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J Appl Physiol 101:477-485, 2006. First published Apr 6, 2006; doi:10.1152/japplphysiol.00042.2006

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Submitted 13 January 2006; accepted in final form 27 March 2006

Rezende, Enrico L., Fernando R. Gomes, Jessica L. Malisch, Mark A. Chappell, and Theodore Garland, Jr. Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. *J Appl Physiol* 101: 477–485, 2006. First published April 6, 2006; doi:10.1152/japplphysiol.00042.2006.—We studied relations between maximal O_2 consumption ($\dot{V}O_{2\,max}$) during forced exercise and subordinate traits associated with blood O_2 transport and cellular respiration in four lines of mice selectively bred for high voluntary wheel running (S lines) and their four nonselected control (C) lines. Previously, we reported $\dot{V}O_{2\,max}$ of 59 females at three P_{O_2} (hypoxia = 14% O_2, normoxia = 21%, hyperoxia = 30%). Here, we test the hypothesis that variation in $\dot{V}O_{2\,max}$ can be explained, in part, by hemoglobin concentration and P_{O_2} necessary to obtain 50% O_2 saturation of Hb (an estimate of Hb affinity for O_2) of the blood as well as citrate synthase activity and myoglobin concentration of ventricles and gastrocnemius muscle. Statistical analyses controlled for body mass, compared S and C lines, and also considered effects of the mini-muscle phenotype (present only in S lines and resulting from a Mendelian recessive allele), which reduces hindlimb muscle mass while increasing muscle mass-specific aerobic capacity. Although S lines had higher $\dot{V}O_{2\,max}$ than C, subordinate traits showed no statistical differences when the presence of the mini-muscle phenotype was controlled. However, subordinate traits did account for some of the individual variation in $\dot{V}O_{2\,max}$. Ventricle size was a positive predictor of $\dot{V}O_{2\,max}$ at all three P_{O_2}. Blood Hb concentration was a positive predictor of $\dot{V}O_{2\,max}$ in S lines but a negative predictor in C lines, indicating that the physiological underpinnings of $\dot{V}O_{2\,max}$ have been altered by selective breeding. Mice with the mini-muscle phenotype had enlarged ventricles, with higher mass-specific citrate synthase activity and myoglobin concentration, which may account for their higher $\dot{V}O_{2\,max}$ in hypoxia.

cardiac output; experimental evolution; hemoglobin; hypoxia tolerance; myoglobin

A MAJOR GOAL OF EVOLUTIONARY PHYSIOLOGY is to understand how particular types of selection may cause changes at multiple levels of biological organization. For instance, when selection acts at the level of whole-animal performance, which of the many lower-level traits that potentially affect organismal ability change in concert? Do all potentially relevant subordinate traits change or only a few key ones? Although interspecific comparative studies are more traditional, physiologists are now routinely employing selection experiments of various types to study the evolution of complex traits, such as organismal performance or susceptibility to disease (34).

As an example, laboratory rats have been bidirectionally selected for treadmill endurance-running performance (19), which led to significant differences in maximal O_2 consumption ($\dot{V}O_{2\,max}$) during forced treadmill exercise between the high-capacity runner and low-capacity runner lines. At *generation 7*, the high-capacity runner line attained mass-specific $\dot{V}O_{2\,max}$ 11.8 and 21.0% higher than the low-capacity runner line in normoxia and hypoxia (~10% O_2), respectively (10). These differences were mainly related to differences in O_2 extraction by skeletal muscle, in association with higher capillary-to-fiber surface ratios in muscles of high-capacity runners (10, 14), and apparently less related to differences in cardiac output (15).

We have used selective breeding to produce four replicate lines of laboratory house mice (S lines) that, at an apparent selection limit (plateau), run voluntarily on wheels ~170% more than four nonselected lines that serve as controls (C lines; e.g., Refs. 7, 12, 13, 28–31). These lines are an interesting counterpart to those of Koch and Britton (10, 14, 15, 19) because they have been selected for voluntary rather than forced locomotor behavior. Whereas it makes intuitive sense that their selection protocol resulted in a substantial divergence in $\dot{V}O_{2\,max}$, it is not obvious that our selection for high voluntary locomotor activity over a 24-h period would lead to an increase in $\dot{V}O_{2\,max}$ (or locomotor endurance per se) relative to unselected control (C) lines. Indeed, some previous studies have suggested that voluntary wheel-running distance and $\dot{V}O_{2\,max}$ or treadmill running endurance are largely distinct phenotypes in mice (e.g., Refs. 5, 21, and references therein).

Nevertheless, results to date indicate that mass-specific $\dot{V}O_{2\,max}$ in normoxia has increased in response to selection for high voluntary wheel running: 6.7% in males from *generation 10* (35); 33% for males from *generation 33* (29); 14.5% in females from *generation 35* (28, 29). Most recently, measurements of females from *generation 36* at different P_{O_2} showed that the S lines achieved mass-specific $\dot{V}O_{2\,max}$ on average 20.7% higher than C lines in hypoxia (P_{O_2} = 14%), 24.0% higher in normoxia, and 25.8% higher in hyperoxia (P_{O_2} = 30%; Ref. 30).

Relations between a voluntary behavior, such as wheel running, and the ability to engage in that behavior (e.g., locomotor endurance) can be complicated, especially when both the behavior and underlying abilities are evolving across generations (5, 11, 36). At first thought, it may not be obvious why physiological limitations (e.g., related to $\dot{V}O_{2\,max}$) would affect voluntary wheel running. For a given sample of mice, one possibility is that some individuals have extremely high levels of motivation for wheel running but lack the physiological ability to express that "desire." Other individuals might

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have a high ability to run on wheels but lack the motivation to do so. Depending on the prevailing phenotypes in the starting population for a selection experiment, improvement in average wheel running within a line might require increased motivation, increased ability, or both. Moreover, the components of wheel running (i.e., motivation and ability) might evolve at different rates and somewhat differently among replicate lines. For our S lines of mice, the data on $\dot{V}O_{2\,max}$ summarized in the previous paragraph suggest that S mice may also exhibit significantly higher exercise ability (endurance), a possibility that has not yet been tested. Moreover, S mice were able to increase their $\dot{V}O_{2\,max}$ in hyperoxia, relative to normoxia, to a greater extent than C lines (30). We therefore proposed that differences in $\dot{V}O_{2\,max}$ observed between S and C mice are probably associated with differences in diffusive and convective components of the O_2 cascade (30).

The present study is an initial investigation of subordinate traits that might account for the higher $\dot{V}O_{2\,max}$ of S lines compared with C and/or account for individual variation in $\dot{V}O_{2\,max}$ within the S and C groups. We used the same sample of mice employed by Rezende et al. (30). We focused primarily on morphological and biochemical properties of skeletal and cardiac muscles, including organ size, citrate synthase (CS) activity as an indicator of cellular aerobic capacity, and myoglobin concentration. We also measured two traits involved in blood O_2 transport, hemoglobin concentration ([Hb]) and hemoglobin-O_2 affinity. As in previous studies of these lines, we employ our standard nested analysis of covariance (ANCOVA) model (with body mass as a covariate) to compare S and C lines with respect to various phenotypes. Then, to test for statistical effects of the candidate subordinate traits (e.g., blood hemoglobin content) on $\dot{V}O_{2\,max}$, we employ the same statistical model but with the addition of a candidate subordinate trait. If an S vs. C difference in $\dot{V}O_{2\,max}$ disappears (or is substantially reduced) when a candidate subordinate trait is entered into the model, and the subordinate trait is itself a statistically significant predictor of $\dot{V}O_{2\,max}$, then we infer that the trait accounts for an important amount of the difference in $\dot{V}O_{2\,max}$ between S and C lines. If an S vs. C difference in $\dot{V}O_{2\,max}$ is not diminished by addition of a candidate subordinate trait, then whether the subordinate trait is itself a statistically significant predictor of $\dot{V}O_{2\,max}$, we infer that other traits must account for the difference in $\dot{V}O_{2\,max}$ between S and C lines.

An unexpected discovery in our selection experiment has been the presence of a Mendelian recessive allele that halves hindlimb muscle mass while doubling muscle mass-specific aerobic capacity (7, 13, 38). This allele has increased in frequency in two of the four S lines, indicating that it has been favored by the selection protocol (the other two S lines apparently lost it by chance via random genetic drift; see Ref. 7). Subsequently, we have found that the allele has gone to fixation in one of the two S lines (all individuals now express the phenotype) and remained polymorphic in the other (38). Unexpectedly, individuals with the "mighty mini-muscle" phenotype (homozygous for the allele) achieved significantly higher $\dot{V}O_{2\,max}$ in hypoxia but not in normoxia or hyperoxia (30). Therefore, we also test whether the mini-muscle phenotype is associated with subordinate traits that may affect $\dot{V}O_{2\,max}$.

MATERIALS AND METHODS

Animals and experimental protocol. As described previously (30), we sampled 59 females from *generation 36* of the artificial selection experiment for high voluntary wheel-running behavior. We chose females because they run both more and faster in absolute terms compared with males (29). Hence, it seemed more likely that $\dot{V}O_{2\,max}$ could be a limiting factor to wheel running in females. The subordinate predictors of $\dot{V}O_{2\,max}$ may well differ between males and females, and indeed we plan to explore that possibility in future studies. In brief, the selective breeding experiment has used within-family selection for high voluntary wheel running (quantified as total revolutions during *days 5* plus *6* of a 6-day trial) in four independent lines (S lines) derived originally from an outbred, genetically variable population of laboratory house mice. Four C lines are also maintained, in which parents are chosen randomly with respect to wheel running. Sib matings are disallowed in all lines to minimize inbreeding.

Maximum aerobic capacity during forced exercise ($\dot{V}O_{2\,max}$) was measured in hypoxia (14% O_2), normoxia (21% O_2), and hyperoxia (30% O_2) using open-flow respirometry, as described previously (30). Summarizing, for logistical reasons, mice were separated at random into five measurement batches of 12 individuals and were tested in an enclosed motorized treadmill during 2 consecutive days in each atmospheric P_{O_2} (randomized order). Trials lasted $<$15 min and ended when mice could no longer keep pace with the treadmill and/or O_2 uptake did not increase with further increases in treadmill speed.

The day after the 6 days of measurements (i.e., 2 days at each P_{O_2} and three different P_{O_2}), individuals were anesthetized with isofluorane, and blood samples were obtained with infra-orbital puncture using heparinized microhematocrit tubes (75 μl), which were then transferred to 20-μl tubes. Mice were then killed by cervical dislocation, and left and right gastrocnemius muscles (lateral plus medial heads) and the ventricles of the heart were dissected, weighed, and immediately frozen in liquid nitrogen and kept at −80°C until biochemical analyses. To minimize potential errors caused by inter-individual differences in methodology, each researcher performed the same task throughout the experiment, from measurements of $\dot{V}O_{2\,max}$ on the treadmill to biochemical and hematological assays. Age at death averaged 102 days, with a range of 86–117 days. All animal procedures are in compliance with the Univeristy of California, Riverside, Institutional Animal Care and Use Committee and US laws.

Hematology and biochemistry. Hb concentration ([Hb]) was estimated in duplicate or triplicate with a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA) at 540 nm, employing a protocol slightly modified from the one described by Sigma Diagnostics (33). Briefly, 20 μl of blood was transferred into a test tube containing 5 ml of Drabkin's reagent, incubated for 30 min while exposed to room light and at room temperature, and then maintained in the dark at ~4°C until reading at the end of the day. Concentrations were interpolated from calibration curves obtained with standard hemoglobin solutions (J. T. Baker, catalog no. 3075, baker.com/clinical/clinical_cyanide.html) diluted in Drabkin's reagent in the following concentrations: 0.0, 5.0, 10.0, 15.0, and 20.0 g/100 ml.

Hemoglobin O_2 affinity was estimated with a Hemox analyzer (TCS, Medical Products Division, Southampton, PA) connected to a thermoregulated water bath at 37°C (RTE 111, Neslab Instruments, Newington, NH). Affinity was estimated by plotting P_{O_2} vs. the degree of Hb saturation with O_2 (monitored by dual-wavelength spectrophotometry at 560 and 576 nm) (9) and is expressed here as the P_{O_2} at which 50% of Hb is saturated with O_2 (P_{50}; obtained by regression). Samples of not less than 30 μl of blood were diluted in 5 ml of buffer (pH = 7.4 ± 0.1) at 37°C. The sample was then oxygenated to 100% with air, P_{O_2} was adjusted, and the deoxygenation curve was recorded while the sample was flushed with N_2. Measurements of P_{50} were performed in duplicate for each individual.

We measured CS activity and myoglobin concentration in both skeletal (medial gastrocnemius) and cardiac muscles with spectrophotometry (Spectramax Plus, Molecular Devices, Sunnyvale, CA). Because tissue samples were taken the day after the last treadmill measurement, any short-term effects of exercise on CS activity (within minutes or hours, e.g., Ref. 20) or myoglobin concentration would be minimized. Activity of CS was estimated using the same protocol described in Houle-Leroy et al. (12), measuring the rates of transfer of sulfhydryl groups to 5,5-dithiobis(2-nitrobenzoic acid) at 412 nm. Final dilution of gastrocnemius and ventricle tissues for CS assays was 1:20,000 (wt/vol). Myoglobin concentration was calculated from the difference between absorbances obtained at 538 and 568 nm, following the protocol developed by Reynafarje (26). Measurements of myoglobin concentration were performed in quadruplicate, except for two individuals with the mini-muscle phenotype, where measurements were performed in triplicate because of their remarkably smaller gastrocnemius (about half of the size of the same muscle in a normal mouse; Refs. 7, 38).

Statistical analyses. Analyses were performed with SPSS for Windows or SAS PROC MIXED (SAS Institute). We first compared subordinate traits of S and C lines (line type effect) by nested ANCOVA models with type III tests of fixed effects. In these models, line type was the grouping variable (main effect), replicate lines ($n = 8$ in total) were nested within line type as a random factor, mini-muscle phenotype was a fixed effect, and body mass and age were included as covariates. Tests of line-type effects were always performed with 1 and 6 degrees of freedom. We also performed the analyses without body mass as a covariate because *1*) for such traits as [Hb] or enzyme activity measured per gram of tissue it is not clear that body mass should be used as a covariate (e.g., Ref. 12) and *2*) mice from S lines are smaller than C (36). The likelihoods of models estimated with and without replicate line (random effect) in the model were used to test the significance of line effects (twice the difference in log likelihoods was compared with a χ^2 distribution with 1 degree of freedom). In a separate set of analyses, we analyzed effects of the mini-muscle phenotype within the one polymorphic line (laboratory designation is *line 6*; $N = 12$ and 5 with normal and mini-muscle phenotype, respectively). We used ANCOVA with mini-muscle as a fixed effect and age as a covariate, and ran models both with and without body mass as a covariate.

Apparent outliers in our enzymatic and hematological data were removed before final analyses, as in Rezende et al. (Ref. 29 and references therein): we created a 0–1 dummy variable for a putative outlier (assigning 1 to the datum that is a potential outlier) and computed the significance as the nominal P value multiplied by the number of data points in the analysis. When the resulting P value is <0.05, then the datum is considered to represent a statistical outlier and is removed from further analyses. Based on this procedure, we deleted one individual for P_{50} and one for cardiac myoglobin concentration.

Finally, we tested whether subordinate traits were significant predictors of individual variation in $\dot{V}O_{2\,max}$ once other factors had been

Table 1. *Effects of selection, lines, body mass, and the mini-muscle phenotype*

	N	Selected	Control	S/C	$P_{selection}$	P_{line}	$P_{body\ mass}$	P_{mini}
Gastrocnemius mass, g	55	0.0924±0.0026	0.1010±0.0028	0.915	0.0831	0.0568	<0.001*[+]	<0.0001*[−]
		0.0852±0.0104	0.1102±0.0105	0.773	0.1460	<0.0001*	0.0200*[+]	
Gastrocnemius CS, U/muscle	54	5.449±1.101	6.559±1.124	0.831	0.5230	0.0007*	0.2850 [−]	0.5702 [−]
		5.367±1.047	6.670±1.063	0.805	0.4290	0.0010*	0.3011 [−]	
Gastrocnemius myoglobin, mg/muscle	53	0.1059±0.0061	0.1029±0.0069	1.029	0.7816	1	0.1272 [+]	0.6114 [−]
		0.1049±0.0057	0.1041±0.0064	1.008	0.9289	1	0.1370 [+]	
Mass-specific CS activity, U/g†	54	59.3 (43.4–81.0)	60.8 (44.2–83.6)	0.975	0.9190	0.0001*	0.1351 [−]	<0.0001*[+]
		62.6 (47.5–82.4)	57.4 (43.3–76.0)	1.090	0.6878	0.0028*		<0.0001*[+]
		69.6 (56.4–85.8)	49.9 (40.4–61.6)	1.394	0.3075	<0.0001*		
Mass-specific myoglobin, mg/g†	53	1.15 (1.02–1.29)	1.02 (0.90–1.16)	1.123	0.2914	1	0.5346 [+]	<0.0001*[+]
		1.13 (1.02–1.25)	1.04 (0.94–1.17)	1.080	0.3628	1		<0.0001*[+]
		1.26 (1.11–1.43)	0.92 (0.81–1.05)	1.369	0.1321	0.0269*		
Ventricle mass, g	55	0.1269±0.0029	0.1228±0.0032	1.033	0.4294	0.2766	0.0006*[+]	0.0789 [+]
		0.1282±0.0030	0.1212±0.0032	1.058	0.1793	0.2401	0.0005*[+]	
Ventricle CS, U/muscle	54	38.45±2.03	36.77±2.30	1.045	0.6414	0.2314	0.0953 [+]	0.4588 [+]
		38.94±1.83	36.23±2.08	1.075	0.4049	0.3356	0.0705 [+]	
Ventricle myoglobin, mg/muscle	54	0.7310±0.0239	0.6493±0.0263	1.126	0.1537	0.3087	<0.0001*[+]	0.0680 [+]
		0.7239±0.0219	0.6350±0.0242	1.140	0.0454*	0.4292	<0.0001*[+]	
Mass-specific CS activity, U/g	54	299.8±14.32	303.6±16.18	0.987	0.8802	0.3315	0.6714 [−]	0.9546 [+]
		302.7±12.50	300.1±13.73	1.008	0.8988	0.3660		0.9917 [−]
Mass-specific myoglobin, mg/g	54	5.60±0.099	5.28±0.120	1.061	0.1205	1	0.0270*[+]	0.4805 [+]
		5.50±0.091	5.43±0.107	1.013	0.6512	1		0.2680 [+]
[Hb], g/dl blood	53	14.12±0.187	14.68±0.204	0.962	0.1152	0.2007	0.1007 [−]	0.2988 [−]
		14.24±0.159	14.53±0.172	0.980	0.2726	0.3557		0.1806 [−]
P_{50}, Torr	49	53.07±1.246	52.27±1.351	1.015	0.7163	1	0.7016 [+]	0.2005 [−]
		52.85±1.091	52.53±1.168	1.006	0.8544	1		0.2140 [−]

Values are adjusted (least squares) means calculated for all variables from SAS PROC MIXED for a female mouse 95 days of age (range was 79–110 days) and 27.5 g body mass (±0.1g difference among analyses because sample size varied slightly). The model tested for effects of line type [$P_{selection}$; selected (S) vs. control (C) groups] nested over random line effects (P_{lines}), while controlling for body mass and presence of the mini-mucle phenotype ($P_{body\ mass}$ and P_{mini}). Values are means ± SE, except for traits that were log-transformed, where we report within parentheses the asymmetrical 95% confidence interval obtained after back-transformation. For effects of body mass and the mini-muscle, we also report sign of the partial regression coefficient (+ indicates mini > normal). [Hb], Hb concentration; P_{50}, P_{O_2} necessary to obtain 50% O_2. *Significant values at $P < 0.05$ (2-tailed and unadjusted for multiple comparisons). †Log-transformed for statistical analyses to improve normality of residuals.

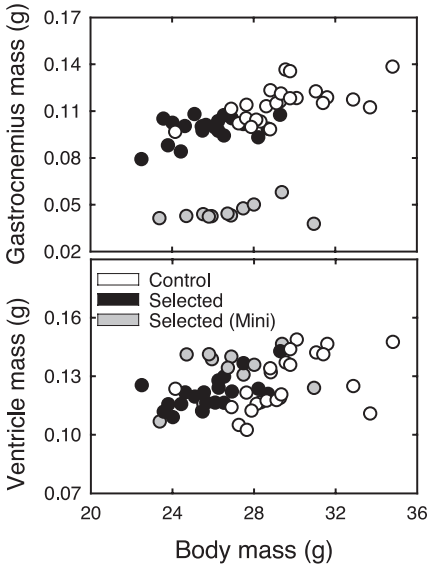

Fig. 1. Relation between body mass and wet muscle mass of gastrocnemius and ventricles for females from 4 lines selected for high wheel running (S) and 4 control lines (C) (N = 59 total). Within S lines, individuals with the "mini-muscle" phenotype (see text) are highlighted in gray.

controlled statistically. We used the full nested ANCOVA models as described above, and as presented in Rezende et al. (30), but added the subordinate traits studied here as additional covariates. Because the mini-muscle phenotype (coded as 0 = normal, 1 = mini) was highly correlated with gastrocnemius mass ($r = -0.9$), we excluded muscle mass from the analyses to avoid problems with multicollinearity. Preliminary analyses showed that P_{50} was never a significant predictor of $\dot{V}O_{2\,max}$ at any P_{O_2}, and data were missing for several individuals, so this trait was excluded from final analyses. To test for possible differences between S and C mice in the relations of subordinate traits to $\dot{V}O_{2\,max}$, we added interaction terms of the trait*line type, which were tested for significance over the interaction of trait*line(line type) with 1 and 6 degrees of freedom.

RESULTS

Effects of line type and mini-muscle. S and C lines showed no statistically significant difference for any subordinate trait when the presence of the mini-muscle phenotype was controlled statistically (Table 1). However, replicate lines showed significant variation in CS activity, as has been reported previously for mixed hindlimb muscle of animals from *generation 14* (13). As expected, mini-muscle gastrocnemius were ~50% smaller in mass compared with normal mice (Fig. 1). Also as expected (13), mini-muscles have a mass-specific CS activity that was about twofold higher than normal muscles (Fig. 2, Table 1). Myoglobin concentration was also significantly higher in mini muscles (Fig. 2, Table 1). In addition, mini-muscle individuals tended to have enlarged ventricles (Fig. 1), as has been reported previously (7, 37). Analyses of only the individuals within selected *line 6* were consistent with all of these effects of the mini-muscle phenotype (Table 2).

$\dot{V}O_{2\,max}$ and subordinate traits. Which subordinate traits were significant predictors of $\dot{V}O_{2\,max}$ depended on both line type and the atmospheric P_{O_2} during treadmill trials (Table 3). The nested ANCOVAs pooling S and C mice indicated that $\dot{V}O_{2\,max}$ was positively correlated with ventricle mass, regardless of P_{O_2} (Fig. 3, Table 3). When line types were analyzed separately, the correlation was always statistically significant for S mice, but not for C mice, which may simply reflect the smaller sample size and hence fewer degrees of freedom for the latter (Table 3).

In the pooled analyses of S and C lines, $\dot{V}O_{2\,max}$ was positively related to [Hb] in hypoxia, mainly because of the strong correlation in S lines (Table 3). The separate analyses of

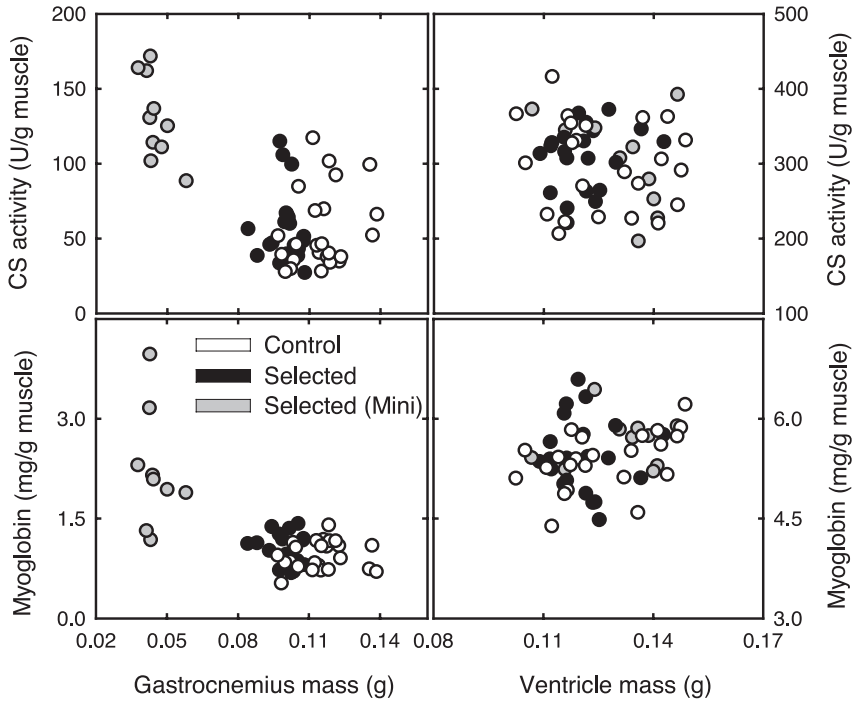

Fig. 2. Wet muscle mass of gastrocnemius (*left*) and ventricles (*right*) vs. citrate synthase (CS) activity and myoglobin concentration for mice shown in Fig. 1. Raw values are shown, not controlled for effects of body size or other factors (see text).

Table 2. *Effects of the mini-muscle phenotype*

	N	Normal	Mini-muscle	Mini/Normal	$P_{body\ mass}$	P_{mini}
Body mass, g	16	26.70±0.50	26.39±0.87	0.988		0.7634
Gastrocnemius mass, g	13	0.0992±0.0016	0.0477±0.0024	0.481	0.0376*	<0.0001*
Gastrocnemius CS, U/muscle	13	6.818±0.729	5.542±1.106	0.813	0.3005	0.3666
Gastrocnemius myoglobin, mg/muscle	13	0.1012±0.0075	0.1000±0.0114	0.988	0.0831	0.9328
Mass-specific CS activity, U/g†	13	65.3 (53.8–79.1)	118.3 (88.4–158.4)	1.813	0.0826	0.0093*
	13	63.7 (51.3–79.0)	125.0 (90.4–172.9)	1.964		0.0069*
Mass-specific myoglobin, mg/g†	13	0.992 (0.844–1.165)	2.028 (1.588–2.589)	2.044	0.2500	0.0011*
	13	1.004 (0.853–1.183)	1.969 (1.539–2.519)	1.961		0.0012*
Ventricle mass, g	13	0.1227±0.0028	0.1340±0.0043	1.092	0.0058*	0.0603
Ventricle CS, U/muscle	13	39.77±1.52	46.55±2.31	1.170	0.0037*	0.0389*
Ventricle myoglobin, mg/muscle	13	0.6652±0.0203	0.7632±0.0308	1.147	0.0130*	0.0276*
Mass-specific CS activity, U/g	13	324.0±11.6	348.2±17.6	1.075	0.1702	0.2868
	13	326.2±12.2	343.2±18.3	1.052		0.4593
Mass-specific myoglobin, mg/g	13	5.428±.136	5.676±0.206	1.046	0.7959	0.3462
	13	5.433±.128	5.666±0.193	1.043		0.3380
[Hb], g/dl blood	12	14.07±.22	13.89±0.32	0.987	0.3752	0.6628
	12	14.05±.22	13.93±0.31	0.992		0.7629
P_{50}, Torr	13	51.18±1.65	50.64±2.36	0.989	0.3811	0.8571
	13	51.40±1.62	50.20±2.29	0.977		0.7752

Effects were calculated within the only line where it remains polymorphic [selected *line 6* (lab designation)], obtained from regular analysis of covariance with or without body mass as a covariate (see METHODS). Values are adjusted means ± SE (except for traits that were log-transformed, where we report within parentheses the asymmetrical 95% confidence interval around the mean obtained after back-transformation), obtained from SAS PROC MIXED including mini-muscle as a fixed effect (P_{mini}), and body mass ($P_{body\ mass}$) and age as covariates. *Statistically significant values (2-tailed, $P < 0.05$, unadjusted for multiple comparisons). †Log-transformed for statistical analyses to improve normality of residuals.

Table 3. *Analysis of covariance for $\dot{V}O_{2max}$ of mice from S and C lines in hypoxia, normoxia, and hyperoxia*

	$\dot{V}O_{2max}$ Hypoxia	$\dot{V}O_{2max}$ Normoxia	$\dot{V}O_{2max}$ Hyperoxia
		S + C (N = 48)	
S adjusted mean ± SE, ml/min	4.894±0.107	5.494±0.194	6.276±0.281
C adjusted mean ± SE, ml/min	4.571±0.117	5.243±0.215	5.362±0.309
S/C ratio	1.071	1.048	1.170
S vs. C	$F(1,6) = 3.12$, $P = 0.1277$ [+]	$F(1,6) = 0.75$, $P = 0.4183$ [+]	$F(1,6) = 6.01$, $P = 0.0497$* [+]
Body mass, g	$F(1,27) = .50$, $P = .4867$ [+]	$F(1,27) = 0.01$, $P = 0.7107$ [−]	$F(1,27) = 0.30$, $P = 0.5901$ [+]
Gastrocnemius CS activity, U/g	$F(1,27) = 3.02$, $P = 0.0934$ [+]	$F(1,27) = 0.20$, $P = 0.6556$ [+]	$F(1,27) = 1.62$, $P = 0.2135$ [+]
Gastrocnemius myoglobin, mg/g	$F(1,27) = 1.57$, $P = 0.2204$ [+]	$F(1,27) = 0.15$, $P = 0.7010$ [−]	$F(1,27) = 0.17$, $P = 0.6876$ [−]
Ventricle mass, g	$F(1,27) = 16.54$, $P = 0.0004$* [+]	$F(1,27) = 9.97$, $P = 0.0039$* [+]	$F(1,27) = 9.73$, $P = 0.0043$* [+]
Ventricle CS activity, U/g	$F(1,27) = 0.32$, $P = 0.5772$ [+]	$F(1,27) = 0.23$, $P = 0.6320$ [−]	$F(1,27) = 0.43$, $P = 0.5199$ [+]
Ventricle myoglobin, mg/g	$F(1,27) = 1.08$, $P = 0.3084$ [+]	$F(1,27) = 2.10$, $P = 0.1587$ [+]	$F(1,27) = 0.02$, $P = 0.8868$ [+]
[Hb], g/dl blood	$F(1,27) = 5.82$, $P = 0.0229$* [+]	$F(1,27) = 0.10$, $P = 0.7526$ [+]	$F(1,27) = 0.02$, $P = 0.8966$ [−]
Mini-muscle [0–1]	$F(1,27) = 0.40$, $P = 0.5318$ [−]	$F(1,27) = 0.07$, $P = 0.7955$ [−]	$F(1,27) = 0.56$, $P = 0.4623$ [−]
		S (N = 27)	
Body mass, g	$F(1,10) = 0.19$, $P = 0.4125$ [+]	$F(1,10) = 2.00$, $P = 0.1877$ [+]	$F(1,10) = 0.97$, $P = 0.3482$ [−]
Gastrocnemius CS activity, U/g	$F(1,10) = 4.28$, $P = 0.0654$ [+]	$F(1,10) = .30$, $P = .5947$ [+]	$F(1,10) = 1.96$, $P = 0.1922$ [+]
Gastrocnemius myoglobin, mg/g	$F(1,10) = 0.25$, $P = 0.6245$ [+]	$F(1,10) = 0.07$, $P = 0.8002$ [+]	$F(1,10) = 1.15$, $P = 0.3088$ [−]
Ventricle mass, g	$F(1,10) = 14.33$, $P = 0.0036$* [+]	$F(1,10) = 9.98$, $P = 0.0102$* [+]	$F(1,10) = 11.32$, $P = 0.0072$* [+]
Ventricle CS activity, U/g	$F(1,10) = 3.63$, $P = 0.0857$ [+]	$F(1,10) = 0.92$, $P = 0.3601$ [+]	$F(1,10) = 1.31$, $P = 0.2784$ [+]
Ventricle myoglobin, mg/g	$F(1,10) = 0.05$, $P = 0.8273$ [+]	$F(1,10) = 1.65$, $P = 0.2274$ [+]	$F(1,10) = 1.85$, $P = 0.2035$ [+]
[Hb], g/dl blood	$F(1,10) = 10.62$, $P = 0.0086$* [+]	$F(1,10) = 16.26$, $P = 0.0024$* [+]	$F(1,10) = 0.40$, $P = 0.5404$ [+]
Mini-muscle [0–1]	$F(1,10) = 1.09$, $P = 0.3212$ [−]	$F(1,10) = .05$, $P = 0.8258$ [−]	$F(1,10) = 0.42$, $P = 0.5322$ [−]
		C (N = 21)	
Body mass, g	$F(1,5) = 1.03$, $P = 0.3563$ [+]	$F(1,5) = 1.58$, $P = 0.2649$ [+]	$F(1,5 = 3.25$, $P = 0.1315$ [+]
Gastrocnemius CS activity, U/g	$F(1,5) = 0.02$, $P = 0.9015$ [+]	$F(1,5) = 0.01$, $P = 0.9169$ [−]	$F(1,5) = 2.43$, $P = 0.1795$ [+]
Gastrocnemius myoglobin, mg/g	$F(1,5) = 0.30$, $P = 0.6053$ [+]	$F(1,5) = 0.46$, $P = 0.5273$ [−]	$F(1,5) = 2.75$, $P = 0.1584$ [−]
Ventricle mass, g	$F(1,5) = 5.73$, $P = 0.0620$ [+]	$F(1,5) = 5.97$, $P = 0.0584$ [+]	$F(1,5) = 2.90$, $P = 0.1494$ [+]
Ventricle CS activity, U/g	$F(1,5) = 0.85$, $P = 0.3994$ [−]	$F(1,5) = .76$, $P = 0.4245$ [−]	$F(1,5) = 0.49$, $P = 0.5131$ [+]
Ventricle myoglobin, mg/g	$F(1,5) = 0.17$, $P = 0.6997$ [+]	$F(1,5) = 0.10$, $P = 0.7682$ [+]	$F(1,5) = 0.38$, $P = 0.5665$ [−]
[Hb], g/dl blood	$F(1,5) = 0.24$, $P = 0.6466$ [+]	$F(1,5) = 9.05$, $P = 0.0298$* [−]	$F(1,5) = 4.00$, $P = 0.1020$ [−]

Hypoxia, 14% O_2; normoxia, 21% O_2; hyperoxia, 30% O_2. Analyses of covariance were performed controlling for effects of subordinate traits (shown as partial *F* and 2-tailed *P* values). Measurement batch and replicate line were included as random effects in these analyses (results not shown), and age was an additional covariate (results not shown). Signs of partial regression coefficients for selection history (S vs. C), body mass, and subordinate traits are in brackets (for selection history, + indicates S > C). *Statistically significant (2-tailed, $P < 0.05$, unadjusted for multiple comparisons).

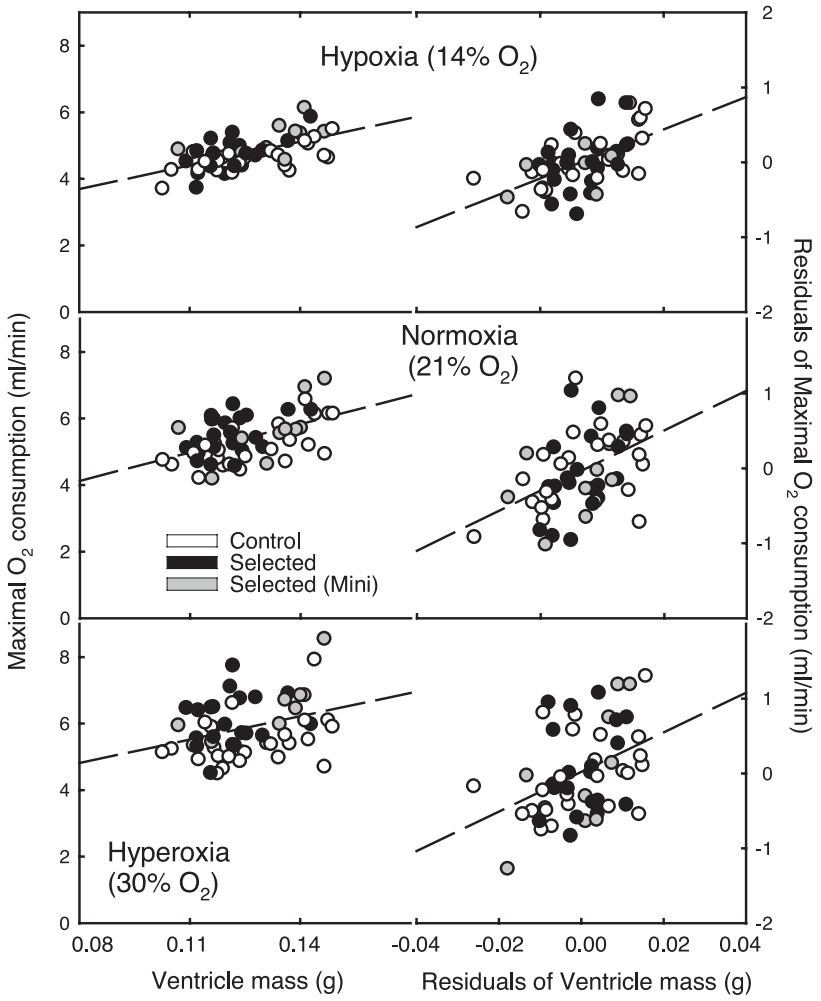

Fig. 3. Relationship between ventricle mass and $\dot{V}O_{2\,max}$ obtained during forced-exercise treadmill trials performed in hypoxic (14% O_2), normoxic (21% O_2), and hyperoxic atmospheres (30% O_2), plotted as raw data (*left*) or residuals from nested analysis of covariance obtained separately for $\dot{V}O_{2\,max}$ and ventricle mass controlling for selection history, size, age, batch, additional subordinate traits, and the mini-muscle phenotype (*right*; see Table 3 for full models). Dashed lines indicate simple linear regressions. Mini-mice (all from S lines) are shown in gray for illustrative purposes.

S and C lines indicated positive correlations between $\dot{V}O_{2\,max}$ and [Hb] at all P_{O_2} for S lines but negative correlations for C lines in normoxia and hyperoxia (Table 3). The interaction between [Hb] and line type tested over the [Hb]*line(line type) interaction was statistically significant in normoxia and close to significance in hypoxia and hyperoxia (Fig. 4).

Intercorrelations of subordinate traits. Few subordinate traits were significantly correlated at the level of individual variation after removing effects of line type, age, the mini-muscle phenotype, and body mass (Rezende EL, unpublished observations). These results are of interest in and of themselves but are also important because they indicate that multicollinearity is not a problem for the analyses presented in Table 3. After accounting for body mass, selection, age, and the mini-muscle phenotype, ventricle mass residuals were positively correlated with residuals of gastrocnemius mass ($r = 0.427$, 2-tailed, $P = 0.003$) and negatively with CS activity ($r = -0.331$, $P = 0.023$).

Residuals of CS activity in the ventricles were negatively correlated with CS activity residuals in the gastrocnemius, being significant in different models with activity expressed per gram of muscle (above) or per the entire muscle (both ventricles and gastrocnemius, $r = -0.428$, $P = 0.001$). This negative correlation remained significant after removing individuals with the mini-muscle phenotype, regardless of how CS activity was expressed ($N = 36$, $r = -0.332$, $P = 0.024$ with values per gram of muscle).

DISCUSSION

Although mice from lines selectively bred for high voluntary wheel running have evolved higher $\dot{V}O_{2\,max}$ than their nonselected C lines (28–30, 35), our results show that some potentially important subordinate traits involved in O_2 transport or aerobic metabolism do not reflect this difference (Table 2). Myoglobin concentrations of both S and C lines in both gastrocnemius and ventricles are within the range reported for other rodents (e.g., Refs. 23, 26, 27). However, ventricle myoglobin was ~70% higher than has been reported for other strains of laboratory mice (8). In our mice, myoglobin concentration and CS activity in ventricles did not differ between mice with and without mini-muscles (Tables 1 and 2), contrasting with results for the gastrocnemius. Values obtained in the gastrocnemius can serve as "controls" for ventricle measurements, ensuring that the latter are accurate, because *1*) assays were performed together, *2*) results for CS activity are in accordance with previous published results (12, 13), *3*) myoglobin and CS activity in both organs were qualitatively similar (e.g., compare results for CS and myoglobin in Fig. 2, *left*), and *4*) myoglobin levels were between two- and fivefold higher in

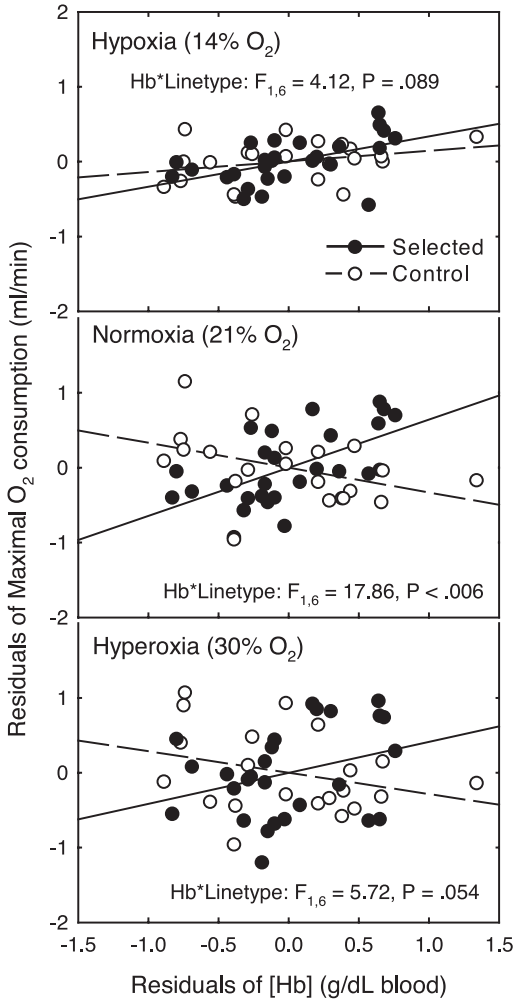

Fig. 4. Relationship between residuals of Hb concentration and $\dot{V}O_{2\ max}$ during forced-exercise in hypoxia, normoxia, and hyperoxia, from nested analyses of covariance controlling for selection history, size, age, batch, presence, or absence of the mini-muscle and all remaining subordinate traits (for full model, see Table 3). Lines represent linear regressions performed separately for S and C lines, and significance of the Hb*line type interaction (the only interaction term included in the model) is reported for each P_{O_2}.

the cardiac muscle, as with previous studies in rodents (Ref. 27, but see Ref. 23).

Although [Hb] was within the range reported in the literature for other strains of laboratory mice (e.g., Refs. 8, 25), our estimates were ~15% lower than reported for these same lines at *generation 14* (16.8–16.9 g/dl for sedentary females; Table 3 in Ref. 37). One possible explanation for this discrepancy is age, which averaged 79 days in Swallow et al. (37) but 102 days in the present study. In any case, both studies found no statistical difference in [Hb] between S and C lines when housed without wheel access.

Similar values for P_{50} between S and C lines indicate that this trait has not evolved in concert with increased wheel running in S mice. In contrast, Henderson et al. (10) reported that P_{50} differed significantly between divergent lines of rats selected for high- and low-endurance-running capacity, although they concluded that it was unlikely that this difference would have any substantial effect on O_2 transport. Our values of P_{50} were slightly higher than those reported in the literature for mice (~40 Torr; Ref. 18) or other mammalian species (between 15 and 45 Torr; e.g., Refs. 2, 10). Replicates of P_{50} measurements, however, were highly repeatable (N = 49, Pearson's r = 0.945), with low variation between measurements (coefficient of variation = 2.1%).

Although P_{50} measured in vitro under controlled conditions at constant pH did not differ, it is possible that hemoglobin affinity in vivo differs between S and C lines. Mice from S lines run voluntarily on wheels at substantially higher speeds (with correspondingly higher O_2 uptake and CO_2 production), have higher body temperatures when running on wheels (31), and have increased CO_2 production concomitantly with $\dot{V}O_{2\ max}$ during treadmill trials (28, 30). Thus it is possible that blood CO_2 concentration differs between S and C mice during voluntary wheel running, which could affect hemoglobin directly or indirectly through changes in blood pH in peripheral tissues (2, 24). Although S and C lines could also differ in blood buffering capacity, we have no data with which to address this possibility.

Central and peripheral limitations on $\dot{V}O_{2\ max}$. The importance of cardiac output for aerobic capacity and endurance has been widely acknowledged in the exercise physiology literature (e.g., Refs. 1, 3, 32, 39). Although we did not measure cardiac output, heart size has often been used as a predictor of cardiac output and ultimately $\dot{V}O_{2\ max}$ (e.g., Refs. 1, 3, 15, 32; but see Ref. 10). Everything else being equal, size alone should be a significant predictor of the heart's pumping capacity, as has been shown for heart mass and stroke volume or cardiac output (1, 17). The highly significant positive correlations between $\dot{V}O_{2\ max}$ and ventricle mass (after correcting for correlations with body mass) at different P_{O_2} in the present study (Table 3) suggest that cardiac output is indeed an important factor in determining individual differences in $\dot{V}O_{2\ max}$ (Fig. 3). Thus our results support the hypothesis that $\dot{V}O_{2\ max}$ in these lines is centrally limited to some extent (32), i.e., that organs involved in O_2 acquisition and delivery are important in setting an upper limit to whole animal maximal O_2 consumption.

Because $\dot{V}O_{2\ max}$ in S lines increased approximately linearly with increased P_{O_2}, whereas C lines did not increase $\dot{V}O_{2\ max}$ in hyperoxia, Rezende et al. (30) hypothesized that the contribution of O_2 convective components (i.e., ventilatory convection and cardiac output) to $\dot{V}O_{2\ max}$ differed between line types. The present results offer some support for this hypothesis. First, the relations between ventricle mass and $\dot{V}O_{2\ max}$ were stronger in S lines than in C lines, as judged by lower P values. (However, the lower P values in S lines must partly reflect the greater degrees of freedom.) Second, in C lines, the correlation between ventricle mass and $\dot{V}O_{2\ max}$ was close to significance in hypoxia and normoxia, but not in hyperoxia (Table 3). Third, the line-type effect in hyperoxia remained significant after subordinate traits were included in the model, and the magnitude of the difference was similar (~17% higher in S lines in Table 3 vs. 21% higher in Ref. 30). Thus additional factors other than the measured traits must contribute to the elevated $\dot{V}O_{2\ max}$ of S females.

The positive relation between $\dot{V}O_{2\ max}$ and [Hb] within S lines (Table 3, Fig. 4) presumably reflects the positive effect of higher arterial O_2 content associated with higher [Hb], under the assumption that the cardiac output of S mice has "excess capacity" and can accommodate the increased blood viscosity associated with higher hematocrit. (Although we did not mea-

sure hematocrit, the correlation between [Hb] and hematocrit is high in these lines. For example, considering the mice reported in Ref. 37, $r = 0.843$ for the 41 sedentary females and $r = 0.902$ for the 40 females with 8 wk of wheel access.) In contrast, the negative relation within C lines might indicate that their cardiac output is insufficient to accommodate increased blood viscosity associated with higher hematocrit. Interestingly, body mass residual [Hb] and ventricle mass were correlated negatively in S lines ($N = 27$, $r = -0.105$) but positively in C lines ($r = 0.143$), resulting in an overall correlation of almost zero ($r = 0.048$). None of these correlations are strong enough to suggest that multicollinearity could account for the different relation between $\dot{V}O_{2\,max}$ in normoxia and [Hb] within S vs. within C lines (Table 3). Another possibility that could account for the difference, not mutually exclusive with any differences in maximal cardiac output, would be lower peripheral resistance in S lines, e.g., associated with increased capillarity in skeletal muscles. In any case, selection for high voluntary wheel running seems to have altered the physiology underlying aerobic performance in these lines of mice.

Previous studies have shown that both S and C mice can achieve $\dot{V}O_{2\,max}$ on average 32% higher during cold exposure in a He-O_2 atmosphere than during forced treadmill exercise, demonstrating that the pulmonary and cardiovascular system could provide more O_2 to the muscles than is required during forced exercise (28). We have also shown that, at least in S mice, mitochondrial oxidative capacity was not limiting during $\dot{V}O_{2\,max}$ in normoxia, as they were able to significantly increase $\dot{V}O_{2\,max}$ when running in a 30% O_2 atmosphere. Hence, constraints on $\dot{V}O_{2\,max}$ in normoxia probably occur at the level of O_2 transport (30). Taken as a whole, our results support current models that indicate whole organism $\dot{V}O_{2\,max}$ is influenced by multiple factors rather than a single one (4, 22). In this context, it is possible that the four S lines have increased $\dot{V}O_{2\,max}$ in different ways (i.e., different solutions for the same selective "problem"), which could explain why most subordinate traits did not differ between line types (i.e., when comparing all four S lines with all four C lines). As shown in Table 1, differences among replicate lines were statistically significant for gastrocnemius CS activity even in analyses that included the mini-muscle phenotype as a main effect. (However, these combined analyses do not separate possible differences among the S lines from those among the C lines.)

It is important to emphasize that the experimental protocol we used cannot completely determine which differences between S and C lines are strictly genetic ("innate") vs. a result of "training" effects (i.e., phenotypic plasticity). Nevertheless, given that we had to measure both wheel running and $\dot{V}O_{2\,max}$ (at three atmospheres), any possible protocol will allow for some possibility of training effects, and those could differ in magnitude between S and C lines because of differences in performance on the wheels or the treadmill, or even because of innate differences in "trainability" between S and C lines (for discussion of these and related issues, see Ref. 6). [In addition, S mice also tend to be more active than C in normal housing (Garland T, unpublished observations).] However, the protocol we used should reasonably reflect the actual conditions encountered by mice during the selection protocol (6 days of wheel access) as well as their physiological status after such access. The phenotype under selection is running behavior on *days 5 + 6* of a 6-day test, which inherently may reflect variation in both motivation and ability (see Introduction), both of which may be affected by the prior 4 days of wheel access. Possible short-term exercise effects (within minutes or hours, e.g., Ref. 20) were circumvented because samples were obtained 1 day after the last treadmill measurement. In any case, we did not observe statistically significant differences between S and C mice with respect to CS activity or any of the other candidate subordinate traits measured (Table 1).

Results for mice with the mini-muscle phenotype offer further support for the notion that the S lines have evolved higher $\dot{V}O_{2\,max}$ in different manners. These individuals achieve higher $\dot{V}O_{2\,max}$ in hypoxia compared with mice that have normal muscles (30), although it is unclear whether this increase may result primarily from larger hearts (Tables 1 and 2) or from more efficient O_2 extraction in peripheral tissues during exercise associated, for instance, with potentially higher intracellular O_2 transport and storage [e.g., higher myoglobin concentrations (Tables 1 and 2); e.g., Refs. 11, 16, 40, 41] and aerobic capacity (Ref. 13; Tables 1 and 2). Our results support the idea that increased cardiac outputs may account for significantly higher $\dot{V}O_{2\,max}$ in hypoxia for mini-mice, as the mini-muscle effect becomes nonsignificant ($P_{mini} = 0.2679$, $P_{ventricle\ mass} = 0.0003$) when ventricle mass is included as the only subordinate trait in the model (see also Table 3). In this context, more direct measurements of cardiac output (as well as heart rate and chamber dimensions) would allow additional insights concerning the relative importance of central mechanisms (and their components) in determining aerobic capacity in these lines of mice.

ACKNOWLEDGMENTS

The authors thank L. Karpinski, J. Sinclair, and the undergraduates who helped with the mouse colony throughout generations. We are thankful to S. Kelly, P. del Agua, and R. Hepple for discussions, comments, and insights on early versions of the manuscript, and two anonymous referees for comments and suggestions.

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GRANTS

This work was supported by National Science Foundation Grants IBN-0212567 (T. Garland) and IBN-0111604 (K. A. Hammond and M. A. Chappell).

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