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Effects of voluntary activity and genetic selection on muscle metabolic capacities in house mice *Mus domesticus*

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Houle-Leroy, Philippe, Theodore Garland, Jr., John G. Swallow, and Helga Guderley. Effects of voluntary activity and genetic selection on muscle metabolic capacities in house mice *Mus domesticus. J Appl Physiol* 89: 1608–1616, 2000.—Selective breeding is an important tool in behavioral genetics and evolutionary physiology, but it has rarely been applied to the study of exercise physiology. We are using artificial selection for increased wheel-running behavior to study the correlated evolution of locomotor activity and physiological determinants of exercise capacity in house mice. We studied enzyme activities and their response to voluntary wheel running in mixed hindlimb muscles of mice from *generation 14*, at which time individuals from selected lines ran more than twice as many revolutions per day as those from control (unselected) lines. Beginning at weaning and for 8 wk, we housed mice from each of four replicate selected lines and four replicate control lines with access to wheels that were free to rotate (wheel-access group) or locked (sedentary group). Among sedentary animals, mice from selected lines did not exhibit a general increase in aerobic capacities: no mitochondrial [except pyruvate dehydrogenase (PDH)] or glycolytic enzyme activity was significantly ($P < 0.05$) higher than in control mice. Sedentary mice from the selected lines exhibited a trend for higher muscle aerobic capacities, as indicated by higher levels of mitochondrial (cytochrome-*c* oxidase, carnitine palmitoyltransferase, citrate synthase, and PDH) and glycolytic (hexokinase and phosphofructokinase) enzymes, with concomitant lower anaerobic capacities, as indicated by lactate dehydrogenase (especially in male mice). Consistent with previous studies of endurance training in rats via voluntary wheel running or forced treadmill exercise, cytochrome-*c* oxidase, citrate synthase, and carnitine palmitoyltransferase activity increased in the wheel-access groups for both genders; hexokinase also increased in both genders. Some enzymes showed gender-specific responses: PDH and lactate dehydrogenase increased in wheel-access male but not female mice, and glycogen phosphorylase decreased in female but not in male mice. Two-way analysis of covariance revealed significant interactions between line type and activity group; for several enzymes, activities showed greater changes in mice from selected lines, presumably because such mice ran more revolutions per day and at greater velocities. Thus genetic selection for increased voluntary wheel running did not reduce the capability of muscle aerobic capacity to respond to training.

artificial selection; genotype-environment interaction; quantitative genetics; muscle metabolic capacities; exercise adaptations; aerobic capacity

SELECTIVE BREEDING HAS PROVEN to be one of the most useful analytic tools in behavioral genetics and is emerging as an important tool in evolutionary physiology (9, 12) but has rarely been used in exercise physiology (20, 25). We are using artificial selection for increased voluntary wheel-running behavior (38) to study the correlated evolution of locomotor activity and the physiological capacity for exercise in house mice (*Mus domesticus*). We chose to select on wheel running, because it may be physiologically demanding and ecologically relevant, possibly reflecting exploration for necessary resources (24, 33). In addition, voluntary wheel running has a great potential to elicit intense exercise by rodents and causes a variety of morphological and physiological modifications, including decreased body mass (2, 13, 23, 31, 32, 40), increased maximal O_2 consumption (23, 39), changes in muscle enzyme activity (31, 42), vascular adaptations (32), shifts in muscle fiber-type composition (19), and greater reliance on fat metabolism, as indicated by a lower respiratory exchange ratio (RER) in mice given long-term access to running wheels (39).

We have used within-family selection to create four replicate lines of mice that exhibit high voluntary wheel running compared with four random-bred control lines (37, 38). After 14 generations, wheel running in the selected and control lines differed by more than twofold (40). In the present study, our main goal was to establish whether mice from the selected lines also have a higher muscle aerobic capacity. After 10 generations, the selected lines had a significantly higher maximal rate of O_2 consumption during forced treadmill exercise than the control lines (39) but did not show an increase in contractile performance, isotonic endurance, or succinate dehydrogenase activity in the medial gastrocnemius muscle (42). Because chronic wheel use reduces RER in these mice (39), selection for

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increased wheel running may also affect fuel use during exercise.

Our second goal was to determine whether selection for high activity levels has decreased the capacity of mice to enhance their muscle aerobic capacity when they are given access to running wheels for 8 wk (i.e., training via voluntary activity). The small body size of mice is associated with high tissue aerobic capacities as expressed per gram of tissue. Given the constraints for the allocation of intracellular space between myofibrils and mitochondria, increases in muscle aerobic capacity may be difficult in such small mammals. In fact, the training response of mice has been shown to be less pronounced than that of larger mammals, such as rats, guinea pigs, and rabbits, after chronic nerve stimulation of the tibialis anterior muscle (34).

Because high levels of voluntary activity may require high endurance and aerobic capacity, we hypothesized that selection for increased wheel running would be accompanied by an increase in the aerobic capacity of muscles directly implicated in locomotor activity. Here, we studied simultaneously the effects of "nature" (genetic endowment for high activity) and "nurture" (chronic access to a running wheel) on enzyme markers of main metabolic pathways. Inasmuch as ours are the first lines of mice specifically selected for high levels of wheel running, we can view the effects of wheel access as a sort of "positive control." That is, metabolic adaptations to voluntary exercise are well known in rats. Do they also occur in mice? If we can demonstrate such responses in the present study, then it can be argued that our protocols for measuring enzyme activities should also be adequate for demonstrating effects of genetic selection, if they do indeed occur. A previous study (42) of these mice from *generation 10* reported that access to running wheels for 8 wk increased succinate dehydrogenase activity in the medial gastrocnemius but that selected and control lines did not differ. However, that study considered only male mice, which run less than female mice after several weeks of wheel access (Table 1 and Fig. 1 in Ref. 40), and, in the present *generation 14*, mice from the selected lines show a substantially greater increment in wheel running (Table 1) than was apparent at *generation 10*. Therefore, we measured activities of multiple enzymes in male and female mice.

Because overall metabolic flux through a pathway may be determined by a limited number of enzymes, their activities can reflect changes in metabolic capacities (26). To have a comprehensive view of the pathways implicated in energy metabolism, we need to measure enzymes in different pathways. Therefore, we measured three enzymes that regulate major metabolic pathways: phosphofructokinase (PFK; glycolysis), pyruvate dehydrogenase (PDH; mitochondrial pyruvate oxidation), and carnitine palmitoyltransferase (CPT; fatty acid oxidation). We also measured enzymes that deliver glucose to glycolysis: hexokinase (HK; blood glucose) and glycogen phosphorylase (GP), in the activated (GP_a), inactivated (GP_b), and total forms ($GP_{tot} = GP_a + GP_b$). As markers of mitochondrial abundance, located in the matrix and the inner mitochondrial membrane, respectively, we measured citrate synthase (CS) and cytochrome-*c* oxidase (CCO). Finally, we measured lactate dehydrogenase (LDH), the terminal enzyme of anaerobic glycolysis.

METHODS

Selection experiment. Mice used in this study were sampled from *generation 14* (2nd litters) of an artificial selection experiment for increased voluntary activity levels on running wheels. Full details of the selection experiment are provided elsewhere (37, 38), and only a brief overview will be repeated here. Body mass data for the same individuals studied here were reported in a previous study (40), which also gives further information on housing and maintenance.

For the selection experiment, the original progenitors were outbred Hsd:ICR house mice (*M. domesticus*) obtained from Harlan Sprague Dawley (Indianapolis, IN). The ICR strain shows substantial genetic variation, with levels of heterozygosity similar to those reported for wild populations of house mice (5), and has served as a model system for numerous quantitative genetic analyses (7, 8, 11, 37).

At each generation, 10 pairs (families) of mice were used to propagate each of eight lines, four selected for high wheel running and four randomly bred as control lines. At each generation, we tested ~600 mice (all individuals from selected lines and a random sample from control lines) for wheel running by housing them individually with access to running wheels for 6 consecutive days (beginning at 5.5–8 wk of age). The selection criterion was the total number of revolutions run on *days 5* and *6* of the 6-day test: in the selected lines, the highest-running male and female mice from each family were chosen as breeders. In the control lines, one male and one female mouse from each family were chosen randomly. The 10 male and 10 female animals from each line were paired randomly within the line, with the provision of no matings between siblings. Pups were weaned

Table 1. *Wheel running during the final week of access to wheels*

	Line Type					
	Control (n = 38)		Selected (n = 40)		Selected/Control	
Variable	M	F	M	F	M	F
Total revolutions/day*	3,999 ± 550	4,985 ± 438	8,384 ± 928	12,921 ± 754	2.1	2.6
Average rpm*	12.1 ± 1.0	13.4 ± 0.8	20.2 ± 1.5	26.8 ± 1.2	1.7	2.0
1-min intervals/day*†	306 ± 24	372 ± 21	405 ± 19	481 ± 17	1.3	1.3

Values are means ± SE. M, male mice; F, female mice. The analysis used the number of toes clipped for individual identification and wheel freeness as covariates; 1 revolution = 1.12 m; 1-min intervals/day is the number of 1-min intervals during which any wheel revolutions occurred. *$P < 0.05$ for effects of line type (i.e., genetic selection); †$P < 0.05$ applies only to female mice.

at 21 days of age, weighed, and toe clipped for identification. In the present study, they were housed with three siblings until the following day, when they were housed individually with access to running wheels.

Animal husbandry. Routine housing was in standard clear plastic cages (27 × 17 × 12.5 cm deep) with metal or wire tops and wood shavings as bedding. Throughout the selection experiment and during the study, water and food [Harlan Teklad Laboratory Rodent Diet (W) 8604] were available ad libitum. A constant 12:12-h photoperiod was maintained, and room temperature was controlled at ~22°C.

Voluntary wheel-running behavior. In the selection experiment and for the mice used in this study, voluntary wheel running was measured on Wahman-type activity wheels (1.12-m-circumference, 35.7-cm-diameter, 10-cm-wide running surface of 10-mm mesh bounded by clear Plexiglas walls; model 86041 with modifications, Lafayette Instruments, Lafayette, IN). Standard housing cages were attached to the wheels via a 7.7-cm-diameter hole and steel tube, which allowed mice continuous access to the wheels. Attached to each wheel was a photocell counter, which was interfaced to an MS DOS-compatible personal computer. Customized software from San Diego Instruments (San Diego, CA) measured the number of revolutions during every 1-min interval for each wheel. Data were downloaded every 24 h, at which time wheels were checked to remove food pellets and wood shavings and to ensure freedom of rotation. As recorded in this study, wheel running (revolutions/day) can be divided into two components: number of 1-min intervals during which any activity occurred (1-min intervals/day) and mean revolutions per minute (rpm) during the minutes of activity. For the sedentary group (see *Sampling strategy*), wheels were prevented from rotating with a wire tie. In the wheel-access group, voluntary activity was monitored every day for each mouse from 22 days of age until the day before death (mean age at death 78.9 days, range 75–82 days).

Sampling strategy. Our study aimed to determine activities of muscle enzymes (HK, GP, PFK, LDH, CS, CPT, CCO, and PDH) on 10 mice per line without access to wheels (sedentary group) and on 10 mice per line with access to wheels. Within each family and gender, one individual was assigned to a free wheel and one was assigned to a locked wheel (sedentary group). Therefore, each line (4 selected and 4 control) was represented by five wheel-access male, five wheel-access female, five sedentary male, and five sedentary female. Within each line, one mouse in each of these four subgroups came from the same family to obtain a balanced design within family and gender. When, as occurred in two cases, an individual died, the corresponding same-gender sibling was removed to retain a balanced design.

Enzyme extraction and assay. Mice were killed by cervical dislocation to avoid effects of pharmaceuticals. Within ~10 min of death, all the muscles of the left hindlimb (except the triceps surae, which includes the lateral and medial heads of the gastrocnemius, soleus, and plantaris) were frozen on dry ice and then placed at −80°C. The muscle samples were transported in a container with liquid nitrogen to Laval University for enzymatic determinations and measurements of protein concentrations. Subsequent storage was at −80°C.

Enzyme activities were measured using a UV/Vis spectrophotometer (Beckman DU 640) equipped with a temperature-controlled cell holder and a circulating refrigerated water bath (Haake G8). All enzymatic assays were carried out at 37°C using saturating concentrations of substrates and cofactors as determined in preliminary analyses. CS and CPT activities were measured at 412 nm to detect the transfer of sulfhydryl groups to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). CCO activity was measured at 550 nm to follow the oxidation of reduced cytochrome-*c*. PDH activity was monitored at 500 nm to follow NADH production by coupling it to the reduction of *p*-iodonitrotetrazolium violet (INT) via an intermediate electron carrier (lipoamide dehydrogenase). PFK, LDH, HK, and GP activities were measured at 340 nm by following the disappearance of NADH or the production of NADPH. The extinction coefficients for NAD(P)H, DTNB, cytochrome-*c*, and INT were 6.22, 13.6, 19.1 and 15.4 $ml \cdot cm^{-1} \cdot \mu mol^{-1}$, respectively. For all enzymes, reaction rates were linear for ≥4 min. All assays were run in duplicate, and means were analyzed. Specific activities were expressed in international units (μmol substrate transformed to product/min) per gram of tissue wet mass. The pH of all solutions was adjusted at room temperature (20°C) to standardize the pH value at extraction (4°C) and assay (37°C) temperature.

Chemicals and biochemicals were purchased from Sigma Chemical (St. Louis, MO), Boehringer Mannheim (Montréal, PQ, Canada), or ICN Pharmaceuticals (Montréal, PQ, Canada).

The frozen muscle samples were homogenized 1:10 (wt/vol) in 100 mM K_2HPO_4/KH_2PO_4, 5 mM EDTA, 0.1 mM fructose-2,6-bisphosphate, 0.1% Triton X-100, and 1 mM dithiothreitol, pH 7.2 (slightly modified from 30), by use of a Polytron instrument (Brinkmann Instruments, Rexdale, ON, Canada) at half-maximal power for 10 s. Homogenization was finished manually with a 5-ml ground glass tissue grinder.

For CCO (EC 1.9.3.1) assay, 100 mM KH_2PO_4/K_2HPO_4 and 0.1 mM cytochrome-*c* reduced with sodium hydrosulfite ($Na_2S_2O_4$), pH 7.0, were used. After reduction of cytochrome *c* with sodium hydrosulfite, the excess was removed by bubbling with air (15). Reactions were run against a control of 0.1 mM cytochrome-*c* oxidized with 50 μM $K_3Fe(CN)_6$.

For CS (EC 4.1.3.7) assay, 100 mM Tris·HCl, 0.2 mM acetyl CoA, 0.1 mM DTNB, and 1 mM oxaloacetate (omitted for control), pH 8.0, were used.

For PFK (EC 2.7.1.11) assay, 50 mM triethanolamine·HCl, 5 mM $MgCl_2$, 50 mM KCl, 4 mM ATP, 0.28 mM NADH, 3.5 mM fructose 6-phosphate (omitted for control), and excess levels of aldolase (1 U), triosephosphate isomerase (50 U), and α-glycerophosphate dehydrogenase (8 U), pH 7.6, were used.

For HK (EC 2.7.1.1) assay, 50 mM triethanolamine·HCl, 8 mM $MgCl_2$, 0.5 mM NADP, 8 mM ATP, excess levels of glucose-6-phosphate dehydrogenase (4 U), and 4 mM glucose (omitted for control), pH 7.6, were used.

For LDH (EC 1.1.1.27) assay, 50 mM triethanolamine·HCl, 0.28 mM NADH, and 2.4 mM pyruvate (omitted for control), pH 7.6, were used.

For CPT (EC 2.3.1.21) assay, 75 mM Tris·HCl, 1.5 mM EDTA, 0.05 mM palmitoyl CoA, 0.2 mM DTNB, and 2 mM carnitine (omitted for control), pH 8.0, were used.

For GP_a (EC 2.4.1.1) assay, 50 mM KH_2PO_4/K_2PO_4, 0.25 mM EDTA, 15 mM $MgCl_2$, 0.4 mM NADP, 4 μM glucose-1,6-bisphosphate, glycogen (2 mg/ml; omitted for control), and excess levels of phosphoglucomutase (5 U) and glucose-6-phosphate dehydrogenase (5 U), pH 7.0, were used. For measurement of total phosphorylase, 1.6 mM AMP was added.

For PDH (EC 1.2.4.1) assay, 50 mM Tris·HCl, 0.5 mM EDTA, 0.2% Triton X-100, 1 mM $MgCl_2$, BSA (1 mg/ml), 2.5 mM NAD, 0.1 mM CoA, 10 mM oxalate, 0.6 mM INT, 0.2 mM thiamine pyrophosphate, lipoamide dehydrogenase (6 U), and 6 mM pyruvate (omitted for control), pH 7.8, were used.

Protein concentrations. The myofibrillar and sarcoplasmic protein fractions were separated by centrifugation of a 1:10 (wt/vol) homogenate of muscle samples in 100 mM potassium

phosphate buffer (pH 7.0) at 13,200 rpm in a Micromax centrifuge at room temperature for 3 min (3). The pellet was washed twice, and the supernatants of the washings were combined with the initial supernatant to isolate the sarcoplasmic fraction; the pellet represented the myofibrillar fraction. To facilitate protein solubilization, urea and acetic acid were added (36). The protein concentration of the different fractions was determined using bicinchoninic acid (35) and BSA as the standard.

Statistical analysis. The General Linear Models (GLM) procedure in JMP (SAS Institute) was used to apply analysis of covariance (ANCOVA) models to our data. A cross-nested two-way ANCOVA model was used to test simultaneously effects of line type (selected vs. control mice) and activity group (sedentary vs. wheel-access mice) on enzyme activities. All analyses were performed separately for female and male mice *1*) to simplify analyses and interpretation, *2*) because the genders differ in wheel running (21, 22, 38, 40), and *3*) because preliminary analyses of the enzymatic data indicated some statistically significant interactions between gender and line type or activity group.

The two main grouping factors, line type and activity group, were considered fixed effects. Replicate line ($n = 8$ total), nested within line type, was a random effect. In the two-way ANCOVA models, family nested within line was also included as a random effect. In the foregoing mixed models (i.e., with random and fixed effects), we tested the effects over appropriate error terms as follows: in the two-way ANCOVA models, effects of line type were tested over the mean squares of line, and effects of line were tested over the mean squares of family. Effects of activity and the activity $\times$ line type interaction were tested over the mean squares of the activity $\times$ line interaction.

Several covariates were used in the ANCOVA models. Body mass, age, time of death, and (z-transformed time of death)^2 were included as covariates in all models of enzyme activities and protein fraction concentrations. As reported previously, body mass of these mice was affected by line type and activity (40). Within many species, tissue metabolic capacities change with body mass (10, 36). Nevertheless, it is not always clear that adjusting for body mass is the most biologically appropriate statistical approach. Therefore, analyses were also performed without body mass as a covariate. In all cases, adjusted means were calculated by using the least-squares means command in JMP GLM; all covariates in the model, regardless of statistical significance, were used to calculate adjusted means. For a given variable, outliers were removed from ANCOVA models when their studentized residual was >2.5; to retain a balanced design, the corresponding same-gender sibling was removed.

RESULTS

Wheel running. As expected on the basis of results from *generations 10* (21, 37–39) and *13* (21), mice from selected lines (for both genders) covered significantly ($P < 0.05$) more distance (revolutions/day) and ran at higher average velocities (rpm) than controls (Table 1). Mice from the selected lines also spent more time (1-min intervals/day) running, although the difference was statistically significant ($P < 0.05$) only for female mice (40). In selected and control lines, female mice ran more minutes per day and at higher velocities than male mice (Table 1). As reported for previous generations (21, 22, 37–39), the difference in running distance between selected and control lines was still caused primarily by mice from selected lines running faster, not more minutes per day.

Enzyme activities. Many muscle enzyme activities (U/g muscle) were affected by access to running wheels that were free to rotate, as revealed by two-way ANCOVAs (P values in Table 2, adjusted group means in Table 3). Three mitochondrial enzymes had significantly higher activities in free-wheel-access groups for both genders: CCO and CPT ($P < 0.01$ and $P < 0.05$ for male and female mice, respectively) and CS ($P < 0.01$ and $P < 0.001$ for male and female mice, respectively). The level of PDH was significantly greater ($P < 0.001$) only for male mice; for female mice, this trend was apparent only when body mass was not included as a covariate ($P < 0.05$). Similar to the mitochondrial markers, the level of HK was higher ($P < 0.001$) in wheel-access groups for both genders. An additional enzyme of carbohydrate metabolism, GP (as GP_a and GP_{tot}), was decreased by wheel access, but only in female mice ($P < 0.05$). The percentage of phosphorylase *a* did not differ between activity groups (sedentary vs. wheel access) for either gender (data not shown). LDH activities were higher in wheel-access groups for male mice ($P < 0.05$) but tended to be lower in female mice.

As shown in Table 2, no enzyme activity (expressed in U/g muscle) was significantly affected by the selection protocol, except PDH activity, for which the effect was significant ($P = 0.036$) only for female mice and only when body mass was not included as a covariate. However, mitochondrial enzymes (CCO, CS, CPT, and PDH) and two enzymes of carbohydrate metabolism (HK and PFK) tended to be higher for both genders in selected lines than in control lines (Table 3, Fig. 1). Also, we frequently observed significant differences among the replicate lines within line type (Table 2; CCO, CS, HK, GP_{tot}, and PFK).

In our analysis, interactions between line type and activity constitute genotype $\times$ environment interactions. Because ANOVAs (and ANCOVAs) have relatively low power to detect interactions (41), we considered an interaction significant if $P < 0.1$. For female mice, CCO, CS, CPT, HK, GP_a, and GP_{tot} showed significant line type $\times$ activity interactions ($P < 0.1$). For male mice, only CS, CPT, and GP_{tot} showed evidence of such interaction, and the P values were marginally not significant ($P = 0.11$, $F_{1,6} = 3.41$, 3.45, and 3.51, respectively).

To explore the genotype-dependent training response in more detail, we analyzed the measurement groups (control sedentary vs. control wheel access; selected sedentary vs. selected wheel access) with a posteriori tests, which add contrasts between groups, after the two-way ANCOVA (Table 3). An improvement in aerobic capacity with voluntary running was shown by a significant increase of CS activities ($P < 0.01$) in wheel-access mice from the selected lines for both genders compared with their sedentary counterparts (30 and 20% for female and male mice, respectively). These changes were similar to the increases of CCO activities ($P < 0.05$) for both genders in selected lines (26 and

Table 2. *Values for F tests from two-way ANCOVA of enzyme activities of hindlimb muscles*

Enzyme	Line-Within-Line Type		Line Type		Activity		Line Type × Activity	
	M	F	M	F	M	F	M	F
CCO								
All covariates	$F_{6,29} = 4.73$†	$F_{6,30} = 3.73$†	$F_{1,6} = 0.26$	$F_{1,6} = 0.14$	$F_{1,6} = 18.43$†	$F_{1,6} = 6.28$*	$F_{1,6} = 0.18$	$F_{1,6} = 4.45$§
Without body mass	$F_{6,29} = 5.37$‡	$F_{6,30} = 3.85$†	$F_{1,6} = 1.26$	$F_{1,6} = 2.19$	$F_{1,6} = 30.29$†	$F_{1,6} = 10.09$*	$F_{1,6} = 0.28$	$F_{1,6} = 5.71$§
CS								
All covariates	$F_{6,30} = 8.60$‡	$F_{6,28} = 7.49$‡	$F_{1,6} = 0.26$	$F_{1,6} = 0.29$	$F_{1,6} = 19.60$†	$F_{1,6} = 30.07$‡	$F_{1,6} = 3.41$	$F_{1,6} = 10.54$*
Without body mass	$F_{6,30} = 8.05$‡	$F_{6,28} = 8.43$‡	$F_{1,6} = 0.89$	$F_{1,6} = 2.11$	$F_{1,6} = 28.17$†	$F_{1,6} = 48.50$‡	$F_{1,6} = 3.64$	$F_{1,6} = 14.81$*
CPT								
All covariates	$F_{6,30} = 2.14$	$F_{6,31} = 1.62$	$F_{1,6} = 0.93$	$F_{1,6} = 0.003$	$F_{1,6} = 18.16$†	$F_{1,6} = 7.86$*	$F_{1,6} = 3.45$	$F_{1,6} = 4.34$§
Without body mass	$F_{6,30} = 2.38$	$F_{6,31} = 1.48$	$F_{1,6} = 2.99$	$F_{1,6} = 2.47$	$F_{1,6} = 24.10$†	$F_{1,6} = 12.52$*	$F_{1,6} = 3.45$	$F_{1,6} = 5.43$§
PDH								
All covariates	$F_{6,29} = 1.37$	$F_{6,30} = 1.72$	$F_{1,6} = 4.51$	$F_{1,6} = 0.01$	$F_{1,6} = 59.10$‡	$F_{1,6} = 3.72$	$F_{1,6} = 0.58$	$F_{1,6} = 1.04$
Without body mass	$F_{6,29} = 1.30$	$F_{6,30} = 1.62$	$F_{1,6} = 4.66$	$F_{1,6} = 7.18$*	$F_{1,6} = 50.12$‡	$F_{1,6} = 12.11$*	$F_{1,6} = 0.38$	$F_{1,6} = 3.17$
HK								
All covariates	$F_{6,28} = 8.95$‡	$F_{6,31} = 9.68$‡	$F_{1,6} = 0.95$	$F_{1,6} = 0.35$	$F_{1,6} = 66.35$‡	$F_{1,6} = 146.90$‡	$F_{1,6} = 0.63$	$F_{1,6} = 13.52$†
Without body mass	$F_{6,28} = 8.01$‡	$F_{6,31} = 8.22$‡	$F_{1,6} = 1.90$	$F_{1,6} = 2.39$	$F_{1,6} = 81.48$‡	$F_{1,6} = 277.15$‡	$F_{1,6} = 0.81$	$F_{1,6} = 25.12$†
GP_a								
All covariates	$F_{6,30} = 0.80$	$F_{6,31} = 1.41$	$F_{1,6} = 3.01$	$F_{1,6} = 0.10$	$F_{1,6} = 0.50$	$F_{1,6} = 6.45$*	$F_{1,6} = 0.93$	$F_{1,6} = 4.50$§
Without body mass	$F_{6,30} = 1.12$	$F_{6,31} = 1.34$	$F_{1,6} = 0.23$	$F_{1,6} = 1.82$	$F_{1,6} = 0.05$	$F_{1,6} = 10.14$*	$F_{1,6} = 0.51$	$F_{1,6} = 5.64$§
GP_{tot}								
All covariates	$F_{6,31} = 2.96$*	$F_{6,31} = 3.30$*	$F_{1,6} = 0.53$	$F_{1,6} = 0.90$	$F_{1,6} = 0.04$	$F_{1,6} = 9.13$*	$F_{1,6} = 3.51$	$F_{1,6} = 4.21$§
Without body mass	$F_{6,31} = 3.10$*	$F_{6,31} = 3.52$†	$F_{1,6} = 0.04$	$F_{1,6} = 4.21$	$F_{1,6} = 1.42$	$F_{1,6} = 12.58$*	$F_{1,6} = 2.11$	$F_{1,6} = 4.93$§
PFK								
All covariates	$F_{6,31} = 11.11$‡	$F_{6,31} = 3.93$†	$F_{1,6} = 0.02$	$F_{1,6} = 0.07$	$F_{1,6} = 0.33$	$F_{1,6} = 0.006$	$F_{1,6} = 0.008$	$F_{1,6} = 0.003$
Without body mass	$F_{6,31} = 9.89$‡	$F_{6,31} = 3.91$†	$F_{1,6} = 0.39$	$F_{1,6} = 0.01$	$F_{1,6} = 0.06$	$F_{1,6} = 0.113$	$F_{1,6} = 0.002$	$F_{1,6} = 0.036$
LDH								
All covariates	$F_{6,30} = 0.91$	$F_{6,30} = 1.30$	$F_{1,6} = 0.11$	$F_{1,6} = 0.25$	$F_{1,6} = 7.63$*	$F_{1,6} = 2.87$	$F_{1,6} = 1.30$	$F_{1,6} = 0.002$
Without body mass	$F_{6,30} = 0.89$	$F_{6,30} = 1.31$	$F_{1,6} = 1.29$	$F_{1,6} = 1.57$	$F_{1,6} = 4.80$	$F_{1,6} = 3.81$	$F_{1,6} = 0.78$	$F_{1,6} = 0.013$

Covariates used in these models were body mass, age, time of euthanasia, and (z-transformed time of death)^2. Enzyme activity was measured in U/g muscle. ANCOVA, analysis of covariance; CCO, cytochrome-c oxidase; CS, citrate synthase; CPT, carnitine palmitoyltransferase; PDH, pyruvate dehydrogenase; HK, hexokinase; GP_a, glycogen phosphorylase form a; GP_{tot}, total glycogen phosphorylase; PFK, phosphofructokinase; LDH, lactate dehydrogenase. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$; §$P < 0.1$ (noted only for line type × activity).

22% for female and male mice, respectively). However, the lack of a line type × activity group interaction for CCO in male mice (Table 2) indicates that wheel-access male mice from control and selected lines ran enough to elicit significant increases in CCO activities ($P < 0.05$) relative to their sedentary counterparts (21 and 22% in control and selected lines, respectively).

PDH activities also increased in wheel-access groups compared with their sedentary counterparts (19 and 21% in selected and control lines, respectively), but the difference was statistically significant ($P < 0.01$) only in male mice (for both line types). Significant increases in CPT activities ($P < 0.05$ and $P < 0.01$ for female and male mice, respectively) with wheel access were shown for both genders (25 and 44% in female and male mice, respectively) only in selected lines.

Similar to the results for mitochondrial markers, wheel access for either line type significantly increased HK activities ($P < 0.01$) for both genders relative to their sedentary counterparts (19 and 26% in control female and male mice; 30 and 18% in selected female and male mice, respectively). Access to running wheels significantly decreased ($P < 0.05$) GP activities (by 13 and 18% for GP_a and GP_{tot}, respectively) in wheel-access female mice from the selected lines compared with their sedentary counterparts. Wheel-access male mice showed only slight variation in GP levels. Glycolytic capacity, as indicated by PFK activities, decreased slightly in wheel-access mice from both line types compared with sedentary mice.

The effect of wheel access on LDH activities was quite different between genders. For female mice, LDH activities decreased by 7% in wheel-access mice from both line types compared with their sedentary counterparts, whereas wheel-access male mice showed the opposite trend. However, a posteriori tests revealed that the 9% increase in LDH activities in selected male mice with wheel access, compared with their sedentary counterparts, was only marginally significant ($P = 0.046$).

When the foregoing analyses were repeated without body mass as a covariate, the same effects were found, except two significant relationships appeared for PDH in female mice with respect to line type and activity (Table 2). Moreover, for all enzymes (except PDH in female mice) and protein fractions (sarcoplasmic, myofibrillar, and total proteins) tested, body mass was never a statistically significant ($P < 0.05$) covariate. Therefore, body mass does not seem to be a predictor of enzyme activities and protein fractions within the range of masses in this experiment. Nonetheless, our ANCOVAs corrected for any differences in body mass among the line types and activity groups.

The analyses described above are for enzyme data as units per gram of muscle, but activities can also be expressed per total muscle mass. Because the two line

Table 3. *Enzyme activities of hindlimb muscles of the four measurement groups*

	Control Lines				Selected Lines			
	Sedentary (n = 38)		Wheel access (n = 38)		Sedentary (n = 40)		Wheel access (n = 40)	
Enzyme	M	F	M	F	M	F	M	F
CCO								
Mean	56.9 ± 2.3	62.0 ± 2.8	71.9 ± 2.3	65.9 ± 4.0	69.6 ± 4.1	66.7 ± 3.0	87.2 ± 6.1	87.2 ± 4.2
Adjusted mean	59.7 ± 5.3	66.4 ± 6.1	74.1 ± 4.3*	68.7 ± 5.4	68.6 ± 4.2	64.3 ± 4.9	85.8 ± 5.6*	83.2 ± 6.9*
CS								
Mean	59.9 ± 1.6	62.7 ± 1.7	67.9 ± 1.9	70.2 ± 2.0	70.0 ± 5.1	67.9 ± 4.0	87.2 ± 6.1	91.8 ± 5.5
Adjusted mean	62.2 ± 3.8	65.6 ± 3.9	69.7 ± 3.1	72.4 ± 3.5	70.5 ± 3.0	67.7 ± 3.4	86.4 ± 3.9†	91.3 ± 4.8†
CPT								
Mean	0.39 ± 0.03	0.46 ± 0.04	0.51 ± 0.05	0.49 ± 0.04	0.50 ± 0.04	0.49 ± 0.04	0.75 ± 0.06	0.65 ± 0.05
Adjusted mean	0.41 ± 0.07	0.51 ± 0.04	0.53 ± 0.05	0.53 ± 0.03	0.48 ± 0.05	0.46 ± 0.03	0.75 ± 0.07†	0.59 ± 0.04*
PDH								
Mean	3.6 ± 0.2	3.8 ± 0.2	4.3 ± 0.2	4.2 ± 0.2	4.2 ± 0.2	4.2 ± 0.2	5.0 ± 0.2	5.5 ± 0.2
Adjusted mean	3.4 ± 0.1	4.3 ± 0.4	4.2 ± 0.1†	4.6 ± 0.4	4.3 ± 0.1	4.0 ± 0.3	5.2 ± 0.2†	4.8 ± 0.5
HK								
Mean	6.2 ± 0.1	6.3 ± 0.2	8.1 ± 0.3	7.8 ± 0.3	8.2 ± 0.7	7.9 ± 0.5	9.8 ± 0.6	10.8 ± 0.8
Adjusted mean	6.0 ± 0.3	6.6 ± 0.2	7.8 ± 0.3†	8.0 ± 0.2†	8.7 ± 0.3	7.8 ± 0.2	10.4 ± 0.4†	10.5 ± 0.3†
GP_a								
Mean	47.6 ± 2.8	53.3 ± 3.4	44.4 ± 2.3	52.4 ± 2.4	48.8 ± 2.7	55.1 ± 2.7	48.5 ± 2.6	43.1 ± 2.5
Adjusted mean	41.8 ± 4.1	52.7 ± 3.3	41.5 ± 3.3	51.4 ± 2.9	50.4 ± 2.9	54.3 ± 2.5	54.6 ± 3.8	44.2 ± 3.5*
GP_{tot}								
Mean	125 ± 3	130 ± 4	116 ± 4	126 ± 3	119 ± 6	128 ± 4	118 ± 4	105 ± 2
Adjusted mean	117 ± 5	131 ± 6	110 ± 4	126 ± 6	122 ± 3	126 ± 5	127 ± 4	105 ± 6*
PFK								
Mean	58.5 ± 6.5	58.3 ± 7.1	54.3 ± 4.6	57.4 ± 6.1	74.4 ± 8.0	68.2 ± 8.7	72.6 ± 6.6	63.3 ± 7.4
Adjusted mean	64.2 ± 9.8	57.9 ± 11.5	61.0 ± 8.0	57.6 ± 10.2	69.7 ± 7.3	66.8 ± 8.7	65.4 ± 9.5	65.9 ± 12.4
LDH								
Mean	1,002 ± 32	1,060 ± 29	1,027 ± 34	1,009 ± 25	940 ± 31	1,027 ± 37	989 ± 35	941 ± 21
Adjusted mean	953 ± 36	1,064 ± 60	996 ± 31	995 ± 56	956 ± 26	1,023 ± 45	1,050 ± 35*	950 ± 64

Values are means and adjusted means ± SE, expressed in U/g muscle. *$P < 0.05$; †$P < 0.01$ according to a posteriori tests (after 2-way ANCOVAs reported in Table 2) of effect of wheel access within a given line type and gender. Covariates used in these models were body mass, age, time of death, and (z-transformed time of death)^2.

types and the activity groups differ in body mass (40) and because hindlimb muscle mass was related to body mass (18), enzyme activities expressed per total muscle mass are more apt to be correlated with body mass, leading to misleading interpretations of the significance of line type and activity factors. Inasmuch as no additional insight was offered by the analysis of enzyme activities expressed per total hindlimb mass, we concluded that the best way to examine differences among groups was to express data in units per gram of muscle.

Inasmuch as the protein fractions (sarcoplasmic, myofibrillar, and total proteins) expressed by milligrams per gram of muscle in the hindlimb muscles did not differ significantly between line types or with wheel access, with the exception of sarcoplasmic proteins for wheel-access male mice (18), the differences in enzyme activity were unlikely caused by nonspecific changes in muscle hydration or protein concentration.

DISCUSSION

Access to running wheels for 8 wk led to major modifications in the metabolic profile of hindlimb muscles, but effects on specific enzyme activities differed between male and female mice and between our control and selected lines of mice. In particular, we found several statistically significant interactions between line type (selected or control) and activity group (access to freely rotating or locked wheels) in female mice (Table 2). Such interactions mean that the effects of nature (genotype) and nurture (voluntary exercise) are neither independent nor simply additive.

Nevertheless, the interpretation of the interactions seems relatively straightforward. Wheel access changed enzyme activity levels to a greater extent in the selected than in the control lines (especially in female mice), presumably because mice from selected lines ran more revolutions per day and at greater velocities (Table 1). In other words, where an interaction between line type and activity was significant for a given enzyme (Table 2), the selected lines exhibited a greater training response than was observed in the control lines. Thus selection for increased voluntary wheel running clearly has not reduced the capability of muscle aerobic capacity to respond to training. Moreover, a recent study of *generation 13* mice housed as in the present study revealed that selected individuals with access to locked wheels, as in our "sedentary" group, may actually climb in the wheels more than their unselected counterparts (21). Hence, greater training effects of voluntary running might be revealed if the sedentary group were housed in ordinary cages with no access to even locked wheels.

The generalized improvement in aerobic capacity in response to wheel access (Table 2) is consistent with what is commonly observed in response to endurance

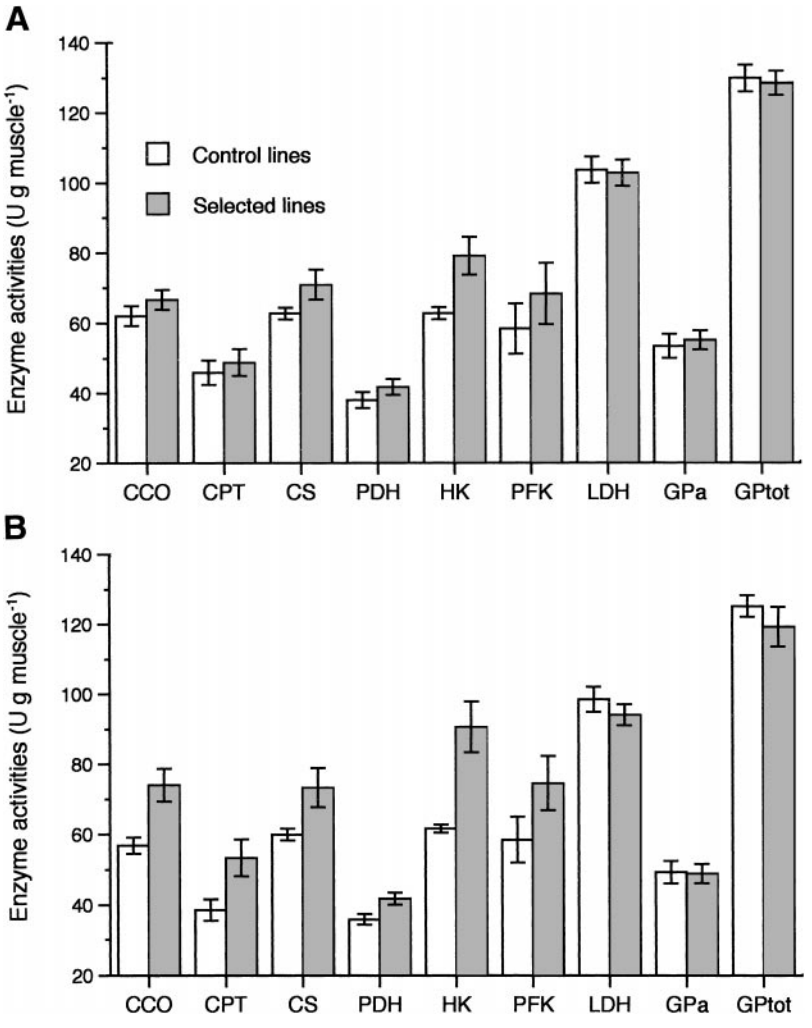

Fig. 1. Enzyme activities (means ± SE) of hindlimb muscles of sedentary female (*A*) and male (*B*) mice. Activities were multiplied by 100 [carnitine palmitoyltransferase (CPT)], 10 [pyruvate dehydrogenase (PDH) and hexokinase (HK)], and 0.1 [lactate dehydrogenase (LDH)]. CCO, cytochrome-*c* oxidase; CS, citrate synthase; PFK, phosphofructokinase; GP_a, glycogen phosphorylase form a; GP_b, inactive form of glycogen phosphorylase; GP_{tot}, total glycogen phosphorylase (GP_a + GP_b).

training in rats and humans via forced treadmill exercise (14, 16, 17) or voluntary wheel running (31, 32). The activities of three mitochondrial enzymes, CCO, CS, and CPT, increased with wheel access for both genders. The significant line type × activity group interactions observed for female but not male mice may simply reflect the greater difference in wheel use (distance and mean velocity) between selected and control female mice compared with male mice (see Table 1 and Fig. 1 in Ref. 40). Consistent with this hypothesis, a previous study of male mice from *generation 10*, in which a similar exercise protocol was used except the sedentary group was housed four per cage with no access to locked wheels, reported that succinate dehydrogenase activity was increased by a similar amount in wheel-access mice from control and selected lines in medial gastrocnemius muscle (42).

The activity of PDH, another mitochondrial enzyme, and one suspected to be a key controller of the relative rates of lactate accumulation and carbohydrate oxidation (4), also increased in wheel-access groups, although this increase was statistically significant only for male mice (Table 3). These results suggest an enhanced capacity for mitochondrial pyruvate oxidation in response to wheel access.

The increased CPT levels in wheel-access mice of both genders (significant only in selected lines) would enhance transport of free fatty acids into mitochondria for their eventual β-oxidation. This rise in CPT activities is consistent with studies on *generation 10*, in which increased fat metabolism is suggested to explain the lower RER in wheel-access mice (39). Enhanced capacity for muscular lipid uptake after voluntary running is also suggested in rats, because the activity of lipoprotein lipase, an enzyme necessary for tissue uptake of plasma triglyceride fatty acids, increased in red vastus lateralis of rats after 7 wk of voluntary wheel running (2).

Higher HK activities were found in wheel-access groups than in their sedentary counterparts for both genders and for selected and control lines. Again, this finding is consistent with previous studies in which HK was increased in rat muscle after voluntary wheel running (31) and forced treadmill endurance training (14). Because voluntary running decreased the activities of GP in wheel-access female mice from selected lines and inasmuch as male mice showed little impact of wheel access on GP levels, we conclude that wheel access enhanced the hindlimb muscles' capacity for aerobic mobilization of blood glucose compared with

the mobilization of muscle glycogen. Thus wheel-access mice, especially female mice, may spare muscle glycogen while the use of blood glucose is enhanced. These changes in capacities of carbohydrate metabolism, particularly those in female mice, are consistent with studies on rat and human muscle after endurance training on motorized treadmills (14, 17). The enhanced use of blood glucose does not require an enhanced glycolytic capacity, as reflected by the relative stability of PFK activities between wheel-access and sedentary mice. Because glycolytic capacities were similar in mice with and without access to wheels (Table 3), the energetic support of higher activity levels in wheel-access mice was likely provided by the greater reliance on fat oxidation during voluntary running (see above).

The changes in certain enzyme activities, particularly those involved in carbohydrate metabolism, in response to wheel access differed markedly between male and female mice. The increase in LDH activities for wheel-access male mice was unexpected. Generally, after endurance training on treadmills, rat and human muscle exhibits lower LDH activities (14, 17). Because male mice ran fewer minutes per day and at lower revolutions per minute than female mice (Table 1), the difference between genders in levels of enzymes of anaerobic metabolism (LDH) may be explained by the volume of daily training (14).

Such differences could also be explained by gender differences in energy expenditure. From studies on *generations 10* (22) and *13* (37), it was concluded that total energy budgets of the mice were more affected by the amount of time active (number of 1-min intervals showing any wheel revolutions) than by the distance traveled (revolutions/day) or average running velocity (rpm). Because selected female mice spent more time (481 min/day) on wheels than did selected male mice (405 min/day; Table 1), one could expect energy expenditure to be greater in female than in male mice. However, studies of rats showed that female rats were less affected than male rats by the energy deficit caused by voluntary wheel running (6). It was found that voluntary wheel running reduced body mass, body fat, and protein mass in male but not in female rats, despite a more negative estimated energy balance in female rats (6). Possible mechanisms include gender-specific hormonal responses. The estrous cycle in female animals induces fluctuations in wheel-running activity, especially at proestrus, where female animals exhibit higher levels of activity on wheels (1). On the other hand, female animals can delay sexual maturation (to the point of total inhibition) when energetic costs of obtaining food do not allow body growth, whereas male animals maintain considerable reproductive development (27–29).

In contrast to our prediction, 14 generations of selection for increased voluntary activity levels did not generally improve muscle aerobic capacities, inasmuch as no significant effect of line type was observed for enzyme levels (except that of PDH in female mice when body mass was not included as covariate). Nonetheless, inspection of the values in Table 3 and Fig. 1 shows that selected lines tended to have higher muscle aerobic capacities, as indicated by the higher levels of mitochondrial enzymes (CCO, CS, CPT, and PDH), HK (a glycolytic enzyme that is associated with mitochondria), and PFK (an indicator of glycolytic aerobic capacity) with concomitant lower anaerobic capacities, as indicated by LDH (especially in male mice). Moreover, heterogeneity in the responses of the replicate lines within the line types, as indicated by the statistically significant line-within-line type effects for CCO, CS, HK, GP_{tot}, and PFK (Table 2), may have masked the anticipated effect of selection for high voluntary wheel running.

Although a main effect of selection per se on enzyme activities of locomotor muscles was not statistically demonstrable, the locomotor behavior of the mice from selected lines was obviously very different from that of the control mice (Table 1). Thus, for these mice, the constraints on the use of muscle metabolic capacities were apparently not sufficient to require their modification by selection that has more than doubled voluntary wheel running. This supports the idea that behavior can evolve substantially without changes in underlying morphological and physiological traits that facilitate the behavior. Comparisons of Hsd:ICR mice with wild mice from a Wisconsin population also support this idea: measurements of behavior and/or whole organism performance show greater differences than do lower level traits (8, 11).

Although selection for increased voluntary wheel running has not led to a general increase in muscle aerobic capacity, mice from selected lines, when given access to running wheels for several weeks, do experience training effects that substantially increase muscle aerobic capacities, and these increases tend to be greater than those exhibited by mice from unselected control lines (Tables 2 and 3). Thus selection, via the intermediary of enhanced voluntary running behavior, has modified the realized aerobic capacities of hindlimb muscles of house mice. This may be viewed as an epigenetic phenomenon in which a behavior affects lower level phenotypic traits that, in turn, affect the ability to express the behavior. Such examples will complicate and enrich our understanding of the ways in which behavior and physiology evolve in a correlated fashion.

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