate for analysis of body mass.

*$P<0.05$. This statistical difference indicates that the higher values exhibited by mice from the HR lines can be explained as a simple linear function of their greater amount of wheel running during the final week of wheel access.

harder', i.e. run more. Corresponding tests for all traits are reported in Table 3.

Finally, each line type (HR and C) comprises four replicate lines that have been genetically separate for many generations and, hence, may themselves differ in phenotypic expression depending on the environment (active or sedentary). To assess the effects of environment (active versus sedentary) on replicate lines within line types, we utilized separate one-way ANCOVAs for the active (as discussed above) and sedentary groups. We considered the active (wheel access) and sedentary (no wheel access) groups separately to avoid complications stemming from additional covariance parameter estimates (random effects). In the two-way ANCOVA, two covariance parameters are estimated [line nested within line type and the wheel access×line(line type) interaction], while in the one-way ANCOVA only the line(line type) parameter is estimated. To determine whether significant variation among replicate lines was present, the −2 log REMLs of the first and last iteration evaluations within each ANCOVA were examined. The difference in −2 REMLs can be compared with a $\chi^2$ distribution with 1 d.f., for which the critical value for $P=0.05$ is 3.841. Note that this test, if significant, does not indicate whether the source of line heterogeneity is among the HR lines, among the C lines, or both.

## RESULTS

As expected, HR lines ran more than C lines for the duration of wheel access. Detailed wheel-running results were previously reported in Kelly et al. (2014; see their table 1, fig. 1). For all body composition characteristics, *P*-values are reported in Tables 1–3 and least-squares adjusted means and standard errors corresponding to two-way ANCOVAs in Table 1 are reported in Table S1. Covariates in the analyses presented in Tables 1–3 included log body mass, age, time of day and time of day$^2$.

### Body composition

HR lines were smaller than C lines, and wheel access reduced body mass of both HR and C lines. When body length was used as a covariate, the effects of wheel access and line type (HR versus C) on body mass became marginally non-significant. When examining only mice that had wheel access, HR lines were significantly smaller than C lines, and the difference was not a function of the greater amount of wheel running exhibited by the HR lines (Table 3). Additionally, when examining only the sedentary mice (no wheel access), HR lines were significantly smaller (Table 2). Considering all mice, body length was significantly shorter in the HR lines ($P=0.0368$; Table 1), and the *P*-value for the line type-by-wheel access interaction was 0.0772. Separate one-way ANCOVAs indicated no effect of line type for the wheel-access group, but HR lines were shorter than C when housed without wheel access (Table 2). Body mass index (BMI, kg m$^{-2}$) was significantly lower in the HR lines and wheel access reduced the BMI of both HR and C lines, without a significant interaction (Fig. 1).

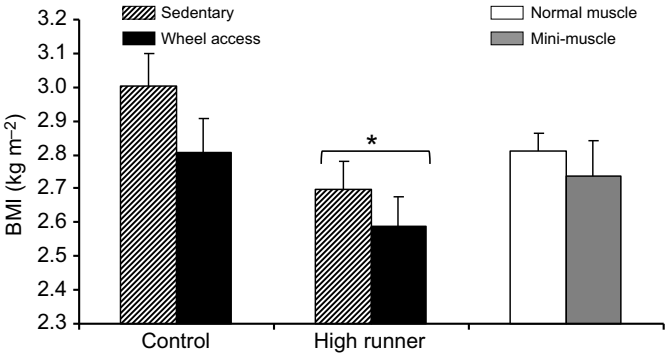


**Fig. 1. Group differences for body mass index (BMI, kg m$^{-2}$).** Least-squares means and standard errors corresponding to statistical tests are shown in Table 1. Covariates included, age, time of day of euthanasia and (*z*-transformed time of day)$^2$. ANCOVA revealed high runner (HR) mice had significantly lower BMI relative to control (C) lines (*$P=0.0458$), wheel access tended to lower BMI ($P=0.0535$) and no effect of the mini-muscle phenotype was observed ($P=0.4891$).

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### Organ masses, hematocrit, myoglobin and citrate synthase

HR lines had significantly larger ventricles (Fig. 2A) with more myoglobin on a mass-specific basis ($P$=0.0332) than C lines. Wheel access significantly increased ventricle mass of both groups (HR and C; $P$=0.0028), but with an interaction $P$=0.0648, indicating that, as compared with C mice, HR mice with wheels had a larger increase in ventricle mass relative to their sedentary counterparts. This interaction effect was explainable statistically by their greater amount of running (for further explanation, see Materials and methods and Fig. 2B).

Mice from HR lines had significantly higher Hct ($P$=0.0415, mass adjusted; $P$=0.0125, not mass adjusted; Fig. 3). When the active and sedentary groups were analyzed separately (Tables 2 and 3), HR lines had significantly higher Hct than C lines within the active group (two-tailed $P$=0.0122), but within the sedentary group there was no statistical difference.

CS activity in the gastrocnemius tended to be higher in the HR lines ($P$=0.0657) and was significantly increased by wheel access in both HR and C lines ($P$=0.0469, Table 1). Separate one-way ANCOVAs of the active and sedentary groups indicated that HR lines housed with wheels had significantly higher gastrocnemius CS activity (Table 3),

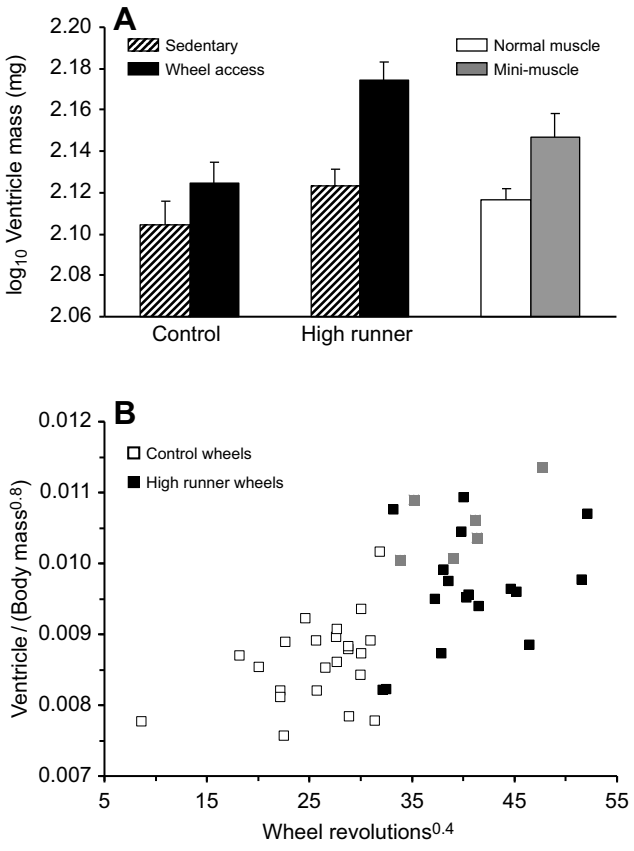


**Fig. 2. Effects of genetic selection, activity and muscle phenotype on ventricle mass.** (A) Group differences for mass-adjusted $\log_{10}$ ventricle mass and (B) relationship between ventricle mass [controlling for body mass (transformed with an exponent of 0.8)] and the amount of running exhibited during the final week of exposure to wheels. (A) ANCOVA revealed that line type [high runner (HR) versus control (C)], wheel access (wheel presence versus absence) and mini-muscle (presence versus absence) all had significant positive effects on ventricle mass, with a significant line type-by-activity interaction ($P$=0.0648). (B) The greater phenotypic values for the HR lines (black squares) as compared with C lines (white squares) are statistically explainable by their greater amount of running ('more pain, more gain'). Individuals with the mini-muscle phenotype are in gray.

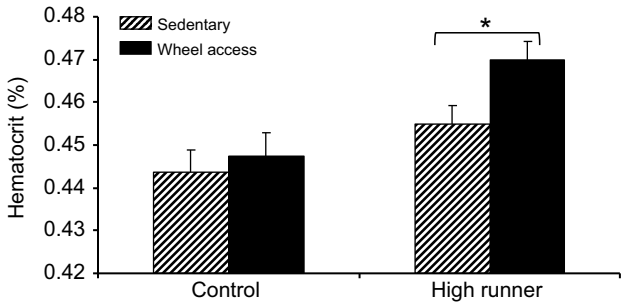


**Fig. 3. Group differences for mass-adjusted hematocrit (%).** ANCOVA revealed HR mice had significantly elevated hematocrit (*$P$=0.0125) while activity (wheels versus no wheels) tended to increase hematocrit in both groups ($P$=0.0726). The line type-by-activity interaction was not significant ($P$=0.2342).

but no statistical differences when housed without wheels (Table 2). The difference with wheel access could be explained by the greater amount of wheel running exhibited by the HR lines.

Wheel access significantly increased kidney mass ($P$=0.0261) and small intestine length ($P$=0.0331) in HR and C lines (Table 1). Additionally, HR lines (as compared with C when housed with wheels) had significantly longer large intestines ($P$=0.0286), and this difference was not explainable by the elevated running exhibited by the HR lines (Table 3). Finally, spleen, liver, lung (wet and dry), stomach (wet and dry) and cecum (wet and dry) mass were not significantly affected by line type (HR versus C) or wheel access in any analysis (two- or one-way ANCOVA).

### Replicate line variation

Line effects were examined utilizing separate one-way ANCOVAs for the active and sedentary groups (see Materials and methods). Within the sedentary group, even with the effect of mini-muscle phenotype controlled, significant line differences were found in body mass (with or without body length as a covariate), BMI, soleus and plantaris muscle mass, spleen mass and wet lung mass (see Table 2). Within the active group, no HR line 6 animals had the mini-muscle phenotype, so 'line' can be heavily influenced by HR line 3, which is fixed for the mini-muscle gene. Line differences were found for soleus muscle mass, gastrocnemius muscle mass, peritoneal fat pad mass, spleen mass, kidney mass, wet lung mass, gastrocnemius CS activity and myoglobin concentration (see Table 3).

### Effects of the mini-muscle phenotype

Mice with the mini-muscle phenotype had significantly smaller gastrocnemius and plantaris muscles, but significantly larger soleus muscles (Table 1). Additionally, the triceps surae, a composite of all three previously mentioned muscles, was significantly reduced in mini-muscle individuals. As documented in previous studies (e.g. see Garland et al., 2002; Swallow et al., 2005; Kolb et al., 2010), the relative mass of the ventricles was increased in individuals with the mini-muscle phenotype (Fig. 2A). Several additional effects of the mini-muscle phenotype were also observed: increases in relative spleen mass, kidney mass, liver mass, stomach mass (dry), lung mass, myoglobin concentration and CS activity in the gastrocnemius muscle (see Table 1).

## DISCUSSION

Both voluntary physical activity (leading to phenotypic plasticity) and selective breeding (leading to evolutionary adaptation) for high



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activity levels on wheels alter organ masses and muscle metabolic properties of laboratory house mice, and the effects are generally in the same direction. Surprisingly, traits hypothesized to facilitate nutrient processing and absorption (e.g. stomach, small and large intestine, and cecum mass) were largely unaffected by either long-term physical activity or selective breeding. The only significant effect of selection history was among active mice (housed with wheel access for 13–14 weeks), where HR lines exhibited longer large intestines as compared with controls.

A two-way ANCOVA revealed body length and ventricle mass (Table 1) exhibited a statistically significant interaction, i.e. non-additive effects of selective breeding and chronic exercise, which constitutes a genotype-by-environment interaction. Some genotype-by-environment interactions (gastrocnemius CS activity, body mass, BMI, Hct and large intestine length) could only be ascertained utilizing separate one-way ANCOVAs for the wheel access (Table 3) and non-wheel access (Table 2) groups, as the conventional two-way ANCOVA examining the effects of line type (HR versus C) and wheel access yielded apparently low statistical power to detect interactions between these two main effects. For ventricle mass and gastrocnemius CS activity, the greater effect of wheel access in the HR lines can be explained statistically by the elevated wheel running of HR individuals, while for others (body mass, BMI, Hct and large intestine length) the differences seem to reflect inherently greater plasticity in the HR lines (see also Garland and Kelly, 2006).

Replicate lines ($N$=8) showed significant variation in a variety of traits, and this variation sometimes depended on whether mice were housed with or without wheel access. Wheel access generally increased a trait's value across replicate lines. Lastly, a Mendelian recessive allele that halves hind limb muscle mass (mini-muscle), and has been favored by the selection protocol, also affects organ masses and muscle metabolic properties (some of these effects have been reported previously). We discuss these effects in turn.

### Wheel access, selective breeding and genotype-by-environment interactions

For both HR and C lines, wheel access caused a significant reduction in body mass and generally reduced BMI. In the absence of wheel access, mice from HR lines were significantly lighter and shorter than C lines, with no difference in BMI. In the presence of wheel access, body mass and BMI were significantly reduced in HR lines and the reduction was not a function of the greater wheel running. These results are only partially consistent with previous findings, which have demonstrated that the HR female mice seem better able to 'protect' their body mass from the effects of wheel access (Swallow et al., 1999).

Wheel access also increased relative ventricle mass in both HR and C lines. A decrease in body mass and an increase in ventricle mass are consistent with previous rodent studies, and specifically with previous results utilizing the current model (Swallow et al., 2005). It is also important to note that the training effects on ventricle mass were considerably greater in the HR lines. Although the two-way ANCOVA did not reveal a statistically significant interaction between line type (HR versus C) and activity (wheel access or not) as defined by $P$<0.05, the relative power to detect such interactions is low (Wahlsten, 1990, 1991). Two separate one-way ANCOVAs examining wheel access and sedentary mice separately revealed that among mice with wheel access HR lines had larger ventricles relative to C lines. Among sedentary mice there was no statistical difference in ventricle mass between HR and C mice (Table 2). Furthermore, the difference among HR and C mice when housed with wheel access could be statistically explained by the greater wheel running in HR mice. Swallow et al. (2010) found a similar interaction between selection history and wheel access for ventricle mass in rats selected for high treadmill endurance capacity. However, Swallow et al. (2010) did not address the basis (greater wheel running versus greater phenotypic plasticity) of the interaction.

In accordance with human training studies (reviews in Scheuer and Tipton, 1977; Sawka et al., 2000) and previous rodent wheel-running studies (Yashiro and Kimura, 1979; Spodaryk et al., 1985; Pfeil, 1988), wheel access did not cause an increase in Hct levels among C lines. However, as revealed by one-way ANCOVA examining only mice with wheel access, HR mice had elevated Hct relative to C lines, indicating that for HR lines, wheel access caused an increase in Hct levels. Moreover, this increase did not appear to be a function of the greater wheel running in the HR lines. This is partially counter to what has been found previously utilizing these lines of mice. Swallow et al. (2005) found (at generation 14) that wheel access increased Hct and hemoglobin levels in both HR and C lines, of both sexes, with larger training effects in the HR lines. In accordance with the present study, it was later found (see table 2 in Garland and Kelly, 2006) that the greater training effects in Hct and hemoglobin levels found in the HR lines reflected greater plasticity and were not a simple function of the elevated wheel running.

Wheel access significantly increased CS activity and myoglobin concentration in the gastrocnemius muscles of both HR and C lines, with no significant interactions. These increases are consistent with human and rat endurance-training studies that have used both forced (Holloszy, 1967; Green et al., 1983; Holloszy and Coyle, 1984) and voluntary (Rodnick et al., 1989; Sexton, 1995; Houle-Leroy et al., 2000) exercise protocols. Again, separate analyses of the sedentary and wheel-access groups revealed that training effects (wheel access) were greater in the HR lines for CS activity only. The greater wheel running of HR lines could explain the greater training effects for CS activity.

Counter to the hypotheses outlined in the Introduction, organs relevant in nutrient processing were relatively unaltered by selection history (HR versus C) or exercise training over 13–14 weeks of wheel access (see also Vaanholt et al., 2007). Wheel running has been previously shown to increase food consumption by 22.4% in HR and C lines (Swallow et al., 2001) (at generation 13), and when housed with locked wheels, HR lines consumed 8.4% more food per day as compared with C lines (Swallow et al., 2001). Additionally, Koteja et al. (1999) previously revealed a positive relationship between the amount of voluntary running and body mass-adjusted food consumption or energy assimilation rate (Koteja et al., 1999). In response to a long-term increased feeding rate, the digestive adjustments of most mammals and birds include increased gut size and increased amount of digestive enzymes (Karasov and McWilliams, 2005; see also Vaanholt et al., 2007). Although we did not measure food consumption in the present study, wheel access (which has previously been associated with elevations in food consumption) increased small intestine length in both HR and C lines, but did not alter small intestine mass. Additionally, when only examining the mice housed with wheels, HR lines had lengthened large intestines relative to C lines. These results are consistent with predictions, but the magnitude of the response was small. One limitation of the present study is that we only measured organ size, but digestion rate can be greatly increased through changes not only in organ size but also in enzyme and transport mechanisms responsible for breaking down and absorbing substrates. For example, in a previous study (Swallow et al.,

2001) we have observed that HR mice consume more food than C lines. However, here we observed no effect of selection history on liver mass, but did not measure CS activity, which may be increased in HR mice in order to compensate for the increase in food consumption. Additionally, changes in the muscular activity that affect contact time between food and the gastrointestinal tract can also affect digestion rate and efficiency (Karasov and McWilliams, 2005; see also Lavin et al., 2008).

One possible explanation for the lack of major alterations to the digestive track is that previous results (Koteja et al., 1999; Vaanholt et al., 2006; Swallow et al., 2001; Rezende et al., 2009) have demonstrated that the incremental cost of wheel running represents only a small portion of the total energy budgets of the mice. Koteja et al. (1999) conclude: 'If wheel running in the selected lines continues to increase mainly by increases in velocity, then constraints related to energy acquisition are unlikely to be an important factor limiting further selective gain.' It is worth noting, however, that these estimates were made on generation 10, and mice in the current experiment are from generation 37. Many traits have changed from generation 10 to 37, including wheel running. The conclusion of Koteja et al. (1999) assumes a relatively small change in the amount of time spent running, whereas in the current experiment females run significantly more minutes per day during days 36–41 and 88–93 (see Kelly et al., 2014). Beyond the current experiment, we have observed females (sometimes) and males increase the amount of time spent running per day, although the increase is much smaller than the increase in running speed (Rezende et al., 2006b, 2009; Garland et al., 2011a). Nonetheless, this increase in total time spent running may be sufficient to further alter food consumption and energy costs associated with wheel running. This increased running time may be sufficient to explain the small changes in large and small intestine length observed in the present study.

### Replicate line differences

One of the most notable examples of line differences is the mini-muscle gene, which is fixed in one of four HR lines and polymorphic in another, while apparently now absent from all other lines (Kelly et al., 2013; Rezende et al., 2006b; Kolb et al., 2010). Beyond this, in the present study significant variation among replicate lines was found for a number of traits. As reviewed elsewhere (Garland, 2003), such differences may reflect random genetic drift (possibly involving different mutations) and/or different adaptive mechanisms (for other examples in these mice, see Swallow et al., 1998a, 2001; Koteja et al., 1999, 2003; Garland et al., 2002, 2011a; Rezende et al., 2006a,b). When housed with wheels, soleus mass, gastrocnemius mass, gastrocnemius CS activity, peritoneal fat mass, spleen mass, kidney mass and lung mass (wet) varied among replicate lines. When housed without wheels, body mass, BMI, soleus mass, plantaris mass, spleen mass and wet lung mass varied across replicate lines. Note that this test, if significant, does not indicate whether the source of line heterogeneity is among the HR lines, among the C lines, or both. It is especially interesting that traits associated with body composition (peritoneal fat mass, body mass and BMI) varied across replicate lines in at least one housing condition.

### Effects of the mini-muscle phenotype

In the present study, mice expressing the mini-muscle phenotype ran faster, but only during the early stages of wheel access (see Kelly et al., 2014). The mini-muscle phenotype also positively affected ventricle mass, spleen mass, liver mass, lung mass, stomach mass, soleus mass, CS activity and myoglobin concentration in the gastrocnemius muscle. Conversely, the mini-muscle phenotype caused a significant reduction in plantaris and gastrocnemius muscle mass. Additionally, the triceps surae, a composite of all three previously mentioned muscles, was significantly reduced in mini-muscle individuals (results not shown). The effects found in the present study support previous findings and provide additional support (e.g. lung mass increases) about the likely adaptive significance of the mini-muscle mutation as a gene of major effect (see Hannon et al., 2008 for further details).

## Conclusions

To our knowledge, these (along with examples found in Garland and Kelly, 2006) are among the first described examples of direct selection for increased activity levels facilitating a larger phenotypic response to a given amount of exercise training. Additionally, the larger responses to exercise training occur in traits that further support locomotion. This concept has been previously termed 'self-induced adaptive plasticity' by Swallow et al. (2005). In previous studies (Houle-Leroy et al., 2000; Belter et al., 2004; Swallow et al., 2005), interactions between line type and physical activity were found, but whether these interactions reflected greater plasticity or were a result of the elevated wheel running in HR lines was not completely analyzed. Examining genetic factors and training effects in concert on the same set of animals (e.g. Koch et al., 2012; Garton et al., 2016) provides a powerful way to examine the evolution of phenotypic plasticity in response to an altered environment. Future studies should also consider both the duration of exercise training and when in the life cycle it occurs, as exposures occurring earlier in life might have greater effects (Garland et al., 2017).

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

S.A.K. designed the project, conducted experiments, analyzed the data and wrote the manuscript; F.R.G., E.M.K., J.L.M. and T.G. designed the project, conducted experiments and revised the manuscript.

#### Funding

This work was supported by US National Science Foundation grants IOB-0543429 and IOS-1121273 to T.G.

#### Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.148759.supplemental

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## SUPPLEMENTARY MATERIAL

**Table S1.** Least-squares means and standard errors from analyses of all mice as presented in Table 1.

| Trait | High Runner Lines | | | | Control Lines | | | | Mini-Muscle | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sedentary | | Active | | Sedentary | | Active | | Sedentary | | Active | |
| | Mean | *SE* | Mean | *SE* | Mean | *SE* | Mean | *SE* | Mean | *SE* | Mean | *SE* |
| log Body Mass (g) | 1.45 | 0.02 | 1.44 | 0.02 | 1.52 | 0.02 | 1.48 | 0.02 | 1.47 | 0.01 | 1.48 | 0.02 |
| log Body Mass[a] (g) | 1.46 | 0.01 | 1.44 | 0.01 | 1.49 | 0.02 | 1.47 | 0.02 | 1.47 | 0.01 | 1.46 | 0.02 |
| log Snout Rump (mm) | 2.01 | 0.00 | 2.01 | 0.00 | 2.02 | 0.00 | 2.02 | 0.00 | 2.01 | 0.00 | 2.02 | 0.00 |
| BMI[b] ($kg/m^2$) | 2.70 | 0.08 | 2.59 | 0.09 | 3.00 | 0.10 | 2.81 | 0.10 | 2.81 | 0.05 | 2.74 | 0.10 |
| log Soleus (mg) | 0.97 | 0.02 | 0.98 | 0.02 | 0.97 | 0.02 | 0.96 | 0.02 | 0.90 | 0.01 | 1.04 | 0.02 |
| log Plantarus (mg) | 1.12 | 0.02 | 1.13 | 0.02 | 1.14 | 0.02 | 1.15 | 0.02 | 1.16 | 0.01 | 1.11 | 0.02 |
| log Gastroc (mg) | 1.87 | 0.01 | 1.87 | 0.01 | 1.88 | 0.01 | 1.89 | 0.01 | 2.07 | 0.01 | 1.68 | 0.02 |
| log Fat Pad (mg) | 2.73 | 0.06 | 2.64 | 0.07 | 2.66 | 0.08 | 2.58 | 0.08 | 2.60 | 0.04 | 2.71 | 0.08 |
| log Ventricle (mg) | 2.12 | 0.01 | 2.17 | 0.01 | 2.10 | 0.01 | 2.12 | 0.01 | 2.12 | 0.01 | 2.15 | 0.01 |
| log Spleen (mg) | 1.97 | 0.03 | 1.94 | 0.04 | 2.02 | 0.04 | 2.02 | 0.04 | 1.94 | 0.02 | 2.03 | 0.04 |
| log Liver (mg) | 3.18 | 0.01 | 3.20 | 0.01 | 3.20 | 0.01 | 3.19 | 0.01 | 3.17 | 0.01 | 3.21 | 0.01 |
| log Kidney (mg) | 2.63 | 0.01 | 2.67 | 0.02 | 2.64 | 0.02 | 2.66 | 0.02 | 2.63 | 0.01 | 2.68 | 0.02 |
| log Lungs (mg), wet | 2.35 | 0.01 | 2.36 | 0.02 | 2.31 | 0.02 | 2.34 | 0.02 | 2.32 | 0.01 | 2.36 | 0.02 |
| dry | 1.65 | 0.01 | 1.66 | 0.01 | 1.62 | 0.02 | 1.63 | 0.02 | 1.62 | 0.01 | 1.66 | 0.02 |
| log Stomach (mg), wet | 2.53 | 0.01 | 2.53 | 0.01 | 2.55 | 0.01 | 2.54 | 0.01 | 2.52 | 0.01 | 2.55 | 0.01 |
| dry | 1.83 | 0.01 | 1.83 | 0.01 | 1.85 | 0.01 | 1.85 | 0.01 | 1.82 | 0.01 | 1.85 | 0.01 |
| log Sm Intes (mg), wet | 3.35 | 0.02 | 3.38 | 0.02 | 3.37 | 0.02 | 3.39 | 0.02 | 3.36 | 0.01 | 3.38 | 0.02 |
| dry | 2.53 | 0.01 | 2.55 | 0.01 | 2.54 | 0.01 | 2.56 | 0.01 | 2.54 | 0.01 | 2.55 | 0.01 |
| log Sm Intes Lth (cm) | 1.68 | 0.01 | 1.71 | 0.01 | 1.68 | 0.01 | 1.70 | 0.01 | 1.68 | 0.01 | 1.71 | 0.01 |
| log Cecum (mg), wet | 2.41 | 0.02 | 2.35 | 0.02 | 2.41 | 0.03 | 2.41 | 0.02 | 2.37 | 0.01 | 2.42 | 0.03 |
| dry | 1.64 | 0.03 | 1.59 | 0.03 | 1.59 | 0.04 | 1.62 | 0.03 | 1.58 | 0.02 | 1.65 | 0.04 |
| log Lg Intes (mg), wet | 2.61 | 0.01 | 2.65 | 0.01 | 2.60 | 0.02 | 2.61 | 0.02 | 2.61 | 0.01 | 2.63 | 0.02 |
| dry | 1.97 | 0.02 | 2.00 | 0.02 | 1.95 | 0.02 | 1.95 | 0.02 | 1.95 | 0.01 | 1.99 | 0.02 |
| log Lg Intes Lth (cm) | 0.98 | 0.01 | 1.00 | 0.01 | 0.96 | 0.01 | 0.95 | 0.01 | 0.98 | 0.01 | 0.97 | 0.01 |
| Hematocrit (%) | 0.45 | 0.00 | 0.47 | 0.00 | 0.45 | 0.01 | 0.45 | 0.01 | 0.45 | 0.00 | 0.45 | 0.01 |
| no mass | 0.45 | 0.00 | 0.47 | 0.00 | 0.44 | 0.01 | 0.45 | 0.01 | 0.45 | 0.00 | 0.45 | 0.01 |
| *Ventricle* | | | | | | | | | | | | |
| Citrate Synthase | 300.45 | 10.13 | 311.65 | 10.93 | 272.95 | 13.19 | 291.89 | 12.19 | 302.05 | 5.38 | 286.43 | 13.21 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| no mass | 296.27 | 10.08 | 306.43 | 10.76 | 282.85 | 12.18 | 294.04 | 12.54 | 301.42 | 5.63 | 288.38 | 13.71 |
| Myoglobin | 5.56 | 0.11 | 5.52 | 0.12 | 5.14 | 0.14 | 5.20 | 0.13 | 5.50 | 0.06 | 5.21 | 0.14 |
| no mass | 5.51 | 0.12 | 5.44 | 0.12 | 5.25 | 0.14 | 5.21 | 0.14 | 5.49 | 0.06 | 5.22 | 0.15 |
| *Gastrocnemius* | | | | | | | | | | | | |
| Citrate Synthase | 87.35 | 6.60 | 113.27 | 6.82 | 79.46 | 8.25 | 86.47 | 7.76 | 70.93 | 3.52 | 112.34 | 8.05 |
| no mass | 85.58 | 6.73 | 110.35 | 6.85 | 83.56 | 8.04 | 86.82 | 8.09 | 70.81 | 3.87 | 112.34 | 8.62 |
| Myoglobin | 1.42 | 0.06 | 1.74 | 0.06 | 1.36 | 0.08 | 1.48 | 0.07 | 1.01 | 0.03 | 1.99 | 0.08 |
| no mass | 1.41 | 0.06 | 1.72 | 0.06 | 1.39 | 0.07 | 1.49 | 0.07 | 1.01 | 0.03 | 1.99 | 0.08 |

[a]Snout–rump length as the covariate for analysis of body mass.

[b]BMI ($kg/m^2$) is body mass index.


**Fig. S1. Cages with and without wheels placed alternately on racks.** Four groups were compared: mice from C lines housed without wheels (sedentary, *N* = 24); mice from C lines housed with wheels (active, *N* = 24); mice from HR lines housed without wheels (sedentary, *N* = 24) and mice from HR lines housed with wheels (active, *N* = 24).

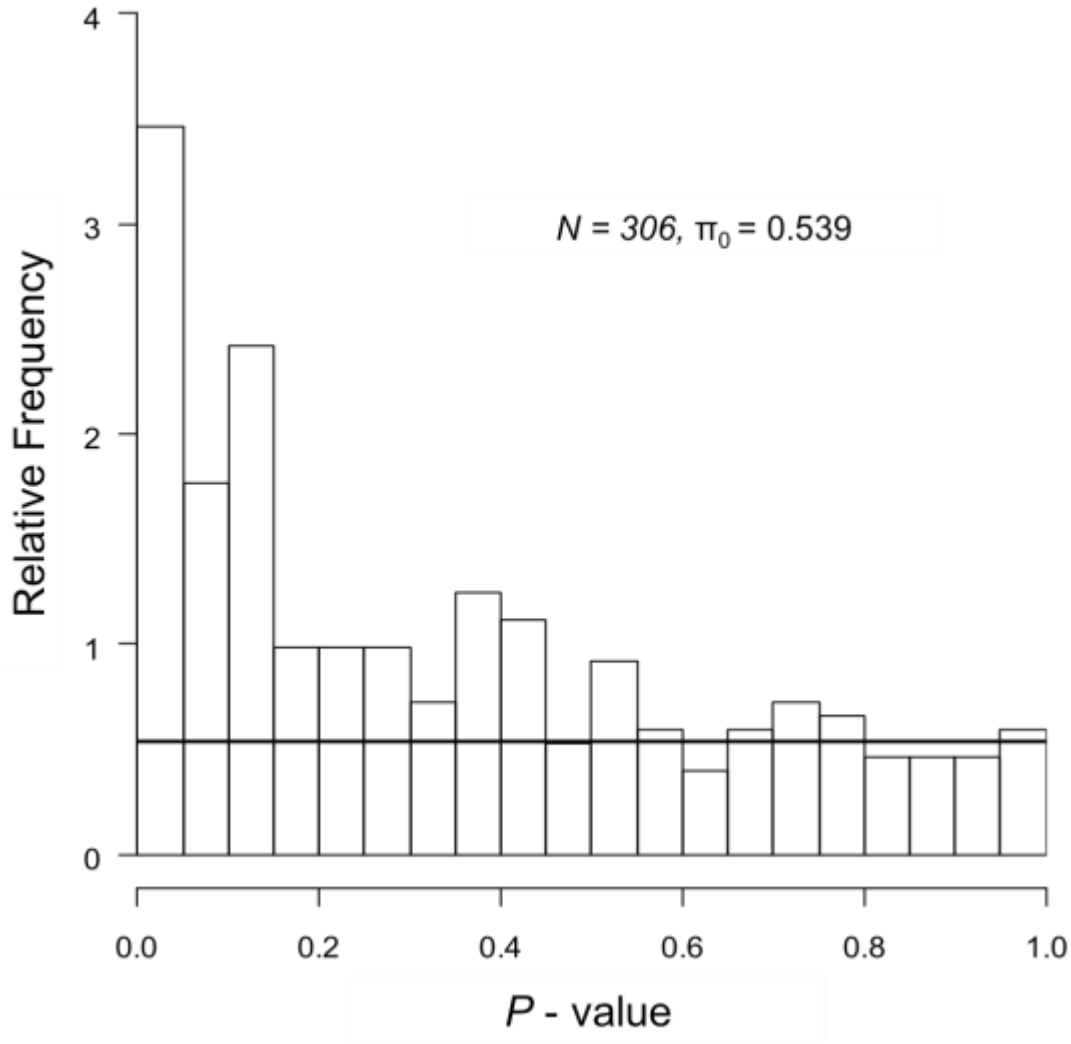


**Fig. S2. Distributions of *P* values in relation to the $\pi_0$ statistic.** The overall proportion of true null hypotheses (Storey and Tibshirani, 2003; Storey, 2003)] for a subset of the *P* –values in Tables 1-3 (see text). The bin size is 0.05; $\pi_0$ is indicated by the solid line. A random set of *P*-values would produce a flat histogram with all bins showing a relative frequency of 1.0; the presence of non-null hypotheses (i.e., significant tests after accounting for Type 1 errors) are indicated by high relative frequencies in the left-most bin. Values of $\pi_0$ were computed with the *qvalue* software package for R.