Do mice bred selectively for high locomotor activity have a greater reliance on lipids to power submaximal aerobic exercise?

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Templeman NM, Schutz H, Garland T, Jr, McClelland GB. Do mice bred selectively for high locomotor activity have a greater reliance on lipids to power submaximal aerobic exercise? Am J Physiol Regul Integr Comp Physiol 303: R101–R111, 2012. First published May 9, 2012; doi:10.1152/ajpregu.00511.2011.—Patterns of fuel use during locomotion are determined by exercise intensity and duration, and are remarkably similar across many mammalian taxa. However, as lipids have a high yield of ATP per mole and are stored in large quantities, their use should be favored in endurance-adapted animals. To examine the capacity for alteration or differential regulation of fuel-use patterns, we studied two lines of mice that had been selectively bred for high voluntary wheel running (HR), including one characterized by small hindlimb muscles (HRmin) and one without this phenotype (HRnorm), as well as a nonselected control line. We evaluated: 1) maximal aerobic capacity (VO2 max); 2) whole body fuel use during exercise by indirect calorimetry; 3) cardiac properties; and 4) many factors involved in regulating lipid use. HR mice achieved an increased VO2 max compared with control mice, potentially in part due to HR cardiac capacities for metabolic fuel oxidation and the larger relative heart size of HRmin mice. HR mice also exhibited enhanced whole body lipid oxidation rates at 66% VO2 max, but HRmin, HRnorm, and control mice did not differ in the proportional mix of fuels sustaining exercise (% total VO2). However, HRmin gastrocnemius muscle had elevated fatty acid translocase (FAT/CD36) sarcob lamellar protein and cellular mRNA, fatty acid binding protein (H-FABP) cytosolic protein, peroxisome proliferator-activated receptor (PPAR)α mRNA, and mass-specific activities of citrate synthase, β-hydroxyacyl-CoA dehydrogenase, and hexokinase. Therefore, high-running mouse lines had whole body fuel oxidation rates commensurate with maximal aerobic capacity, despite notable differences in skeletal muscle metabolic phenotypes.

aerobic capacity; experimental evolution; lipid oxidation; muscles

WHEN ENERGY DEMAND IS INCREASED, as during exercise, a major component of maintaining the essential balance between ATP production and utilization is the ability to select an appropriate mixture of metabolic fuels, in addition to adjusting total flux of substrates through metabolic pathways (50). In exercising mammals, the two predominant substrates sustaining energy expenditure in skeletal muscles are lipids (both intramuscular triglycerides and plasma free fatty acids) and carbohydrates (intramuscular glycogen stores and blood glucose); 50). As exercise intensity increases, carbohydrates support a greater proportion of energy expenditure, as they are capable of sustaining high ATP turnover rates (5, 9, 10, 29, 42, 50).

In fact, it has been long appreciated that exercise intensity largely influences metabolic fuel-use patterns, at least in the few mammalian species in which exercise metabolism has been studied (5, 9, 10, 29, 41). For example, the “crossover concept” describes a theory wherein a human’s fuel use during exercise depends on the interaction between exercise intensity and endurance training status (5). Indeed, when energetic requirements of locomotion are adjusted according to an individual’s maximal capacity for aerobic ATP supply (VO2 max), a strong relationship exists between relative exercise intensity (i.e., intensity relative to VO2 max) and the proportional mix of carbohydrates and lipids fueling exercise (29, 41, 50). Surprisingly, this relationship appears to be widely conserved among the mammalian species examined to date, irrespective of allometric variation or diversity in locomotor capabilities (29, 41, 50). Thus this model of mammalian fuel use represents a valuable tool to formulate hypotheses.

The underlying explanations for this evident unity in mammalian fuel-use patterns remain unknown. Likely, many factors influence the fractional contribution of carbohydrates and lipids to energy supply, including recruitment of different muscle fiber types, hormonal regulation, substrate and oxygen availability, and the regulation of enzyme and substrate transporter quantities and activities (6, 29, 31). For instance, both Roberts et al. (41) and McClelland (29) proposed that one mechanism accounting for the conservation of the fuel-use pattern may be the progressive recruitment of type IIb (fast glycolytic) muscle fibers with increasing exercise intensity (25, 44), which could be instrumental among all mammals in mediating the gradual shift of fuel preference toward carbohydrates (29, 41). However, it is currently unclear what mechanisms cause variation among mammals in absolute fuel oxidation or flux rates, or even if the mechanisms that explain the unity in proportional mix of fuels are common across taxa.

To further explore the underpinnings of the mammalian fuel-use pattern during exercise, we used a model of experimental evolution in which different breeding lines of mice have been selectively bred for high levels of voluntary wheel running (46). This allows for the controlled examination of the evolution of diverse voluntary exercise phenotypes; such models provide opportunities for empirical testing of evolutionary hypotheses without such complications as phylogenetic divergences, as occurs with interspecific comparisons (12). Mice from the high running (HR) lines voluntarily run ~170–200% more revolutions/day on running wheels than those from nonselected control lines and have a higher endurance capacity during forced treadmill exercise (13, 34). This animal model includes four replicate HR lines, providing opportunities for the development of multiple solutions (i.e., differential adaptive changes in subordinate traits) in response to a uniform selective influence (12, 14). For instance, occurrence of a “mini muscle” phenotype has increased in frequency in two of the four HR lines (12, 13). Mice with the “mini muscle” phenotype
(HR_{min}) have a drastically reduced content of type IIB muscle fibers in their hindlimb muscles, resulting in increased mass-specific activities of aerobic enzymes and reduced mass-specific activities of anaerobic enzymes in these muscles, relative to mice with the normal muscle phenotype (3, 18, 19, 22).

In addition to the emergence of the “mini muscle” phenotype, selective breeding for high locomotor activity also led to an increased whole animal V\(_\text{O2}\) max(corrected for differences in type, selective breeding for high locomotor activity also led to mice with the normal muscle phenotype (HR\(_\text{normal}\)) but only under hypoxic conditions (38)). It has been proposed that heart enlargement in HR\(_{min}\) mice could contribute to this increased aerobic performance, especially in hypoxia (39); however, it has not yet been determined whether the hearts of selected mice show significant physical (i.e., hypertrophic) or metabolic remodeling. Therefore, one component of our investigation was to characterize physical and metabolic properties of cardiac muscle in these mice.

Lipids are highly chemically reduced, have a high yield of ATP per mole of fuel (high energy density), and in mammals, are stored in large quantities compared with carbohydrates. So, on first principles, one might predict that mice from selectively bred HR lines should rely more heavily on this fuel to power submaximal locomotion. We hypothesized that HR\(_{min}\) mice in particular would exhibit a shift in the fuel-use pattern toward both a greater absolute lipid flux rate and an increased relative lipid contribution to total aerobic energy expenditure (measured as V\(_\text{O2}\)), considering the enhanced aerobic capacity of their muscles and reduction in type IIB (fast glycolytic) muscle fibers. To test this hypothesis, we assessed whole body fuel use during submaximal exercise and examined key regulatory points of the fatty acid metabolic pathway in the gastrocnemius muscle, a major hindlimb muscle that has notable phenotypic alterations in the HR\(_{min}\) mice (18, 19). Thus, we have a unique opportunity to evaluate the effects of voluntary locomotor behavior and aerobic capacity on the mammalian fuel-use pattern without many of the complications of interspecific comparative studies by using a model of experimental evolution.

**MATERIALS AND METHODS**

*Animals.* All experimental procedures were approved by the University of California Riverside Institutional Animal Care and Use Committee, as well as by the McMaster University Animal Research Ethics Board following Canadian Council for Animal Care guidelines.

We used male mice from an animal model in which four closed lines experience within-family selection for total revolutions run on a custom-built motorized treadmill at an incline of 10°, enclosed in a Plexiglas metabolic chamber (approximate volume 800 ml) and equipped with stimulus electrical grid. Air entering the chamber was scrubbed of H\(_2\)O by Drierite (W. A. Hammond Drierite, Xenia, OH) and of CO\(_2\) by soda lime and Ascarite (Fisher Scientific, Pittsburgh, PA) and maintained at a constant flow of 2,000 ml/min by combined pump and mass-flow controller unit (MFS, Sable Systems International, Las Vegas, NV). Treadmill chamber air was subsampled at 200 ml/min and scrubbed of H\(_2\)O by magnesium perchlorate (Fisher Scientific), and then passed through an O\(_2\) and CO\(_2\) analyzer (FoxBox portable analysis instrument; Sable Systems International). Data were collected with data acquisition software (Expedita; Sable Systems International) at a frequency of 1 sample/s. The accuracy of this setup was determined by measuring the combustion of methanol as described previously (31) and found to be ±5% of theoretical values for absolute rates of V\(_\text{O2}\) and V\(_\text{CO2}\) and ±1% of theoretical values for respiratory exchange ratio (RER = V\(_\text{CO2}\)/V\(_\text{O2}\)). Before V\(_\text{O2}\)max trials, mice were familiarized with forced treadmill running for 10–15 min on 2 consecutive days. For all exercise trials, mice had a 5-min adjustment period in the chamber before exercise began with a starting speed of 10 or 13 m/min and incrementally increased by 3 m/min every 1–2 min. After correction for any baseline drift in V\(_\text{O2}\), CO\(_2\), and V\(_\text{O2}\) max was defined as the maximal steady-state interval at which at least two of the following criteria were satisfied: 1) no change in V\(_\text{O2}\) with increasing speed, 2) RER > 1.0, and 3) the mouse no longer maintained position on the treadmill (24, 31, 38, 43, 47).

Treadmill speed was also assessed subjectively by assigning a score between 1 and 5 (47), and runs deemed unacceptable (scores below 3) were repeated. There was a minimum of 24 h between exercise bouts for any individual.

For all submaximal exercise trials, mice were fasted 6–8 h to ensure a postabsorptive state. The order of the two submaximal exercise intensity trials (target 60% V\(_\text{O2}\)max and 80% V\(_\text{O2}\)max, on average 66% V\(_\text{O2}\)max and 78% V\(_\text{O2}\)max, designated as “low-intensity” and “high-intensity” exercise, respectively) was randomized for each mouse, and the trials were performed between 13:00 and 19:00. Initial treadmill speed for submaximal exercise tests was determined from the relationship between speed and V\(_\text{O2}\) during the V\(_\text{O2}\)max trials; minor adjustments to treadmill speed were made based on real-time monitoring of V\(_\text{O2}\) to maintain a constant relative intensity. Sixty seconds of continuous data from between minutes 9 to 11 of each individual trial were used for analyses. Rates of V\(_\text{CO2}\) and V\(_\text{O2}\) (ml/h) were calculated using Eq. 3b from Withers (52). Lipid and carbohydrate oxidation rates were estimated using indirect calorimetry (11), and estimations were based on the assumption that protein contributions to total oxidation were negligible during aerobic exercise in a postabsorptive state (37). The proportional contribution of lipids or carbohydrates to whole body oxidation was determined by dividing the estimated lipid or carbohydrate oxidation rate (in μmol O\(_2\)-h\(^{-1}\)·g\(^{-1}\)) by the measured total rate of oxygen consumption during the exercise bout (V\(_\text{O2}\), in μmol O\(_2\)-h\(^{-1}\)·g\(^{-1}\)).

**Tissue sampling.** All tissue sampling took place between 13:00 and 18:00 h. Nonexercised mice from generation 57 were fasted for 6–8 h before euthanasia via decapitation. The left and right gastrocnemius...
muscles were removed, weighed, and flash frozen in dry ice with aluminum clamps. The left and right ventricles were separated and sampled as previously described. The right gastrocnemius muscle (genetically selected for high running ability) and left gastrocnemius muscle (genetically selected for low running ability) were chosen for analyses. Total RNA was extracted with TRIzol reagent (Invitrogen, Burlington, ON, Canada), based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentration was measured at 260 nm using a NanoDrop ND-1,000 spectrophotometer (Fisher Scientific). To generate cDNA, 1 μg of total RNA was treated with DNase I (Invitrogen), and reverse transcription was carried out by SuperScript II RNase H reverse transcriptase (Invitrogen) as previously described (48). Five microliters of 1:5 (cDNA:μl water) diluted cDNA were mixed with 12.5 μl SYBR green (Bio-Rad), 5.5 μl RNAse/DNase-free water, and 1 μl each of forward and reverse primers (5 μM) for quantitative real-time PCR reactions. A negative control of water was used on each plate to ensure a lack of contamination. Reactions were performed in duplicate on a Stratagene MX3000P QPCR system (Stratagene, La Jolla, CA), using SYBR green as the reference dye. In all cases, the thermal program consisted of a 3 min initial denaturation at 95°C, then 40 cycles of 95°C for 15 s, 60°C for 45 s, and 72°C for 30 s. A dissociation curve analysis was performed to verify the specificity of PCR products. The relative mRNA levels of each sample were calculated using the comparative Ct method (8). All values are normalized against mRNA levels of the housekeeping gene, which did not differ between groups (P > 0.2 for each housekeeping gene). Statistical analyses for gene expression were performed on values normalized to the housekeeping gene but for the purpose of data

**Immunoblotting.** Fatty acid translocase (FAT/CD36) protein content was measured in sarcolemmal fractions from gastrocnemius muscles using a commercial CD36 (H-300) antibody and a horseradish peroxidase-linked anti-rabbit secondary antibody (sc-9154 and sc-2313, respectively; Santa Cruz Biotechnology, Santa Cruz, CA). Equal amounts of total sarcolemmal protein (5 μg/lane) were separated by electrophoresis in 10% polyacrylamide gels (Bio-Rad Laboratories, Mississauga, ON, Canada). Protein was transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) and blocked with 5% bovine serum albumin (BSA) dissolved in PBS-T (in mM: 1.5 NaH₂PO₄, H₂O, 8.1 Na₂HPO₄, 145.4 NaCl, 0.05% Tween 20, pH 7.4) overnight at 4°C. Membranes were washed with 1% BSA/PBS-T (2 × 5 min), and then primary antibody (diluted 1:200 in 1% BSA/PBS-T) for 1 h, 1% BSA/PBS-T (1 × 15 min, 3 × 5 min), secondary antibody (diluted 1:5,000 in 1% BSA/PBS-T) for 1 h, and, lastly, 1% BSA/PBS-T (1 × 15 min, 3 × 5 min). Chemiluminescence (Perkin Elmer, Waltham, MA) was detected on autoradiographic films (Kodak XAR). Relative protein levels were evaluated based on the measure of band volumes, determined using ImageJ software. All samples are expressed normalized against a standard sample (a mixture of sarcolemmal protein pooled from all three mouse lines) that was loaded on to each gel.
presentation, these normalized values are expressed relative to mean control values (arbitrarily set at 1.0).

Statistics. Statistical analyses were performed using SPSS 11.0 software. Analysis of covariance (ANCOVA) models were used for each dependent variable. Body mass was always included as a covariate, except when it had been accounted for in the dependent variable, or when examining raw ventricular masses independent of body mass. Analyses of FAT/CD36 and H-FABP protein, maximal enzyme activity, and mRNA content were also performed without body mass. Analyses of FAT/CD36 and H-FABP protein, maximal enzyme activity, and mRNA content were also performed without body mass. Analyses of FAT/CD36 and H-FABP protein, maximal enzyme activity, and mRNA content were also performed without body mass. Analyses of FAT/CD36 and H-FABP protein, maximal enzyme activity, and mRNA content were also performed without body mass. Analyses of FAT/CD36 and H-FABP protein, maximal enzyme activity, and mRNA content were also performed without body mass. Analyses of FAT/CD36 and H-FABP protein, maximal enzyme activity, and mRNA content were also performed without body mass.

RESULTS

In vivo: \( \dot{V}O_2 \text{max} \) and fuel use during exercise. Both lines of HR mice exhibited a greater \( \dot{V}O_2 \text{max} \) than nonselected control mice, regardless of whether \( \dot{V}O_2 \text{max} \) was analyzed as a mass-specific variable or, alternately, as a whole animal value with the covariate of body mass (\( P < 0.05 \); Fig. 1, Table 2). Moreover, the mass-specific \( \dot{V}O_2 \text{max} \) of HR\(_{\text{mini}} \) mice was elevated compared with HR\(_{\text{normal}} \) mice (\( P < 0.05 \); Table 2). However, when \( \dot{V}O_2 \text{max} \) was analyzed as whole animal values with body mass as a covariate, the difference between HR\(_{\text{mini}} \) and HR\(_{\text{normal}} \) mice was not statistically significant. The estimated marginal mean whole animal \( \dot{V}O_2 \text{max} \) and SE for control, HR\(_{\text{mini}} \), and HR\(_{\text{normal}} \) mice were 4.353 \( \pm \) 0.058, 4.933 \( \pm \) 0.059, and 4.794 \( \pm \) 0.057 ml/min, respectively, as evaluated at an average body mass of 28.1 g (Fig. 1).

During low-intensity exercise (66 \( \pm \) 1\% for controls, 64 \( \pm \) 1\% for HR\(_{\text{mini}} \) mice, and 69 \( \pm \) 2\% \( \dot{V}O_2 \text{max} \) for HR\(_{\text{normal}} \) mice; overall average 66\% \( \dot{V}O_2 \text{max} \); Table 2), the greater total \( \dot{V}O_2 \) of HR mice (Table 2) was primarily supplied by an increased flux of lipids; HR\(_{\text{mini}} \) mice exhibited higher absolute rates of lipid oxidation than control mice, a difference that approached significance for HR\(_{\text{normal}} \) mice as well (\( P < 0.05 \) for HR\(_{\text{mini}} \) mice, \( P = 0.057 \) for HR\(_{\text{normal}} \) mice; Table 2). Although the concurrent rates of carbohydrate oxidation were not significantly different among the three mouse phenotypes (Table 2), there were no statistically significant differences in the proportional mix of fuels supplying oxidation at 66\% \( \dot{V}O_2 \text{max} \) when fuel oxidation rates were expressed relative to total \( \dot{V}O_2 \) (Fig. 2A). For all mice, \(-70\% \) of energy was supplied via the oxidation of lipids, with the remaining 30\% by the oxidation of carbohydrates (Fig. 2A).

During high-intensity exercise (78 \( \pm \) 1\% \( \dot{V}O_2 \text{max} \) for controls, 76 \( \pm \) 1\% \( \dot{V}O_2 \text{max} \) for HR\(_{\text{mini}} \) mice, and 79 \( \pm \) 1\% \( \dot{V}O_2 \text{max} \) for HR\(_{\text{normal}} \) mice; overall average 78\% \( \dot{V}O_2 \text{max} \); Table 2), the absolute lipid and carbohydrate oxidation rates in control mice were equivalent to those in HR mice (Table 2). At this increased intensity, carbohydrates contributed more than 40\% toward total oxidation in all mice (Fig. 2B).

Cardiac properties. Relative to body mass, HR\(_{\text{mini}} \) mice had significantly larger left ventricles than both control and HR\(_{\text{normal}} \) mice (\( P \leq 0.001 \); Table 3). This was principally due to a reduced body mass in HR\(_{\text{mini}} \) mice compared with the other two lines (\( P < 0.05 \); Table 3); however, a slight enlargement of whole ventricle mass in HR\(_{\text{mini}} \) mice approached statistical significance even without the inclusion of body mass as a covariate (\( P = 0.096 \); Table 3). There were no statistical differences in left ventricle DNA content, whether expressed per gram wet mass or per whole ventricle (Table 3).

In addition to physical changes, both lines of HR mice exhibited some degree of cardiac metabolic remodeling compared with control mice, as indicated by increased maximal HK activity concurrent to reduced maximal CPT II activity (\( P < 0.05 \); Fig. 3). However, there were no statistically significant differences in the mRNA content of PPAR\( \alpha \), MCAD, CPT-1\( \alpha \), or CPT-1\( \beta \) in either the right or left ventricles of HR\(_{\text{mini}} \), HR\(_{\text{normal}} \), or control mice (Table 4).

Skeletal muscle FAT/CD36 and H-FABP. The skeletal muscle of nonexercised HR\(_{\text{mini}} \) mice was characterized by a mark-
Table 2. Mass-specific $\dot{V}O_{2\text{max}}$ and whole body lipid and carbohydrate oxidation rates of control, HR$_{\text{mini}}$, and HR$_{\text{normal}}$ mice during low- and high-intensity submaximal exercise

<table>
<thead>
<tr>
<th>Trait</th>
<th>Control mice</th>
<th>HR$_{\text{mini}}$ Mice</th>
<th>HR$_{\text{normal}}$ Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass-specific $\dot{V}O_{2\text{max}}$, ml O$_2$·h$^{-1}$·g$^{-1}$</td>
<td>9.26 ± 0.12*(10)</td>
<td>10.63 ± 0.12*(10)</td>
<td>10.18 ± 0.13*(9)</td>
</tr>
<tr>
<td>Exercise intensity, % $\dot{V}O_{2\text{max}}$</td>
<td>66 ± 1 (10)</td>
<td>64 ± 1 (10)</td>
<td>69 ± 2 (9)</td>
</tr>
<tr>
<td>$\dot{V}O_{2\text{max}}$, ml O$_2$·h$^{-1}$·g$^{-1}$</td>
<td>6.14 ± 0.09*(10)</td>
<td>7.04 ± 0.09*(10)</td>
<td>6.84 ± 0.10*(9)</td>
</tr>
<tr>
<td>$\dot{V}CO_{2\text{max}}$, ml O$_2$·h$^{-1}$·g$^{-1}$</td>
<td>4.93 ± 0.08*(10)</td>
<td>5.54 ± 0.09*(10)</td>
<td>5.37 ± 0.09*(9)</td>
</tr>
<tr>
<td>Lipid oxidation, ml O$_2$·h$^{-1}$·g$^{-1}$</td>
<td>4.12 ± 0.23*(10)</td>
<td>5.09 ± 0.24*(10)</td>
<td>4.97 ± 0.25*#(9)</td>
</tr>
<tr>
<td>Carbohydrate oxidation, ml O$_2$·h$^{-1}$·g$^{-1}$</td>
<td>2.02 ± 0.20 (10)</td>
<td>1.95 ± 0.21 (10)</td>
<td>1.85 ± 0.23 (9)</td>
</tr>
</tbody>
</table>

Mass-specific maximal aerobic capacity ($\dot{V}O_{2\text{max}}$) is presented as estimated marginal means ± SE, as adjusted for the cofactor of group batch. Exercise intensities (% $\dot{V}O_{2\text{max}}$) are presented as simple means ± SE. All mass-specific oxidation rates are presented as estimated marginal means ± SE, as adjusted for the covariate of individual % $\dot{V}O_{2\text{max}}$. Sample size is shown in parenthesis for each trait. The presence of letters denotes statistical significance, where values not sharing a common letter are significantly different from each other ($P < 0.05$, except an * indicates $P = 0.057$). See text for definitions of abbreviations.

Skeletal muscle metabolic enzyme activities. The mass-specific maximal activities of CS, HOAD, and HK were greatly augmented in the gastrocnemius of HR$_{\text{mini}}$ mice compared with mice with a normal muscle phenotype ($P < 0.05$; Fig. 4A). In fact, because mass-specific enzyme activities nearly double those of control mice (Fig. 4A), in a gastrocnemius less than half the mass ($P < 0.05$; Table 3), for these enzymes HR$_{\text{mini}}$ mice had equivalent total maximal activities as control mice when expressed per whole muscle (Fig. 6B). The mass-specific maximal CPT II activity was not significantly different between HR$_{\text{mini}}$ and control mice, as reflected in the reduced whole muscle maximal CPT II activity in the mini muscles (Fig. 6).

Conversely, although HR$_{\text{normal}}$ mice did not have altered mass-specific enzyme activities compared with control mice, on a whole muscle basis they exhibited significantly greater maximal activities of CS, HOAD, CPT II, and HK ($P < 0.05$; Fig. 6B). These differences were also significant with body mass as a covariate (data not shown). The elevation in total muscle metabolic enzyme activity was likely reflective of a slightly increased gastrocnemius mass in HR$_{\text{normal}}$ mice, which approached statistical significance ($P = 0.053$; Table 3). HR$_{\text{normal}}$ whole muscle CS, CPT II, and HK maximal activities were significantly elevated above those of HR$_{\text{mini}}$ mice as well ($P < 0.05$; Fig. 6B).

Skeletal muscle gene expression and transcriptional regulation. mRNA levels of PGC-1$\alpha$, PPAR$\beta$/$\delta$, and SIRT1 were not significantly different among phenotypes, but PPAR$\alpha$ mRNA content was $\sim$2.5-fold greater in the resting gastrocnemius of HR$_{\text{mini}}$ mice than in HR$_{\text{normal}}$ or control mice ($P < 0.05$; Fig. 7A). Furthermore, transcript levels of the PPAR-regulated gene $FAT/CD36$ were closely correlated with PPAR$\alpha$ among all mice ($r = 0.92$, $p \leq 0.001$; Fig 7B).

DISCUSSION

Our primary goal was to determine whether mice selectively bred for high voluntary running have greater whole body absolute lipid oxidation rates during exercise and a greater contribution of lipids to total aerobic energy expenditure. Our results indicate that selective breeding for high voluntary...
exercise leads to an increase in the absolute rate of lipid oxidation when individuals exercise at the same relative intensity. These oxidation rates are scaled with increases in maximal aerobic capacity in both HR\textsubscript{normal} and HR\textsubscript{mini} mice, resulting in the proportional contributions by lipids and carbohydrates to total exercise energy expenditure being equal among all mice. The two selectively bred HR lines examined showed distinct capacities in the gastrocnemius muscle for cellular lipid uptake (FAT/CD36), cytosolic transport (H-FABP), and mitochondrial oxidation (enzyme \( V_{\text{max}} \)), suggesting that in these distinct high-running phenotypes, the response to selective breeding may have targeted different mechanisms to enhance lipid oxidation rates (e.g., capacity for oxidation of circulatory versus intramuscular sources) over that of the control line.

**Aerobic capacity and cardiac properties.** Our study not only confirms a 10–14% rise in \( V_{O2 \text{max}} \) in HR mice (24, 38, 40, 47) but also indicates that generation 57 HR\textsubscript{mini} mice achieve a greater mass-specific \( V_{O2 \text{max}} \) than HR\textsubscript{normal} mice (at least the line 8 studied here), although this difference was not significantly different on a whole animal \( V_{O2 \text{max}} \) basis with body mass as a covariate, neither left nor whole ventricles are significantly larger in HR\textsubscript{mini} mice, and left ventricles of all mouse phenotypes contain comparable levels of DNA (Table 3). Therefore, this difference in relative heart size could be viewed as predominately reflective of the reduced body masses of HR\textsubscript{mini} mice as opposed to cardiac hypertrophy per se. However, relative heart mass can give a good indication of an animal’s ability to perform sustained aerobic locomotion (4, 51), so it is likely that the increased ratio of heart to body size contributes to the elevated \( V_{O2 \text{max}} \) of HR\textsubscript{mini} mice.

Additionally, both phenotypes of HR mice show evidence of metabolic alterations in the left ventricle. Compared with control mice, HR mice show a reduction in the left ventricular maximal activity of CPT II, an enzyme in the fatty acid catabolic pathway (Fig. 3). Although a nonreversible switch in the basal-preferred fuel substrate from fatty acids to carbohydrates is often regarded as a deleterious hallmark of pressure- or volume-overloaded hypertrophic hearts (36), these mouse show no statistically significant differences in ventricular mRNA levels of several genes involved in fatty acid catabolism, including \( PPAR \alpha \), \( MCAD \), and both muscle- and liver-type \( CPT-1 \). Therefore, it is unlikely that this reduced maximal activity of CPT II in HR left ventricles signifies a diminished capacity for fatty acid oxidation, particularly as there is no change in the maximal activity of HOAD, another fatty acid catabolic enzyme. There is, however, increased maximal activity of HK in the left ventricles of sedentary (i.e., not exercise trained) HR mice compared with control mice (Fig. 3). Upregulated HK is also characteristic of physiologically hypertrophic hearts in exercise-trained rats as a component of the elevated overall metabolic capacity (45). Therefore, differences between HR and control mouse cardiac metabolic enzyme activities may contribute to the enhancement of \( V_{O2 \text{max}} \) and capacity for sustained aerobic exercise in HR mice in
When exercising at a higher intensity (78% \( V\dot{O}_2 \) max), CPT-1 at 1.00). Statistical analyses were performed on raw mRNA levels corrected parenthesis; mRNA expression levels are corrected against

Relative mRNA

icant. See text for definitions of abbreviations.

fuels used across two submaximal relative exercise intensities mice. conjunction with the increased relative heart size of HR\textsubscript{mini} mice.

Whole body fuel use during exercise. The mix of metabolic fuels used across two submaximal relative exercise intensities does not differ statistically among HR\textsubscript{mini}, HR\textsubscript{normal}, and control mice, when fuel oxidation rates are expressed as a percentage of total oxygen consumption (Fig. 2). Rather, HR mice exhibit greater rates of lipid oxidation (i.e., enhanced total lipid flux) than control mice during low-intensity exercise (66% \( V\dot{O}_2 \) max), commensurate with their elevated \( V\dot{O}_2 \) max (Fig. 1, Table 2). When exercising at a higher intensity (78% \( V\dot{O}_2 \) max), the increased energetic demand in all mice is supported by an elevated flux of carbohydrates, and the absolute rate of lipid oxidation in control mice matches the rates in HR mice (Table 2).

HR mice primarily achieve greater levels of voluntary wheel running by running faster than control mice on exercise wheels (13–15, 17, 46). Our findings suggest that due to an increased aerobic capacity, HR mice are able to run at these faster speeds predominately sustained by lipid oxidation, whereas mice from nonselected control lines would require a higher proportion of carbohydrates to achieve equivalent absolute exercise intensities. Therefore, an enhanced capacity for absolute lipid oxidation in conjunction with an increased \( V\dot{O}_2 \) max may have enabled the shift in locomotor speed preference in HR mice, thus allowing them to achieve the greater levels of voluntary exercise for which they are selectively bred (see also Ref. 41). In fact, consumption of a high-fat diet further increases the amount of voluntary wheel running performed by HR (but not control) mice (33). Interestingly, rats from a line selectively bred for high treadmill endurance capacity with an elevated \( V\dot{O}_2 \) max exhibit greater in vitro skeletal muscle palmitate oxidation rates compared with a line selected for low endurance capacity (23, 26), signifying that increased lipid oxidation capacity is a common result of selection for running capacity.

Roberts et al. (41) and McClelland (29) have suggested that conserved mammalian fuel-use patterns are the result of the progression of muscle fiber recruitment, with the increasing proportionate reliance on carbohydrates at higher exercise intensities corresponding to the recruitment of fast glycolytic muscle fibers among all mammals (25, 44). Intriguingly, HR\textsubscript{mini} mice do not have a large quantity of type IIB (fast glycolytic) fibers available for recruitment in their reduced-size hindlimb muscles (3, 18, 19), and yet they exhibit the ability to reach a whole body rate of carbohydrate combustion that is equivalent to mice with the normal muscle phenotype, at least under the submaximal exercise conditions tested here. Moreover, as the proportional reliance on carbohydrates for total oxidation at higher exercise intensities is comparable between HR\textsubscript{mini} mice and HR\textsubscript{normal} mice, it may be possible for mammals to differ in muscle fiber recruitment patterns during exercise without deviating from a conserved whole body pattern of proportionate fuel mix.

From these findings, we investigated other potential regulators of metabolic fuel use to determine whether phenotypically divergent HR mice might utilize different strategies to adjust substrate flux. Specifically, we focused here on several regulators of fatty acid uptake and catabolism in the skeletal muscle, in keeping with our stated hypothesis. However, future studies should explore other pathways (see Refs. 29 and 50), such as carbohydrate or amino acid metabolism or lactate clearance capacity, to further delineate different mechanisms regulating all of the fuels available to power exercise in mammals.

**Table 4. mRNA expression in cardiac muscle of control, HR\textsubscript{mini}, and HR\textsubscript{normal} mice**

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>PPAR\textalpha relative mRNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricle</td>
<td>1.00 ± 0.18 (7)</td>
<td>0.78 ± 0.03 (7)</td>
<td>1.05 ± 0.14 (8)</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>1.00 ± 0.09 (6)</td>
<td>0.88 ± 0.10 (7)</td>
<td>1.28 ± 0.20 (8)</td>
</tr>
<tr>
<td><strong>MCAD relative mRNA</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Left ventricle</td>
<td>1.00 ± 0.13 (7)</td>
<td>0.99 ± 0.11 (7)</td>
<td>0.94 ± 0.13 (8)</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>1.00 ± 0.14 (7)</td>
<td>0.94 ± 0.14 (8)</td>
<td>1.31 ± 0.23 (8)</td>
</tr>
<tr>
<td><strong>CPT-I\textalpha relative mRNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left ventricle</td>
<td>1.00 ± 0.22 (7)</td>
<td>0.74 ± 0.10 (7)</td>
<td>0.79 ± 0.10 (8)</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>1.00 ± 0.17 (6)</td>
<td>0.92 ± 0.18 (7)</td>
<td>1.42 ± 0.32 (8)</td>
</tr>
<tr>
<td><strong>CPT-I\textbeta relative mRNA</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Left ventricle</td>
<td>1.00 ± 0.17 (7)</td>
<td>0.86 ± 0.06 (7)</td>
<td>0.82 ± 0.06 (8)</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>1.00 ± 0.09 (6)</td>
<td>0.88 ± 0.10 (7)</td>
<td>1.28 ± 0.20 (8)</td>
</tr>
</tbody>
</table>

Data are presented as simple means ± SE, with sample size shown in parenthesis; mRNA expression levels are corrected against TBP mRNA; no differences between groups were statistically significant. See text for definitions of abbreviations.
Capacity for circulatory fuel uptake into working muscle. FAT/CD36 is a pivotal sarcolemmal transporter of long-chain fatty acids, and its overexpression can lead to elevated fatty acid oxidation, as well as increased rates of fatty acid transport into giant sarcolemmal vesicles (35). Both cellular mRNA expression and sarcolemmal protein content of FAT/CD36 is substantially augmented in the gastrocnemius muscle of HR_{mini} mice compared with HR_{normal} or control mice (Fig. 4), which suggests that the mini muscles of HR_{mini} mice may have an enhanced capacity for uptake of circulatory fatty acids. Furthermore, the gastrocnemius of HR_{mini} mice exhibit an 80% higher cytosolic content of H-FABP (Fig. 5), a protein that serves as an intracellular sink for fatty acids transported across the sarcolemma, and enables their movement through the cytoplasm (16). Although it seems that H-FABP is not a direct regulator of fatty acid translocation across the sarcolemma, increased H-FABP levels could accommodate rapid changes in substrate flux, such as during the transition from resting to contracting muscle (27). Interestingly, despite these innate biochemical differences in the skeletal muscle, our in vivo results suggest that HR_{mini} mice do not have (or do not capitalize upon) an enhanced whole body capacity to utilize lipids during high-intensity exercise compared with mice with the normal muscle phenotype. Perhaps the muscle phenotype...
differences reflect more an increased reliance on circulatory versus intramuscular lipids in the HR\textsubscript{mini} line.

HR\textsubscript{mini} mice may also have an enhanced capacity for utilizing circulatory glucose in working muscle. During exercise, when the glucose transporter GLUT-4 has been translocated to the plasma membrane, the phosphorylation of glucose by HK becomes a significant functional barrier of glucose uptake into the myocyte (see Ref. 49). While it has previously been shown that there are no differences in constitutive GLUT-4 levels in the gastrocnemius of resting HR\textsubscript{mini}, HR\textsubscript{normal}, or control mice, when housed without access to running wheels (17), HR\textsubscript{mini} mice do have increased mass-specific maximal HK activity in the gastrocnemius (Fig. 6). This elevated HK activity may facilitate enhanced glucose uptake into the gastrocnemius during exercise (see also Refs. 3, 18, 22), given that transgenic mice overexpressing HK II show increased glucose uptake into skeletal muscle during moderate-intensity exercise (20). Indeed, before exercise, HR\textsubscript{mini} mice have larger liver glycogen deposits than the other mouse phenotypes (17), and liver glycogen is an important source of energy during exercise for rodents (2).

Although these data point to variation in the capacity for utilizing circulatory fuels, since neither postexercise intramuscular substrate depletions nor in vivo muscle uptake rates for circulatory fuels during exercise were measured in these mice, further inferences are limited by the complexities of fuel metabolism.

Skeletal muscle capacity for oxidative metabolism. The increased capacity for substrate uptake into the skeletal muscle of HR\textsubscript{mini} mice is accompanied by an enhanced mass-specific capacity to utilize fatty acids. In addition to the increased mass-specific maximal activities of CS, which is a hallmark of the “mini muscle” phenotype’s enhanced aerobic capacity (18, 22), we show that sedentary HR\textsubscript{mini} mice also have an enhanced capacity for \(\beta\)-oxidation at the level of HOAD specific activity in the gastrocnemius (Fig. 6).

On a whole muscle basis, HR\textsubscript{mini} mice tend to have equivalent maximal enzymatic activities as control mice (see also Ref. 22). Conversely, HR\textsubscript{normal} mice (at least the line sampled here) appear to exhibit an enhancement of the overall metabolic capacity of the gastrocnemius. Because of the slight (but statistically nonsignificant) muscle enlargement compared with control mice, HR\textsubscript{normal} mice have significantly greater whole muscle maximal activities of CS, HOAD, CPT II, and HK than control mice (and HR\textsubscript{mini} mice, with the exception of HOAD; Fig. 6). Having a bigger muscle mass as opposed to greater mass-specific activities of metabolic enzymes may be a distinct mechanism that help HR\textsubscript{normal} mice achieve elevated whole body fuel oxidation rates (compared with control mice), commensurate with their increased \(V_{O2}\text{max}\).

Transcriptional regulation of skeletal muscles properties. Constitutive gene expression of PGC-1\textalpha, PPAR\beta/\textalpha, and SIRT1 in muscle are not significantly different among HR\textsubscript{mini}, HR\textsubscript{normal}, and control mice (Fig. 7), suggesting that these transcription factors do not play a major role in the distinct properties of the “mini muscle.” However, HR\textsubscript{mini} mice do exhibit greatly enhanced PPAR\textalpha mRNA expression in the gastrocnemius, and this may be an underlying basis for many of the altered metabolic characteristics of this skeletal muscle. PPAR\textalpha is one of the most predominant PPAR isoforms in skeletal muscle, and it is involved in the upregulation of many genes in the fatty acid metabolic pathway (29). For instance, our results show a strong correlation between mRNA levels of PPAR\textalpha and FAT/CD36 among all mice, which suggests a close relationship between the transcript levels of this transcription factor and a target gene (Fig. 7).

Perspectives and Significance

Despite divergent patterns in daily locomotor behavior and aerobic capacity, the proportionate mix of fuels used across relative exercise intensities was not statistically different for HR\textsubscript{mini}, HR\textsubscript{normal}, and control mice. Instead, this experimental evolution model supports the results of interspecific comparative studies (29, 41, 50) and shows that absolute rates of fuel use during submaximal exercise scale with variation in aerobic capacity among diverse mammals. Furthermore, owing to substantial differences in muscle anatomy and biochemistry, the HR mouse model demonstrates that it may be possible for mammals to differ in muscle fiber recruitment patterns while maintaining a conserved whole body pattern of fuel use, thus emphasizing the robustness of the mammalian fuel use pattern. Based on our findings, we propose that aerobic capacity and locomotor behavior may be evolutionary linked to the capacity for lipid oxidation. Through the course of evolution, mammals may exhibit multiple potential mechanisms or “solutions” [sensu Garland et al. (14)] to enhance lipid oxidation rates commensurate with aerobic capacity; biochemical traits can therefore differ between genetically or phenotypically distinct mammals without causing deviation from a conserved whole body fuel-use pattern.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: N.M.T., T.G., and G.B.M. conception and design of research; N.M.T. and H.S. performed experiments; N.M.T., H.S., and T.G. analyzed data; N.M.T., T.G., and G.B.M. interpreted results of experiments; N.M.T. prepared figures; N.M.T. drafted manuscript; N.M.T., H.S., T.G., and G.B.M. edited and revised manuscript; N.M.T., H.S., T.G., and G.B.M. approved final version of manuscript.

REFERENCES

REGULATION OF FUEL USE IN SELECTIVELY BRED HIGH-RUNNING MICE


