

RESEARCH ARTICLE | *Obesity, Diabetes and Energy Homeostasis*

Acclimation to hypoxia increases carbohydrate use during exercise in high-altitude deer mice

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Lau DS, Connaty AD, Mahalingam S, Wall N, Cheviron ZA, Storz JF, Scott GR, McClelland GB. Acclimation to hypoxia increases carbohydrate use during exercise in high-altitude deer mice. *Am J Physiol Regul Integr Comp Physiol* 312: R400–R411, 2017. First published January 11, 2017; doi:10.1152/ajpregu.00365.2016.—The low O₂ experienced at high altitude is a significant challenge to effective aerobic locomotion, as it requires sustained tissue O₂ delivery in addition to the appropriate allocation of metabolic substrates. Here, we tested whether high- and low-altitude deer mice (*Peromyscus maniculatus*) have evolved different acclimation responses to hypoxia with respect to muscle metabolism and fuel use during submaximal exercise. Using F₁ generation high- and low-altitude deer mice that were born and raised in common conditions, we assessed 1) fuel use during exercise, 2) metabolic enzyme activities, and 3) gene expression for key transporters and enzymes in the gastrocnemius. After hypoxia acclimation, highland mice showed a significant increase in carbohydrate oxidation and higher relative reliance on this fuel during exercise at 75% maximal O₂ consumption. Compared with lowland mice, highland mice had consistently higher activities of oxidative and fatty acid oxidation enzymes in the gastrocnemius. In contrast, only after hypoxia acclimation did activities of hexokinase increase significantly in the muscle of highland mice to levels greater than lowland mice. Highland mice also responded to acclimation with increases in muscle gene expression for hexokinase 1 and 2 genes, whereas both populations increased mRNA expression for glucose transporters. Changes in skeletal muscle with acclimation suggest that highland mice had an increased capacity for the uptake and oxidation of circulatory glucose. Our results demonstrate that highland mice have evolved a distinct mode of hypoxia acclimation that involves an increase in carbohydrate use during exercise.

hypoxia; exercise; carbohydrates; lipids; lactate; hexokinase; glucose transporter

THE LOW O₂ at high altitude poses a significant challenge to effective aerobic locomotion. Animals living in these environments must be able to transport sufficient O₂ to support exercise but also allocate the appropriate metabolic substrates to sustain aerobic ATP production rates. The pattern of metabolic substrate use during exercise has been characterized in a number of mammals (10, 41, 45, 55). In general, as metabolic demand increases with higher work rates, there is a predictable increase in the reliance on carbohydrates to support aerobic ATP production. This pattern is remarkably conserved in

low-altitude native mammals that vary in body size and aerobic capacity, suggesting the basis for a predictive model of exercise fuel use (45). Although exercise fuel use demonstrates phenotypic plasticity to chronic energetic (e.g., exercise training) and environmental (e.g., hypoxia) stress, the observed changes in rates of metabolic substrate oxidation are correlated with changes in aerobic capacity (e.g., Refs. 6, 9, and 34). One of the only documented deviations from this conserved fuel use pattern was observed in mice from the genus *Phyllotis* that are native to the high Andes of Peru. An increased reliance on carbohydrates to power aerobic exercise, independent of aerobic capacity, was observed in two species native to high altitude compared with two low-altitude congeneric species (44).

Substrate allocation is regulated, in part, by the mobilization and transport of circulating fuels for uptake by activated muscle fibers. However, the best-understood mechanisms involve the selective recruitment of intramuscular metabolic pathways. Although membrane transport in the muscle has been proposed to exert a high level of control on substrate utilization, recent evidence suggests that glucose phosphorylation in the cytosol by the enzyme hexokinase (HK) is an important bottleneck for muscle carbohydrate oxidation during exercise (54). Thus, it was surprising that no significant differences in skeletal muscle metabolic phenotype were found between highland and lowland *Phyllotis* mice that could provide a mechanistic explanation for differences in substrate use during exercise (44).

Acclimatization is important for adult animals to adjust on a physiological timescale to changing environments, but these changes are not always in a direction that is considered beneficial (15, 50). Past research has shown that chronic hypoxia can have varying effects on the skeletal metabolic phenotype of lowland mammals, including humans (1, 28). For example, a number of studies on lowland humans have suggested that chronic hypobaric hypoxia reduces muscle aerobic capacity (12, 20), contrary to the predicted benefit of a high mitochondrial content in low-O₂ environments (25). Few studies have examined whether high- and low-altitude natives differ in their acclimation responses to chronic hypoxia, either with regard to whole animal performance or the metabolic phenotype of skeletal muscle. In a previous study on high- and low-altitude deer mice (*Peromyscus maniculatus*) that were born and reared under common conditions, we showed that differences in aerobic capacity under hypoxia were attributable to differences in acclimation responses as well as evolved, genetically based

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changes. However, capillary surface density, tissue oxidative capacity, and transverse areas of oxidative fiber types (type I and IIa) were genetically fixed at higher levels in the gastrocnemius muscle in highland mice compared with lowland mice, and these traits were unaffected by hypoxia acclimation (31). Furthermore, these traits did not vary between wild deer mice and those raised in captivity (47). While a fixed trait in muscle aerobic capacity may reflect the stable low-O₂ conditions at high altitude (50), muscle capacities for substrate oxidation may be more flexible to match appropriate substrate use with changing energetic demands.

Deer mice are well suited to examine mechanisms of local adaptation since they can be found from below sea level to above 4,300 m (36). Estimates of field metabolic rates suggest that highland deer mice expend significantly more energy per day than mice from a lowland population (22), which would require correspondingly high rates of O₂ and substrate consumption. As a likely result, aerobic capacity in hypoxia is under strong directional selection at high altitude (23). Indeed, highland deer mice have higher maximal O₂ consumption ($\dot{V}O_{2\max}$) rates in hypoxia than lowland mice for thermogenesis and locomotion (14–16, 31). Highland deer mice have also been found to have greater capacities for lipid oxidation in muscle, presumably to support higher rates of sustained shivering thermogenesis (14). However, these mice may be expected to rely to a greater extent on carbohydrates to power exercise due to a superior yield of ATP per mole of O₂ consumed (8, 44).

The objective of the present study was to test whether the hypoxia acclimation responses of highland and lowland deer mice differ with respect to muscle metabolic phenotype and fuel use during submaximal exercise. We hypothesized that highland deer mice would have a greater reliance on carbohydrates during submaximal exercise, similar to high-altitude *Phyllotis* mice in the Andes (44). Since hypoxia acclimation does not affect exercise fuel use in laboratory rodents (34), we predicted that population differences in exercise metabolism would be fixed and would not be affected by chronic hypoxia. We assessed fuel use during submaximal exercise, measured metabolic enzyme activities, and gene expression for key membrane transporters and enzymes in the gastrocnemius muscle. We used F₁ generation mice born and raised in common-garden conditions to distinguish between population differences that reflect evolved, genetically based changes and those that reflect differences in acclimation to hypobaric hypoxia.

MATERIALS AND METHODS

Animals and acclimation conditions. All procedures were approved by the McMaster University Animal Research Ethics Board in accordance with guidelines from the Canadian Council on Animal Care. We used F₁ generation deer mice from a captive breeding colony that were offspring of wild-caught parents trapped at 4,350 m above sea level on the summit of Mount Evans, CO (highland mice, *P. m. rufinus*) and at an elevation of 430 m above sea level in Lancaster County, NE (lowland mice, *P. m. nebracensis*), as previously described (14, 31). F₁ mice born and raised at the University of Nebraska were shipped to McMaster University (~100 m above sea level) and kept at ~23°C with a 12:12-h light-dark cycle and provided standard rodent chow and water *ad libitum* for at least 4 wk before experiments started. This common-garden design helps control for both developmental and adult phenotypic plasticity, but we cannot

rule out the possibility that changes in the examined traits are partly attributable to transgenerational epigenetic effects.

Mice from each population were randomly divided into a control (unacclimated) group kept in normoxic laboratory conditions and a hypoxia-acclimated group held in hypobaric chambers at the equivalent of 4,300 m (450 mmHg). These mice were returned briefly to normobaria 1–2 times/wk for cage cleaning and replenishment of food and water. The acclimation period was for a minimum of 6–8 wk, as previously described (4, 31).

Indirect calorimetry. An open-flow respirometry system (Sable Systems, Las Vegas, NV) was used to determine $\dot{V}O_{2\max}$ at ~23°C in mice running on a Plexiglas-enclosed motorized treadmill with a volume of ~800 ml (44). Incurrent air (or 12% O₂-balance N₂) that had been stripped of CO₂ and H₂O was flowed into the treadmill chamber at an average rate of 1,940 ml/min. A subsample of excurrent air was dried using prebaked Drierite (56) and flowed through an O₂ and CO₂ analyzer at ~200 ml/min using a subsampling pump (Sable Systems). The accuracy of the system was periodically verified by burning methanol in a chamber and comparing theoretical and measured $\dot{V}O_2$ and CO₂ consumption ($\dot{V}CO_2$) as previously described (44, 51). The $\dot{V}O_{2\max}$ values presented here are from previously published data (31), with the addition of four unacclimated lowland, five acclimated lowland, and eight acclimated highland mice. $\dot{V}O_{2\max}$ was defined as when at least two of the following three criteria were fulfilled: 1) $\dot{V}O_2$ did not increase with an increase in treadmill speed, 2) the respiratory exchange ratio (RER; $\dot{V}CO_2/\dot{V}O_2$) was ≥ 1.0 , and 3) the mouse demonstrated behavioral signs of exhaustion and could not maintain its position on the treadmill. We have used these same criteria in previous studies (31, 44, 51). To control for the effect of exercise intensity on fuel use (see Ref. 45), mice were exercised at a speed equivalent to ~75% of $\dot{V}O_{2\max}$. For a few mice, we lacked $\dot{V}O_{2\max}$ data and used average treadmill speeds for their acclimation group to perform submaximal running tests on these individuals. Mice were run in the postabsorptive state by fasting for 4–6 h and under their respective acclimation conditions as we have previously done (34, 35). To calculate $\dot{V}O_2$ and $\dot{V}CO_2$, we used the following equations obtained from Withers (57):

$$\dot{V}CO_2 = VI_{\text{stp}}(F_{E_{CO_2}} - F_{I_{CO_2}}) \quad (1)$$

$$\dot{V}O_2 = \frac{VI_{\text{stp}}(F_{I_{O_2}} - F_{E_{O_2}}) - \dot{V}CO_2 \times F_{I_{O_2}}}{(1 - F_{I_{O_2}})} \quad (2)$$

where VI_{stp} is the rate of inflow into the chamber, $F_{E_{CO_2}}$ is the fraction of expired CO₂, $F_{I_{CO_2}}$ is the fraction of inspired CO₂, $F_{I_{O_2}}$ is the fraction of inspired O₂, and $F_{E_{O_2}}$ is the fraction of expired O₂. Cost of transport was calculated by dividing $\dot{V}O_2$ (in ml/min) by treadmill speed (in m/min) to obtain the metabolic cost of moving a meter distance (in ml O₂/m). Rates of carbohydrate and lipid oxidation were calculated according to Frayn (17) assuming that the contribution of protein to overall exercise energy expenditure is minimal in the postabsorptive state (11, 40), but, to our knowledge, this has not been confirmed for mice. Relative rates of carbohydrate and lipid oxidation were calculated by dividing absolute oxidation rates by $\dot{V}O_2$ and expressed as a percentage of total $\dot{V}O_2$. Individual values over 100% or under 0% were set to 100% and 0% (one individual acclimated highland mouse).

Gastrocnemius and liver enzyme activities. Mice were anaesthetized in a small chamber using an isoflurane-soaked cotton ball and euthanized by cervical dislocation. Gastrocnemius muscle and liver samples were collected at rest, crushed between liquid N₂-cooled aluminum plates, and then stored at –80°C until analysis. All enzyme measurements were performed using a Spectromax Plus 384, 96-well microplate reader (Molecular Devices, Sunnyvale, CA). Approximately 30 mg of gastrocnemius and liver samples were powdered in a liquid N₂-cooled mortar and homogenized on ice using a glass on glass homogenizer in 20 volumes of homogenizing buffer containing (in mM) 100 potassium phosphate (pH 7.2), 5 EDTA, and

0.1% Triton X-100. Assays were performed at 37°C in triplicate, and background activities were determined for each assay by omitting substrate.

The apparent V_{\max} of cytochrome *c* oxidase, phosphofructokinase (PFK), β -hydroxyacyl CoA dehydrogenase (HOAD), isocitrate dehydrogenase (IDH), and HK were measured on fresh homogenates. The activities of pyruvate kinase (PK) and citrate synthase (CS) were measured after the homogenates had been frozen and thawed once and three times, respectively. Assay conditions were PFK: 10 mM fructose-6-phosphate (omitted in control), 4 mM ATP, 0.28 mM NADH, 2 mM AMP, 5 mM MgCl₂, 50 mM KCl, 5 mM DTT, 1 unit aldolase, 50 units triose phosphate isomerase, and 8 units α -glycerophosphate dehydrogenase in 50 mM triethanolamine (TEA)-HCl (pH 7.6); HOAD: 0.1 mM acetoacetyl-CoA (omitted in control), 0.28 mM NADH, and 5 mM EDTA in 100 mM TEA-HCl (pH 7.0); IDH: 1.5 mM isocitrate (omitted in control), 2 mM ADP, 0.5 mM NADP, 1 mM MnCl₂, and 8 mM MgCl₂ in 40 Tris-HCl (pH 7.4); HK: 5 mM D-glucose (omitted in control), 8 mM ATP, 8 mM MgCl₂, 0.5 mM NADP, and 4 units glucose-6-phosphate dehydrogenase in 50 mM HEPES (pH 7.6); PK: 5 mM phosphoenol pyruvate (PEP; omitted in control), 5 mM ADP, 10 mM MgCl₂, 0.15 mM NADH, 10 mM fructose-1,6-phosphate, 100 mM KCl, and 9.25 units lactate dehydrogenase (LDH) in 50 mM imidazole (pH 7.4); CS: 0.5 mM oxaloacetate (omitted in control), 0.22 mM acetyl-CoA, and 0.1 mM dithiobisnitrobenzoic acid in 40 Tris-HCl (pH 8.0); phosphoenolpyruvate carboxykinase (PEPCK) in the liver: 1 mM MnCl₂, 0.15 mM NADH, 20 units malate dehydrogenase, 20 mM NaHCO₃, 0.2 mM GDP, and 0.5 mM PEP (omitted in control) in 20 mM imidazole (pH 7.0).

Blood and tissue metabolites. Resting blood samples were obtained from a subset of unstressed mice by placing a lid containing an isoflurane-soaked cotton ball carefully over their home cage. Once lightly anesthetized, mice were quickly removed from the cage and blood was obtained from the facial vein by pricking with a lancet and collecting in a heparinized capillary tube. Mice were then returned to their cage to recover for a minimum of 48 h. Postexercise blood samples were obtained immediately after 15 min of exercise in unanesthetized mice. Whole blood lactate was determined using a lactate analyzer (Lactate Pro LT, Arkray, Kyoto, Japan), and glucose was determined using a handheld glucose meter (Accu-Chek Aviva, Roche Diagnostics) as previously described in mice (7, 30).

Gastrocnemius muscle and liver samples were collected at rest as described above and immediately after 15 min of exercise at ~75% $\dot{V}O_{2\max}$ by cervical dislocation. Tissues were quickly removed, crushed between liquid N₂-cooled aluminum plates, and then stored at -80°C until analysis. A separate group of unacclimated mice from each population was sampled for skeletal muscle at rest as described above for blood sampling. These values were used to compare with postexercise results from all groups. Metabolites were determined by standard methods (5) adapted for a 96-well format using a Spectramax Plus 384 microplate reader (Molecular Devices). Briefly, ~30–50 mg of powdered tissues were homogenized (PowerGen 125, Fisher Scientific, Whitby, ON, Canada) in 600 μ l of 6% perchloric acid on ice. A portion of this homogenate (100 μ l) was removed and stored at

-80°C for glycogen determination. The remaining homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was removed and subsequently neutralized with K₂CO₃ (3 M). Samples were then centrifuged again at 10,000 g for 10 min at 4°C, and the resulting supernatant was used for metabolite quantification. Assay conditions for lactate determinations were 2 mM NAD⁺ in glycine/hydrazine buffer (pH 9.2, Sigma), and the reaction was started using 8 units LDH (Roche Diagnostics). For glycogen determinations, 50 μ l of 1 M K₂HCO₃ and 100 μ l of 400 mM acetate buffer (pH 4.8) were added to the crude homogenate (see above). Half of this homogenate was removed for determinations of free glucose, and the remaining homogenate was combined with 7 μ l (4 U/ μ l) of amyloglucosidase (Roche Diagnostics). All samples were incubated for 2 h at 40°C and then neutralized with 1 M K₂CO₃. Neutralized samples were assayed for glucose under the following conditions: 20 mM imidazole, 1 mM ATP, 0.5 mM NADP, 5 mM MgCl₂, and 1 unit glucose-6-phosphate dehydrogenase (Roche Diagnostics) in buffer containing 300 mM TEA-HCl and 4.05 mM MgSO₄ (pH 7.5) (Roche Diagnostics). The reaction was initiated by adding 1 unit HK (in TRA buffer, Roche Diagnostics).

Gene expression. Gastrocnemius muscles that were sampled from resting animals (with the exception of one unacclimated highland mouse sampled postexercise) were powdered under liquid N₂ as described above, and total RNA was extracted from 30–40 mg tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. One microgram of DNase I-treated total RNA was used to synthesize cDNA using SuperScript II RNase H- reverse transcriptase (Invitrogen). Specific primers (Table 1) were designed by Primer 3 software (43) using available deer mouse sequences from the National Center for Biotechnology Information Sequence Read Archive (Accession Nos. SRA051883 and SRA091630) (14, 15). Each transcript was amplified in duplicate using 4 μ l of cDNA diluted fivefold in RNase and DNase-free water (Invitrogen), 5 μ l of SsoFast EvaGreen Supermix (Bio-Rad, Mississauga, ON, Canada), 10 μ M of forward and reverse primers in 0.4 μ l, and 0.2 μ l of RNAase and DNase-free water. Real-time PCRs were run in the 96-well format using a CFX Connect Real-Time System (Bio-Rad) with a thermal program of 30 s of initial denaturation at 95°C, 40 cycles of 5 s at 95°C, and finally 30 s at 60°C. For each plate, a no-template control was included. Standard curves using one sample were generated for each primer set and used to determine the relative expression of all other samples. Expression levels were normalized to 12S rRNA expression, as previously described (31).

Calculations and statistics. Statistical analyses were performed using GraphPad Prism and SPSS (IBM) software packages. $\dot{V}O_{2\max}$ values were corrected for body mass using allometric regressions, as previously described (31). We also analyzed $\dot{V}O_{2\max}$ data using analysis of covariance (ANCOVA) with body mass as a covariate and obtained similar results as the allometric analysis. ANCOVA using mass as a covariate was also used to analyze cost of transport (COT) and exercise intensity as a covariate for RER (51). All other data were analyzed using two-way ANOVA with population and acclimation as

Table 1. Primer sequences used for real-time PCR analysis of mRNA expression in deer mice gastrocnemius muscle

Gene	Forward Primer	Reverse Primer
<i>Glut4</i>	5'-GCTTTGTGGCCTTCTTTGAG-3'	5'-TCAGGCACTTTGAGGAAGGT-3'
<i>Slc2a1</i>	5'-CATCCAGCCCTGATACAGT-3'	5'-CAACTCCAGGATGGTGACCT-3'
<i>Hk1</i>	5'-AAACCCAGAGAACATCGTG-3'	5'-TTCAGCAGCTTGACCACATC-3'
<i>Hk2</i>	5'-GGTGGAGATGGAGAACCAGA-3'	5'-CCTTCCACACCACTGGACTT-3'
<i>Ndfuc2</i>	5'-ACGAGCCCTTACATTTCTG-3'	5'-AATAGAAGCTGGCGATGCAG-3'
<i>Pck1</i>	5'-CAGGATTGAAGGGGAAGACA-3'	5'-TCTGTCTCAGGGCTTGGAGT-3'
<i>12S</i>	5'-CTGGCCATCGCTTAAACTC-3'	5'-TTGCTTCCCACCTCATAGC-3'

Glut, glucose transporter; Hk, hexokinase. Ndfuc encodes a NADH dehydrogenase (ubiquinone) subunit; Pck1 encodes phosphoenolpyruvate carboxykinase.

main factors. Exercise was also included as a main factor for metabolite data. Data are presented at means \pm SE.

RESULTS

Aerobic capacity and submaximal exercise intensities. There is a strong relationship between exercise intensity relative to maximum aerobic capacity and the proportional use of carbohydrates and lipids (45). Thus, we standardized exercise intensity by first determining running $\dot{V}O_{2\max}$ for animals in their respective acclimation condition. The results show that hypoxic $\dot{V}O_{2\max}$ was $\sim 14\%$ higher in acclimated highland versus lowland mice (3.47 ± 0.05 vs. 3.05 ± 0.20 ml/min, $P = 0.023$). However, highland mice had a significantly higher body mass, and there were no significant population differences in mass-corrected $\dot{V}O_{2\max}$ detected for unacclimated mice tested in normoxia or for acclimated mice tested in hypoxia (ANCOVA, $F_{1,51} = 0.62$, $P = 0.44$; Table 2).

Mice were then run at a target submaximal intensity of 75% $\dot{V}O_{2\max}$. There were no significant differences between populations in relative exercise intensity calculated after the run. However, highland mice were running at a significantly higher absolute treadmill speed, and they also had a significantly lower $\dot{V}O_2$ in normoxia ($F_{1,30} = 4.26$, $P = 0.049$; Table 2). The result of a higher running speed and lower metabolic cost resulted in a significantly lower COT for unacclimated highland mice compared with unacclimated lowland mice ($F_{1,31} = 7.19$, $P = 0.012$; Table 2). A similar difference in COT was seen between populations after acclimation and approached statistical significance ($F_{1,29} = 3.86$, $P = 0.06$).

We determined RERs from $\dot{V}O_2$ and $\dot{V}CO_2$ during submaximal running as an index of metabolic fuel use. There was a significant effect of acclimation on RER during submaximal running ($F_{1,56} = 6.68$, $P = 0.013$) that was driven primarily by an increase in exercise RER values in highland mice from 0.827 ± 0.009 when unacclimated to 0.884 ± 0.018 after hypoxia acclimation (Table 2).

Fuel oxidation with submaximal exercise. To determine rates of whole animal substrate oxidation, we used $\dot{V}O_2$ and $\dot{V}CO_2$ in the oxidation of carbohydrates and lipids (17) to calculate absolute rates of use during exercise. We found that there was a significant interaction between population and acclimation for the absolute rates of carbohydrate oxidation during submaximal running ($F_{1,63} = 4.11$, $P = 0.047$; Fig. 1). Rates of carbohydrate oxidation were significantly lower in

unacclimated highland mice running in normoxia compared with lowland mice ($P = 0.027$). However, acclimation led to a significant 34% increase in carbohydrate oxidation in highland mice ($P = 0.031$; Fig. 1A). There was also a significant interaction effect of acclimation and population for lipid oxidation ($F_{1,63} = 4.14$, $P = 0.041$), which showed a corresponding decline in acclimated highland mice to rates significantly lower than lowland mice ($P = 0.016$; Fig. 1B). To account for any differences in metabolic rate during submaximal exercise, absolute rates of fuel oxidation were divided by total $\dot{V}O_2$. The results show that relative rates of carbohydrate oxidation in highland mice significantly increased with acclimation ($P = 0.004$) to a higher level than rates in acclimated lowland mice (population \times acclimation, $F_{1,63} = 4.35$, $P = 0.041$; Fig. 1C). There was no significant effect of acclimation on either absolute rates or the relative proportion of substrate oxidation in lowland mice (Fig. 1). These results demonstrate that high- and low-altitude mice have different acclimation responses with regard to substrate use during exercise, such that only highland mice respond to chronic hypoxia with an increased reliance on carbohydrates to power submaximal exercise.

Blood and tissue metabolites. To determine if exercise substrate use was reflected in changes in blood and tissue metabolite concentrations, we determined the levels of circulating glucose and lactate at rest and immediately after submaximal exercise. We found a significant effect of exercise on blood glucose ($F_{1,49} = 12.21$, $P = 0.001$) and a significant interaction between population and exercise ($F_{1,49} = 6.08$, $P = 0.018$; Fig. 2A). At rest, glucose levels were significantly lower in highland mice compared with lowland mice but only after acclimation ($P = 0.08$ before acclimation and $P = 0.009$ after acclimation). Fifteen minutes of submaximal exercise led to a significant decline in blood glucose in lowland mice in normoxia ($P = 0.016$) and hypoxia ($P = 0.001$). In contrast, highland mice maintained stable glucose levels with exercise in both conditions ($P > 0.05$).

Blood lactate also showed a main effect of exercise ($F_{1,49} = 95.54$, $P = 0.00$) and an effect of population approached statistical significance ($F_{1,50} = 3.50$, $P = 0.068$; Fig. 2B). Both populations showed a significant accumulation of blood lactate with exercise but to a higher concentration in unacclimated lowland mice in normoxia ($P = 0.006$). However, neither population showed any significant effect of acclimation, but blood lactate concentrations reached equivalent

Table 2. Average body mass, $\dot{V}O_{2\max}$, $\dot{V}O_2$, respiratory exchange ratio, and cost of transport at a target intensity of 75% $\dot{V}O_{2\max}$ in normoxia ($F_{I_{O_2}} = 0.2095$) for unacclimated F_1 deer mice and in hypoxia ($F_{I_{O_2}} = 0.12$) for hypoxia-acclimated F_1 lowland and highland deer mice

	Normoxia		Hypoxia	
	Lowland mice	Highland mice	Lowland mice	Highland mice
Body mass, g	20.5 \pm 0.9 (12)	21.6 \pm 1.2 (13)†	19.0 \pm 0.9 (12)	23.8 \pm 1.4 (13)†
$\dot{V}O_{2\max}$, ml/min	3.91 \pm 0.18	3.79 \pm 0.20	3.05 \pm 0.20*	3.47 \pm 0.05*
Submaximal running				
$\dot{V}O_2$, ml/min	2.99 \pm 0.21 (12)	2.88 \pm 0.15 (20)†	2.60 \pm 0.08 (13)	2.75 \pm 0.11 (18)
% $\dot{V}O_{2\max}$	76 \pm 4	72 \pm 2	82 \pm 2	79 \pm 3
Speed, m/min	18.9 \pm 1.0	23.4 \pm 1.4†	17.0 \pm 1.2	20.3 \pm 0.8*
Respiratory exchange ratio ($\dot{V}CO_2/\dot{V}O_2$)	0.854 \pm 0.011	0.827 \pm 0.009	0.849 \pm 0.016	0.884 \pm 0.018*
Cost of transport, ml/m	0.17 \pm 0.02	0.13 \pm 0.01†	0.16 \pm 0.02	0.14 \pm 0.01

Numbers in parentheses are sample sizes for each group. $\dot{V}O_{2\max}$, maximal O_2 consumption ($\dot{V}O_2$); $\dot{V}CO_2$, CO_2 production; $F_{I_{O_2}}$, fraction of inspired O_2 . *Significant effect of acclimation within a population; †significant effect of population within an acclimation.

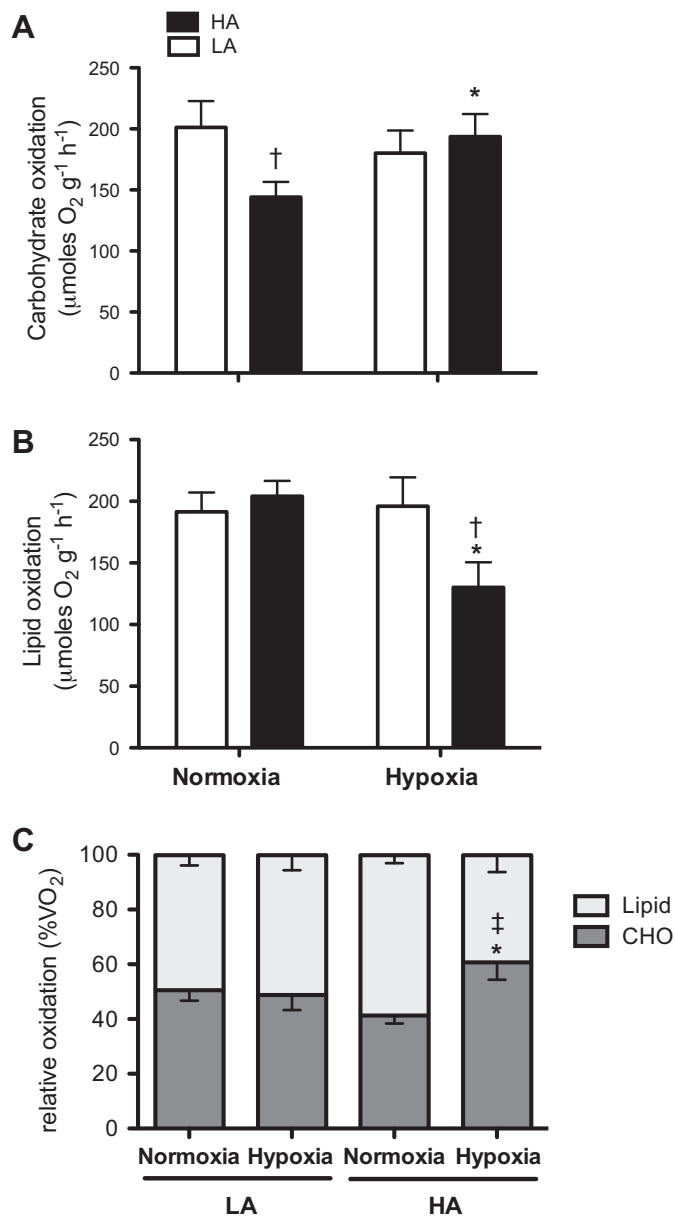


Fig. 1. Absolute rates of carbohydrate (CHO; A) and lipid (B) oxidation at a target intensity of 75% maximal $\dot{V}\text{O}_{2\text{max}}$ (actual intensities appear in Table 2) in lowland (LA) and highland (HA) mice exercising in their acclimation conditions [normoxia [fraction of inspired O_2 ($F_{\text{I}\text{O}_2}$): 0.2095] for unacclimated and hypoxia ($F_{\text{I}\text{O}_2}$: 0.12) for hypoxia acclimated]. The proportional contribution of carbohydrates and lipids as relative oxidation (percent $\dot{V}\text{O}_2$; C) was calculated by dividing absolute oxidation rates by $\dot{V}\text{O}_2$ and multiplying by 100. Results are presented as means \pm SE. *Significant effect of acclimation within a population; †significant effect of population within an acclimation group; ‡significant interaction of population \times acclimation.

levels in lowland and highland mice exercising in hypoxia (Fig. 2B).

Muscle lactate and glycogen and liver glycogen. There was a significant effect of exercise on muscle glycogen, which declined in all groups compared with rest ($F_{1,30} = 65.67$, $P = 0.00$; Fig. 3B). Similarly, muscle lactate increased significantly with exercise in all groups ($F_{1,37} = 10.72$, $P = 0.003$, Fig. 3). However, for both muscle glycogen and lactate, there were no significant main effects of population or acclimation, and there

were no significant interactions between population, acclimation, and exercise. Liver glycogen is an important extramuscular carbohydrate source for locomotion. Resting levels of liver glycogen were not significantly different between the populations and did not change with acclimation (Fig. 4). However, there was a significant reduction in liver glycogen after exercise ($F_{1,37} = 10.72$, $P = 0.003$), but there were no main effects of population or acclimation, and there were no significant interactions between population, acclimation, and exercise.

Gastrocnemius and liver enzyme activities. We previously showed that the gastrocnemius of highland mice has greater oxidative capacity than that of lowland mice, and this trait was not affected by hypoxia acclimation (31). Our results confirm these findings and showed a significant main effect of population for the enzymes HOAD ($F_{1,34} = 8.02$, $P = 0.008$) and IDH ($F_{1,34} = 17.55$, $P = 0.00$), which was higher in highland mice with normoxia ($P = 0.022$) and hypoxia acclimation ($P = 0.01$; Fig. 5, D and E). However, the differences in fuel use in lowland and highland mice after acclimation (Fig. 1) suggest that representative enzymes in glycolysis may be affected by chronic hypoxia. Indeed, there was a significant effect of acclimation for HK activity ($F_{1,34} = 4.15$, $P = 0.05$). Muscle HK activity increased by 35% in highland mice after hypoxia acclimation to activities higher than those in lowland mice ($P = 0.022$; Fig. 5A). Similarly, PK activity showed a significant effect of population ($F_{1,34} = 4.12$, $P = 0.051$) and

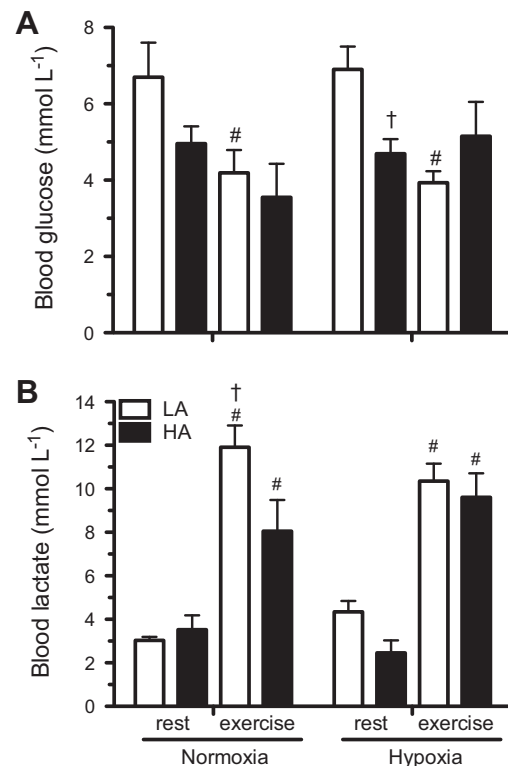


Fig. 2. Blood glucose and lactate concentrations in LA and HA mice at rest and after 15 min of exercise at 75% $\dot{V}\text{O}_{2\text{max}}$ in their acclimation conditions [normoxia ($F_{\text{I}\text{O}_2}$: 0.2095) for unacclimated and hypoxia ($F_{\text{I}\text{O}_2}$: 0.12) for hypoxia acclimated]. Results are presented as means \pm SE. *Significant effect of acclimation within a population; †significant effect of population within an acclimation group; #significant effect of exercise within a population for each acclimation.

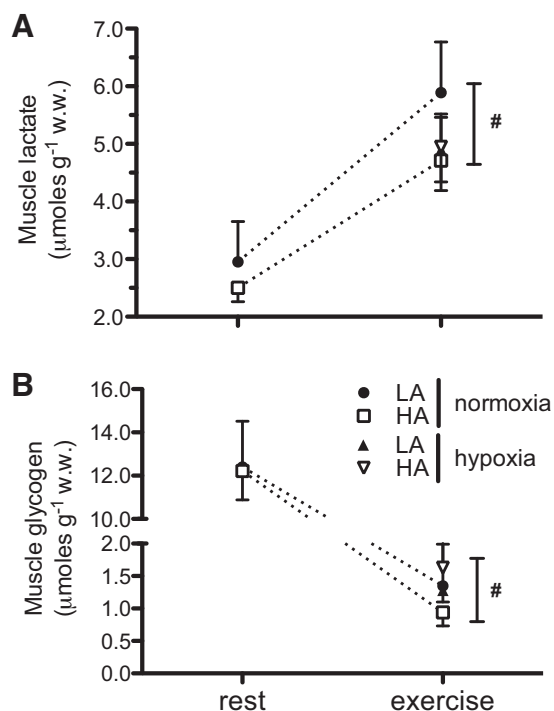


Fig. 3. Gastrocnemius muscle lactate (A) and glycogen concentrations (B) in LA and HA mice at rest and after 15 min of exercise at 75% $\dot{V}O_{2max}$ in their acclimation conditions [normoxia (F_{I_0} : 0.2095) for unacclimated and hypoxia (F_{I_0} : 0.12) for hypoxia acclimated]. Results are presented as means \pm SE. #Significant effect of exercise in all groups.

was higher in lowland mice, but only after acclimation ($P = 0.015$; Fig. 5C). While there was an overall significant population effect for PFK ($F_{1,20} = 5.42$, $P = 0.033$), the slight increase of $\sim 20\%$ in both populations after hypoxia acclimation did not constitute a statistically significant change (Fig. 5B).

Gene expression in gastrocnemius muscle. We tested whether high- and low-altitude mice exhibited differences in the expression of six candidate genes involved in muscle metabolism. Specifically, we tested for differences in constitutive gene expression as well as differences in the acclimation response to hypoxia. Relative transcript levels of Slc2a1 [glucose transporter (GLUT)1] and GLUT4 showed a significant effect of acclimation ($F_{1,22} = 11.51$, $P = 0.003$, and $F_{1,21} = 23.18$, $P = 0.00$, respectively). Slc2a1 significantly increased but only in highland mice ($P = 0.005$), and Glut4 increased in both lowland and highland populations ($P = 0.003$ and $P = 0.004$; Fig. 6, A and B). There was a significant acclimation effect in the expression of the Hk I transcript ($F_{1,20} = 5.42$, $P = 0.033$) and a significant acclimation effect ($F_{1,22} = 4.81$, $P = 0.042$) and nearly statistically significant interaction effect of population and acclimation ($F_{1,22} = 4.07$, $P = 0.059$) for the Hk II expression (Fig. 6, C and D). In unacclimated mice, lowland mice had higher HK II expression ($P = 0.016$), but highland mice showed a significant increase in the expression of this gene with acclimation ($P = 0.007$) to a level equivalent to that in lowland mice (Fig. 6D). Ndfuc2, which encodes a NADH dehydrogenase (ubiquinone) subunit, showed a significant acclimation effect ($F_{1,22} = 8.95$, $P = 0.008$), with acclimation increasing transcript levels in lowland mice to a value greater than highland mice ($P = 0.045$; Fig. 6E). Finally, Pck1, which encodes the

PEPCK enzyme, a component of the gluconeogenic pathway (Fig. 6F), showed a significant effect of population ($F_{1,22} = 5.65$, $P = 0.029$) and tended to be higher in highland mice ($P = 0.065$).

Liver enzyme V_{max} . Glycogenolysis and gluconeogenesis in the liver are major sources of circulatory glucose for working muscle. We examined the V_{max} of PEPCK as an index of gluconeogenic capacity of the liver and CS and cytochrome *c* oxidase for the capacity to supply ATP for liver metabolism. We found that although mean PEPCK activity tended to be higher in highland mice by ~ 8 – 18% , there was no statistically significant effect of population ($F_{1,41} = 2.42$, $P = 0.14$; Fig. 7B). However, activities of cytochrome *c* oxidase showed a significant population effect ($F_{1,42} = 5.30$, $P = 0.027$) and was greater in unacclimated highland mice ($P = 0.04$). There was a significant main effect of population for HOAD activity ($F_{1,42} = 7.22$, $P = 0.011$), which was also higher in highland mice in the unacclimated state ($P = 0.049$; Fig. 7, A and D). Thus, capacities for electron transport and fatty acid oxidation were higher in the livers of unacclimated highland versus lowland mice. Although none of the enzymes measured responded to acclimation, activities of cytochrome *c* oxidase and HOAD were not significantly different between highland and lowland mice in this condition ($P = 0.27$ and $P = 0.085$, respectively).

DISCUSSION

The primary goal of the present study was to characterize differences between highland and lowland mice in the phenotypic plasticity of substrate oxidation during submaximal exercise. Our results show that F₁ generation high-altitude deer mice have increased rates of carbohydrate oxidation after hypoxia acclimation, resulting in a higher proportional contribution of this fuel to total exercise energy expenditure. Highland mice had elevated activities of the mitochondrial enzyme IDH in gastrocnemius muscle, which was unaffected by acclimation. These data confirmed our previous findings that high-altitude mice have evolved a more aerobic skeletal muscle phenotype (31, 47). Similarly, high-altitude mice have a higher capacity for lipid oxidation in skeletal muscle. In contrast, we

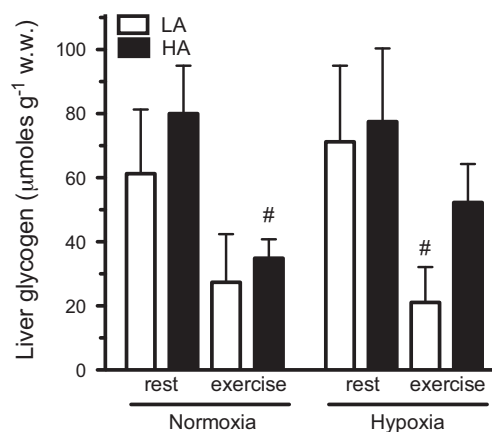


Fig. 4. Liver glycogen concentrations in LA and HA mice at rest and after 15 min of exercise at 75% $\dot{V}O_{2max}$ in their acclimation conditions [normoxia (F_{I_0} : 0.2095) for unacclimated and hypoxia (F_{I_0} : 0.12) for hypoxia acclimated]. Results are presented as means \pm SE. #Significant effect of exercise.

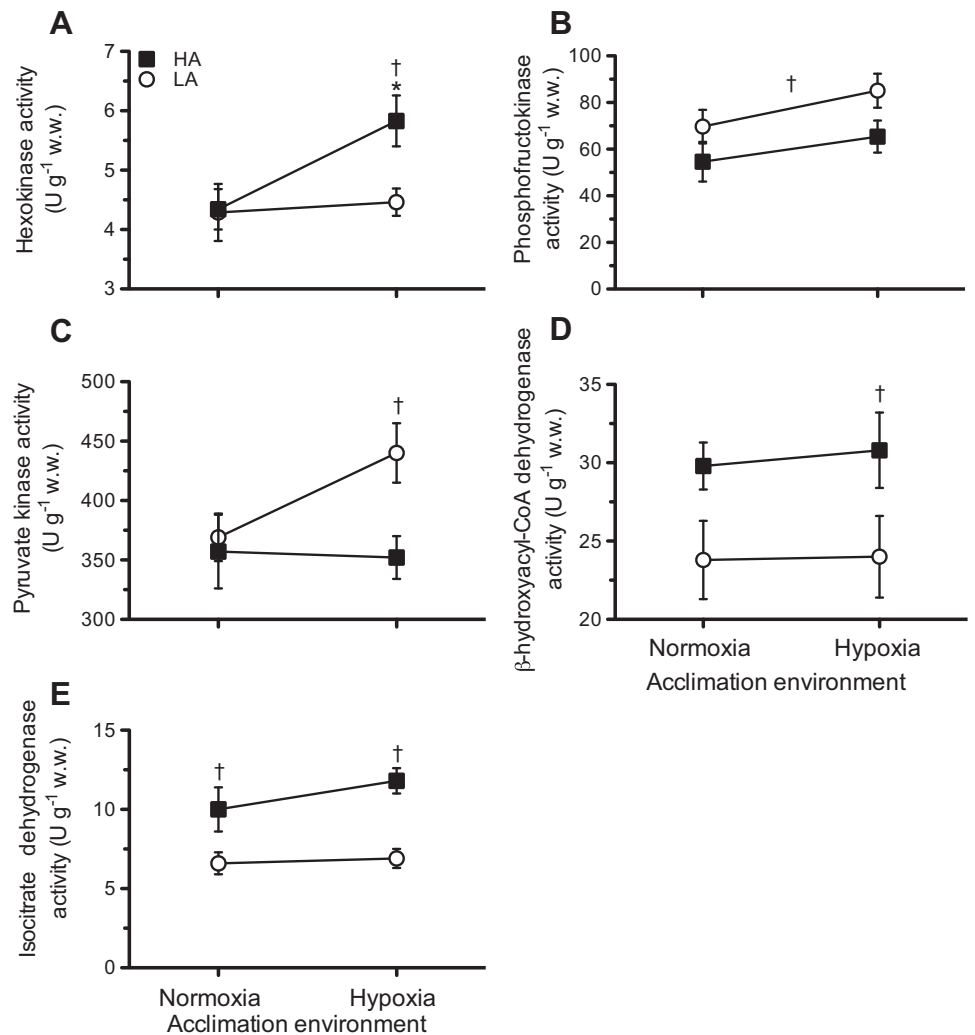


Fig. 5. Apparent V_{\max} (in U/g tissue wet wt) in gastrocnemius for the enzymes hexokinase (A), phosphofructokinase (B), pyruvate kinase (C), β -hydroxyacyl-CoA dehydrogenase (D), and isocitrate dehydrogenase (E) for LA and HA deer mice acclimated to normoxia or hypoxia. Results are presented as means \pm SE. *Significant effect of acclimation within a population; †significant effect of population within an acclimation group.

found that the activities of enzymes involved in glycolysis showed significant phenotypic plasticity in muscle with hypoxia acclimation. In particular, chronic hypoxia increased the activity of HK in highland mice, suggesting an enhanced capacity for circulatory glucose uptake by working muscle during exercise.

Fuel use during submaximal exercise. Our previous studies demonstrated that high- and low-altitude deer mice did not exhibit a significant change in running $\dot{V}O_{2\max}$ in normoxia in response to hypoxia acclimation (31). However, in this study, F₁ generation high-altitude deer mice exhibited increased rates of carbohydrate oxidation during submaximal exercise with hypoxia acclimation (Fig. 1). Thus, highland and lowland mice exhibited independent differences in the phenotypic plasticity of exercise fuel use and aerobic capacity. These results are consistent with cardiac data from highland native humans (27) and whole animal data from other highland mouse lineages (44). High-altitude species of *Phyllotis* mice were found to have a greater reliance on carbohydrates for exercise in hypoxia than did low-altitude species from the same genus (44). It is interesting to note that these mice were wild caught but kept in laboratory conditions for several weeks before being tested. These results suggest a fixed genetic adaptation in the high-altitude *Phyllotis* species is principally responsible for the

observed phenotype. In contrast, we found that, in the unacclimated state, lowland and highland populations of deer mice showed no significant differences in proportional fuel use. Lowland deer mice also showed no change in exercise fuel use with hypoxia acclimation, consistent with data from laboratory rats (34, 35) and lowland humans (9, 32). The differences in acclimation responses documented here demonstrate that high-altitude mice have greater phenotypic plasticity for proportional fuel use than do their low-altitude counterparts. Moreover, these and other data (44) suggest that an increased reliance on carbohydrates to power exercise is a trait unique to highland natives, presumably to optimize the energy yield per unit O₂ in a hypoxic environment (8). The present study also suggests that both genetic adaptation and phenotypic plasticity can affect this trait, but their relative influence is likely a factor of the degree of gene flow with lowland populations or length of colonization at altitude.

Highland mice had a lower COT when tested in normoxia and a similar difference that approached statistical significance when tested in hypoxia after hypoxia acclimation (Table 2). Mechanisms that may lower the cost of moving a unit distance can be either biochemical or biomechanical in nature. Increasing the use of carbohydrates decreases the O₂ cost of ATP production, whereas increased efficiency of generating force

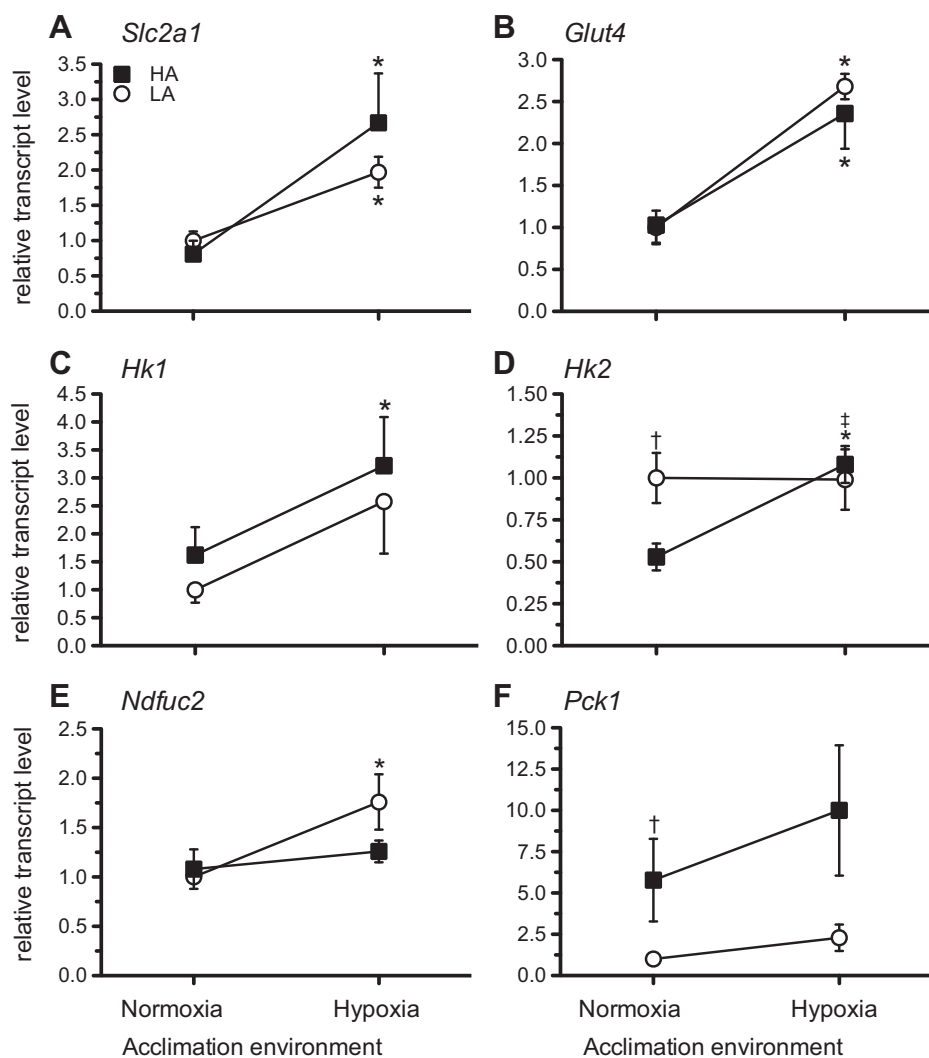


Fig. 6. Relative transcript abundance in gastrocnemius muscle for the following genes involved in carbohydrate metabolism: Slc2a1 (A), glucose transporter (Glut4) (B), hexokinase (Hk1) (C), Hk2 (D), Ndfuc2 [which encodes for a NADH dehydrogenase (ubiquinone) subunit; E], and Pck1 (which encodes phosphoenolpyruvate carboxykinase; F) in LA and HA mice acclimated to normoxia or hypoxia. Results are presented as means \pm SE. *Significant effect of acclimation within a population; †significant effect of population within an acclimation group; ‡significant interaction of population \times acclimation.

decreases ATP consumption at a given work rate. Although the former may contribute to differences in COT in the acclimated state, the lack of variation in exercise fuel use in the unacclimated state does not support this biochemical mechanism in lowering COT (Fig. 1). Highland mice must therefore lower COT by increasing the efficiency of producing muscle force by a yet unknown biomechanical mechanism.

Muscle metabolic phenotype. The gastrocnemius muscle of highland mice showed both higher aerobic capacities and higher capacities for fatty acid oxidation (Fig. 5). Our previous studies have shown that differences in muscle aerobic capacity can be largely attributed to the higher aerial densities of aerobic fibers in highland mice, and these population differences are robust to acclimation conditions (31, 47). The more aerobic phenotype of highland muscle is associated with higher exercise (31) and thermogenic (15) capacities in hypoxia. In fact, there is strong directional selection for increased aerobic capacity for thermogenesis in highland deer mice (14, 23). Lipids are an essential fuel for sustained aerobic heat production in rodents (52), and in deer mice, elevated capacities for thermogenesis in the highland population are associated with elevated capacities for fatty acid oxidation in muscle (14). These data suggest aerobic capacity and lipid metabolism are strongly

linked in this species. However, capacities for muscle uptake and cytosolic transport of fatty acids, two traits associated with increased lipid oxidation in laboratory mice (51), have not been assessed in high-altitude natives.

In contrast to lipid metabolism, the results of this study demonstrate that the capacity for muscle glucose uptake and glycolysis exhibits substantial phenotypic plasticity in response to hypoxia in deer mice. The significant increase in HK activity in highland mice suggests that the capacity for glucose uptake was enhanced in this population. Phosphorylation of glucose by HK has been shown to play an essential role in determining muscle glucose uptake during exercise (54), and elevated muscle HK activity is associated with increased endurance in mice (18). The importance of circulatory fuel to total $\dot{V}O_2$ can be low in humans (42), but it varies across species and with exercise intensity. Rodents exercising at equivalent work rates to those reported here have been shown to support up to 25% of $\dot{V}O_2$ using circulatory glucose (34). In addition, mice lacking the muscle isoform of glycogen synthase showed no decline in endurance (38), suggesting that the ability to use circulating glucose independent of muscle glycogen breakdown is very beneficial. We also found that activities of the glycolytic enzymes PFK and PK were higher after

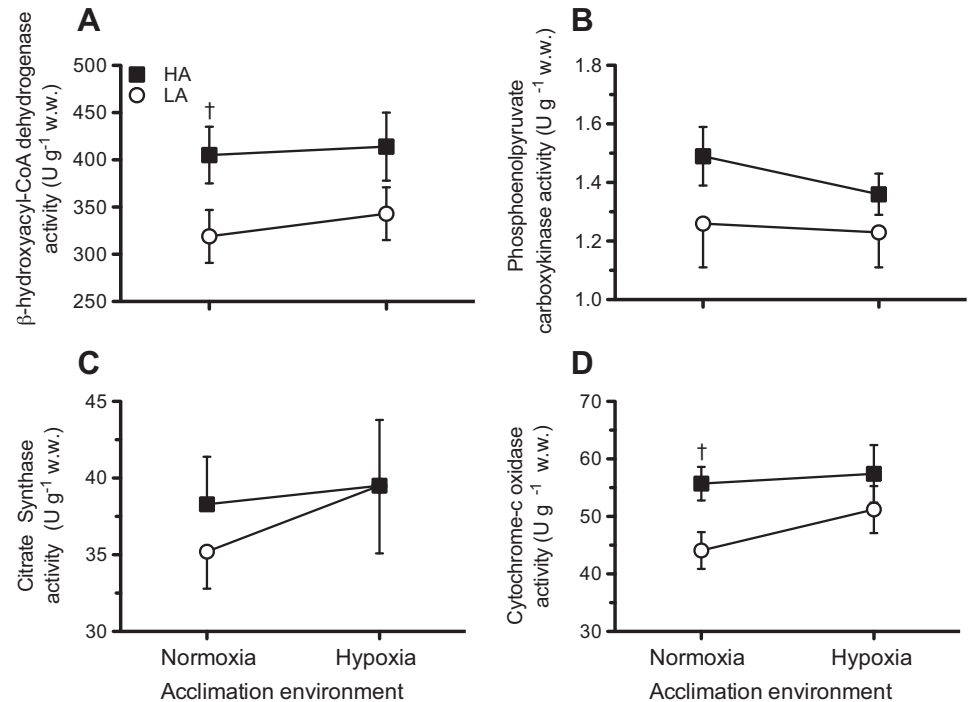


Fig. 7. Apparent V_{\max} (in U/g tissue wet wt) in the liver for the following enzymes: β -hydroxyacyl-CoA dehydrogenase (A), phosphoenolpyruvate carboxykinase (B), citrate synthase (C), and cytochrome *c* oxidase (D) in LA and HA mice acclimated to normoxia or hypoxia. Results are presented as means \pm SE. †Significant effect of population within an acclimation group.

acclimation but in lowland mice. Hypoxia is known to stimulate the increase in glycolytic enzymes including LDH, which was also higher in lowland mice (31). However, after acclimation, both populations had an equivalent capacity of aerobic relative to anaerobic glucose use (PK/LDH: ~ 0.75). These data suggest that the skeletal muscle of hypoxia-acclimated highland mice likely has a uniquely enhanced capacity for using circulatory glucose compared with lowland mice. This would have the combined advantage of yielding more ATP per mole of O_2 and also conserving valuable intramuscular carbohydrate stores. It is important to note that our measurements of enzyme activity indicate the capacity at specific reaction steps but not the *in vivo* rates of pathway flux.

Muscle gene expression. The change in muscle capacity for glucose use with acclimation was associated with increases in mRNA for the genes Hk I and Hk II in highland mice. Expression levels of membrane glucose transporters (Slc2a1 and Glut4) also increased with acclimation but in mice from both populations. However, these data suggest that membrane transport and glucose phosphorylation capacity are both increased in highland mice. Surprisingly, highland mice had higher relative gene expression for Pck1, which encodes the enzyme PEPCK, a component of the gluconeogenic pathway. While skeletal muscles generally exhibit negligible rates of gluconeogenesis, PEPCK has been implicated in enhancing exercise endurance (21, 37). Mice overexpressing this enzyme in locomotory muscles had higher levels of aerobic activity, perhaps by reducing the concentrations of inhibitory tricarboxylic acid cycle end products or by enhancing glycerol synthesis in this nongluconeogenic tissue (21, 58). Previously, we found that expression levels of transcription factors that regulate genes involved in angiogenesis and various metabolic pathways were either unchanged or decreased with hypoxia acclimation regardless of altitude ancestry. The exception was peroxisome proliferator-activated receptor (PPAR)- γ , which

was higher in highland mice muscle at mRNA and protein levels (31). Elevated PPAR- γ may play a role in stimulating Pck1 expression in muscle, as it does in adipocytes. Elevated PPAR- γ has also been associated with increased muscle glucose uptake and insulin sensitivity (2). Alternatively, various other metabolic genes are more highly expressed in the muscle of highland mice (14, 15, 47) and could contribute to the observed differences in metabolic phenotype and exercise fuel use.

Blood and skeletal muscle metabolites. Chronic hypoxia did not significantly alter resting blood glucose levels in either population (Fig. 2). This contrasts with some (19) but not all studies (34) on hypoxia-exposed laboratory rodents and suggests a possible distinct hypoxia response in deer mice. Highland mice maintained constant blood glucose concentrations between rest and immediately postexercise (Fig. 2). This suggests rapid adjustments of hepatic supply and muscle uptake to match changing energetic demands. In contrast, lowland mice showed significant declines in blood glucose with exercise, suggesting a substantial mismatch between substrate supply and demand. Population differences in blood glucose were not associated with muscle or liver glycogen depletion with exercise, which was not significantly different between high- and low-altitude mice or with acclimation (Figs. 3 and 4). The lower blood glucose levels seen in highland mice are consistent with other highland populations, including humans (39), and may be related to elevated PPAR- γ protein expression in skeletal muscle of this population (31). This would be consistent with studies of laboratory mice that demonstrated that overexpression of PPAR- γ conferred an increased insulin sensitivity (2, 3, 24).

Postexercise accumulation of blood lactate was higher in lowland mice in the unacclimated condition but had similar blood concentrations to those in highland mice after hypoxia acclimation. Since muscle lactate was not different between the

populations postexercise, these data suggest that lowland mice may have reduced lactate production with exercise after acclimation, possibly by relying to a lesser extent on anaerobic metabolism. It is also possible that reductions in blood lactate reflect increase uptake by tissues, such as the liver, to serve as a gluconeogenic precursor.

Liver enzymes and glycogen. Neither glycogen nor enzyme activities in the liver were significantly altered by hypoxia acclimation (Figs. 4 and 7). Both populations showed a decline in liver glycogen with exercise but to equivalent levels, suggesting that hepatic glucose production from glycogenolysis did not differ. However, the higher HOAD and cytochrome *c* oxidase activities in the livers of highland mice suggest a greater capacity for energy supply to support processes such as gluconeogenesis. An elevated capacity to generate ATP combined with a trend for higher PEPCK activities is suggestive of enhanced hepatic glucose production (29) in highland mice. A greater capacity for hepatic glucose production and muscle uptake would lead to higher glucose availability for exercise and tighter regulation of blood glucose levels.

Substrate and oxygen transport. Aerobic metabolism involves transport steps that are shared by both substrates and O₂. Adaptations for enhanced O₂ transport observed in highland mice (31) could also enhance substrate transport rates. Highland mice respond to acclimation with a blunted increase in hematocrit compared with lowland mice (31). Under hypoxia, they are able to sustain tissue O₂ delivery by virtue of an increased hemoglobin-O₂ affinity, which safeguards arterial O₂ saturation, thereby enhancing the O₂ capacitance of the blood (the total amount of O₂ unloaded for a given arteriovenous difference in O₂ tension) (48, 49). Circulatory substrate transport should thus be elevated in highland mice, because a lower hematocrit would 1) lead to higher rates of plasma flow at equivalent cardiac outputs and 2) avoid viscosity-related declines in cardiac output that can reduce tissue blood flow (46). As a result, highland mice may be able to avoid depleting valuable intramuscular substrate stores (33). Capillary surface area also plays an essential role in determining net substrate flux from the circulation into muscle (53). Highland mice have significantly higher muscle capillary surface densities (31, 47), which should considerably increase substrate conductance compared with lowland mice. When combined with the capacity for an increased transendothelial glucose concentration difference via increased glucose phosphorylation potential, this increased substrate conductance likely facilitates carbohydrate oxidation at high altitude.

Conclusions and implications. A potential downside to increased carbohydrate use is the exhaustion of limited glycogen stores. We estimate that high-altitude mice could sustain 75% $\dot{V}O_{2\max}$ for ~20 min on available glycogen stores estimated from our muscle and liver measurements. These estimates assume that all of the stored glycogen is available for working muscle and that gluconeogenesis in the liver plays no role in supplying glucose. However, our data suggest that high-altitude mice may have enhanced capacities from gluconeogenesis and circulatory glucose uptake that may extend this estimated running time. When voluntary locomotion on running wheels was examined in a colony of laboratory-reared deer mice, they preferred to run at speeds equivalent to ~70% $\dot{V}O_{2\max}$ but for durations much shorter than 20 min (13), suggesting glucose availability is not limiting to locomotion. Previous work has

demonstrated that some traits in the O₂ delivery pathway exhibit a greater acclimation response to hypoxia in low-altitude mice than in high-altitude mice (31). We show here that given the same environmental acclimation conditions, aspects of substrate delivery and oxidation show greater flexibility in high-altitude mice than low-altitude mice. This suggests that adaptive variation in phenotypic plasticity can be retained for specific subordinate traits while other traits become fixed in a population.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

D.S.L., A.D.C., S.M., and G.B.M. performed experiments; D.S.L., A.D.C., S.M., N.W., Z.A.C., J.F.S., G.R.S., and G.B.M. edited and revised manuscript; D.S.L., A.D.C., S.M., N.W., Z.A.C., J.F.S., G.R.S., and G.B.M. approved final version of manuscript; N.W. and G.B.M. analyzed data; Z.A.C., J.F.S., G.R.S., and G.B.M. interpreted results of experiments; G.B.M. prepared figures; G.B.M. drafted manuscript.

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