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Regulatory changes contribute to the adaptive enhancement of thermogenic capacity in high-altitude deer mice

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In response to hypoxic stress, many animals compensate for a reduced cellular O_2 supply by suppressing total metabolism, thereby reducing O_2 demand. For small endotherms that are native to high-altitude environments, this is not always a viable strategy, as the capacity for sustained aerobic thermogenesis is critical for survival during periods of prolonged cold stress. For example, survivorship studies of deer mice (*Peromyscus maniculatus*) have demonstrated that thermogenic capacity is under strong directional selection at high altitude. Here, we integrate measures of whole-organism thermogenic performance with measures of metabolic enzyme activities and genomic transcriptional profiles to examine the mechanistic underpinnings of adaptive variation in this complex trait in deer mice that are native to different elevations. We demonstrate that highland deer mice have an enhanced thermogenic capacity under hypoxia compared with lowland conspecifics and a closely related lowland species, *Peromyscus leucopus*. Our findings suggest that the enhanced thermogenic performance of highland deer mice is largely attributable to an increased capacity to oxidize lipids as a primary metabolic fuel source. This enhanced capacity for aerobic thermogenesis is associated with elevated activities of muscle metabolic enzymes that influence flux through fatty-acid oxidation and oxidative phosphorylation pathways in high-altitude deer mice and by concomitant changes in the expression of genes in these same pathways. Contrary to predictions derived from studies of humans at high altitude, our results suggest that selection to sustain prolonged thermogenesis under hypoxia promotes a shift in metabolic fuel use in favor of lipids over carbohydrates.

functional genomics | RNA-seq | thermoregulation | transcriptomics

During cold stress, homeothermic endotherms maintain a constant body temperature by increasing metabolic heat production. In small endotherms like mice that have high thermoregulatory demands, thermogenic capacity influences survival in cold environments and therefore has a clear connection to Darwinian fitness (1, 2). Indeed, thermogenic capacity in free-ranging deer mice (*Peromyscus maniculatus*) is subject to strong directional selection at high altitude (3).

Sustaining maximal thermogenic capacities during prolonged periods of cold stress requires a high rate of O_2 flux through oxidative pathways, and this requirement presents a unique challenge for endothermic animals that live under conditions of chronic O_2 deprivation at high altitude. The reduced partial pressure of O_2 (PO_2) at high altitude imposes well-documented constraints on aerobic metabolism (4–8), thereby exacerbating the increased thermoregulatory demands faced by endothermic animals that are native to cold, alpine environments.

In rodents, aerobic thermogenesis is accomplished through both shivering and nonshivering mechanisms, and in deer mice, shivering accounts for roughly 35–50% of total thermogenic capacity (9). As with other forms of strenuous exercise, aerobic shivering thermogenesis requires a precisely regulated flux of fuel supplies to meet the metabolic demands of intensely shivering skeletal muscle (10). In principle, such physiological adjustments can be mediated by coordinated changes in gene expression, which can influence both the overall expression of the metabolic machinery and the utilization of optimal metabolic fuel sources to meet constantly varying thermogenic demands. The challenge of effectively allocating fuel substrates for oxidative metabolism is especially critical at high altitude. Relative to the oxidation of carbohydrates as a fuel source, the oxidation of lipids produces a higher overall yield of ATP per unit of fuel, but it also consumes more O_2 in the process. These considerations have led some workers to suggest that carbohydrates should be the preferred fuel source under hypoxic conditions at high altitude (11–14). However, increased reliance on carbohydrates during periods of prolonged cold exposure may rapidly deplete this critical fuel source. Lipids make up more than 80% of the total energy reserves in mammals, and their energy density is an order of magnitude greater than that of carbohydrates (10), making them a preferred fuel source during periods of sustained submaximal exercise (10, 15, 16) and shivering thermogenesis, even at high intensities (17, 18). Given these tradeoffs, it is unclear which fuel-use strategy is optimal for high-altitude endotherms that are forced to cope with the combined challenges of chronic hypoxia and cold exposure.

Deer mice have the broadest altitudinal distribution of any North American mammal, occurring above 4,300 m in the mountain ranges of western North America to below sea level on the floor of Death Valley in California. Because of this broad altitudinal distribution, deer mice have long been used as a model to study physiological mechanisms of acclimatization and adaptation to high-altitude hypoxia (3–5, 19–31). The well-established connections between thermogenic capacity and fitness (3) make *P. maniculatus* an especially ideal study animal for investigating the mechanistic underpinnings of metabolic adaptation to high-altitude environments.

Here, we integrate measures of whole-organism metabolic performance with measures of metabolic enzyme activities and genome-wide transcriptional profiles of skeletal muscle to gain insight into the regulatory mechanisms that underlie the adaptive enhancement of thermogenic capacity under hypoxia. We show that deer mice that are native to high altitude have an enhanced thermogenic capacity under hypoxia, which is largely driven by an increased capacity to oxidize lipids as a primary fuel source. Differences in metabolic performance between

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highland and lowland deer mice are associated with differences in the activities of enzymes that influence flux through fatty-acid oxidation and oxidative phosphorylation pathways, and with concomitant changes in the expression of genes in these same pathways. These results suggest that changes in gene expression contribute to adaptive differences in thermogenic capacity, and that selection to sustain prolonged thermogenesis may offset the benefits of increased O_2 economy in species with high thermogenic demands.

Results and Discussion

Altitudinal Variation in Thermogenic Performance. We sampled *P. maniculatus* from two localities at the extremes of an altitudinal gradient that spans nearly 4,000 m of vertical relief from the Great Plains of eastern Nebraska (400 meters above sea level, m.a.s.l.) to the summit of Mt. Evans, Colorado (4,350 m.a.s.l.) in the Front Range of the Southern Rocky Mountains (linear distance = 770 km). For comparative purposes, we also sampled a closely related, but strictly lowland species, *Peromyscus leucopus*, in eastern Nebraska. Because we sampled wild-caught adults of unknown age, we cannot control for age-related effects on thermogenic performance. However, there is no evidence of systematic differences in age structure among these three populations that would bias our results.

Deer mice exhibit a high degree of plasticity in thermogenic capacity (4, 5, 21, 32, 33). To control for the effects of physiological plasticity during adulthood, we housed all of the experimental animals under common garden conditions at low altitude (360 m.a.s.l.) for 6 wk before testing. Similar acclimation periods (3–7 wk) are sufficient to induce changes in thermogenic performance in deer mice (21, 34). We did not explicitly control for developmental environment and thus cannot rule out the role of developmental effects on thermogenic performance. However, under common-garden conditions similar to those imposed here, developmental altitude has been shown to have no effect on thermogenic capacity in deer mice (33). Following the acclimation period, we measured thermogenic capacity (cold-induced maximal rate of oxygen consumption, VO_{2max}) using open-flow respirometry in a hypoxic heliox atmosphere (12.6% O_2, 87.4% He), which mimics the atmospheric PO_2 on the summit of Mt. Evans.

After 6 wk of acclimation to normoxic conditions, highland deer mice exhibited significantly higher thermogenic capacities under hypoxia than lowland deer mice and *P. leucopus* (ANCOVA with body mass as a covariate; $F_{2,26} = 14.26$, $P = 6.62 \times 10^{-5}$) (Fig. 1 and Tables S1 and S2). Phylogenetic reconstructions suggest that the common ancestor of *P. maniculatus* and *P. leucopus* was restricted to low altitudes (<1,000 m) (35), and because thermogenic capacity did not differ between the lowland deer mice and *P. leucopus*, the enhanced metabolic performance of high-altitude deer mice appears to be a derived character state.

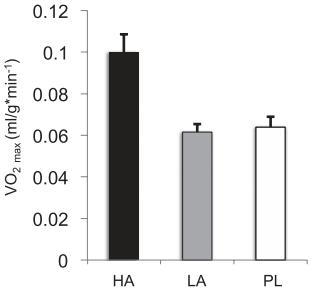

Fig. 1. Mass-specific maximum rates oxygen consumption (VO_{2max}) for highland deer mice (HA), lowland deer mice (LA), and a lowland outgroup species, *Peromyscus leucopus* (PL). Data are presented as means ±1 SEM.

Because the oxidation of lipids consumes more oxygen than the oxidation of carbohydrates, relative rates of CO_2 production and O_2 consumption (quantified as the respiratory exchange ratio, RER) can provide insight into metabolic fuel use (36). Over the course of the experimental trials, the highland and lowland deer mice and *P. leucopus* all relied heavily on lipid oxidation to power thermogenesis. RER values were below 0.8 within minutes of the onset of active thermogenesis in all three groups of mice and remained low throughout the experimental trials (Fig. 2*A*). In principle, the decline in RER could reflect a reduction in body temperature and a resultant change in the solubility of CO_2, but this seems unlikely given that low RERs occur during periods of maximal O_2 consumption (Fig. 2*B*). Instead, these results suggest a primary reliance on lipid catabolism during steady-state thermogenesis. The decline in RER appeared to be more gradual in the highland deer mice, but this result is difficult to interpret, as it may reflect an increased rate of carbohydrate oxidation at the onset of thermogenesis or ventilatory hypocapnia due to an enhanced hypoxic ventilatory response in highland deer mice (37, 38). Nonetheless, all of the mice were apparently relying almost exclusively on lipid oxidation after 10 min of sustained thermogenesis (Fig. 2*A*).

Highland deer mice also sustained much higher rates of O_2 consumption (VO_2) during active thermogenesis (Fig. 2*B*). In all three groups of mice, VO_2 peaked during periods with low RERs, suggesting that maximal rates of aerobic thermogenesis were fueled primarily by lipid catabolism. These results are consistent with studies of Wistar rats, which demonstrated elevated rates of lipid oxidation at low temperature (18). These results also suggest that lipid catabolic capacities in highland deer mice are nearly twofold higher than those of lowland deer mice and *P. leucopus*, given the differences in VO_{2max} (Figs. 1 and 2*B*).

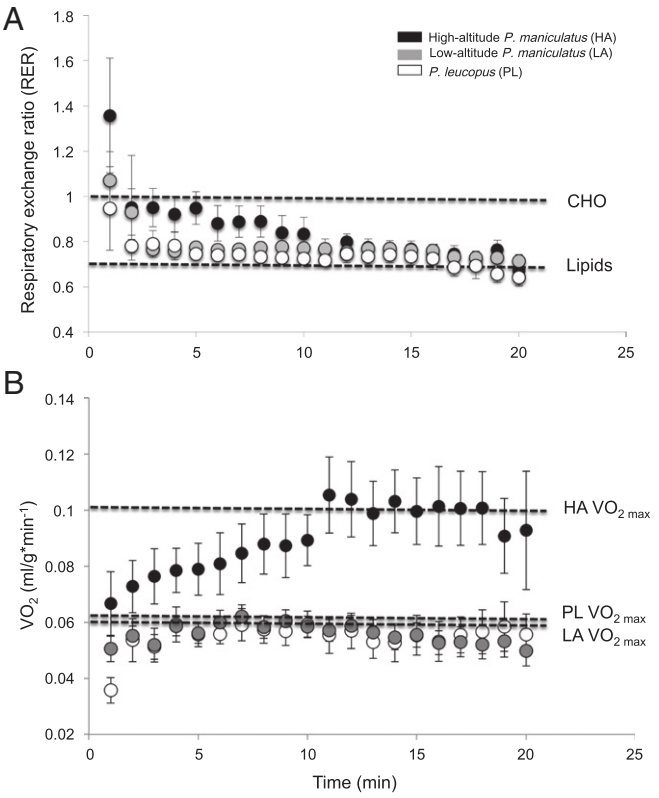

Fig. 2. Respiratory exchange ratios (*A*) and mass-specific rates of O_2 consumption (*B*) during combined cold and hypoxia challenge. Data are presented as 1-min averages ±1 SEM.

Functional Genomics of Thermogenic Performance. Variation in metabolic performance may be manifest at several hierarchical levels of biological organization (39). At the biochemical level, flux through metabolic pathways is jointly determined by the concentrations of metabolic enzymes and their kinetic properties. Changes in gene expression can play an important role in the regulation of pathway flux because mRNA transcript abundance is often positively associated with enzyme concentration (40, 41). We used massively parallel sequencing of skeletal muscle transcriptomes (RNA-seq) (42) to quantify genome-wide patterns of gene expression and to gain insight into the role of transcriptional regulation in tissue-level metabolic adaptation to high altitude. We measured the expression levels of nearly 15,000 genes, hundreds of which were differentially expressed between conspecific populations or between species. After controlling for a 5% genome-wide false discovery rate (FDR), we identified 221 genes that were differentially expressed between the highland and lowland deer mice. Consistent with recent analyses suggesting that divergence in gene expression scales with genetic distance (43, 44), a greater number of genes were differentially expressed in interspecific comparisons (highland deer mice vs. *P. leucopus* = 623 genes; lowland deer mice vs. *P. leucopus* = 424 genes) than in the comparison between conspecific populations of deer mice (highland vs. lowland deer mice = 221 genes).

Functional enrichment analyses revealed that genes involved in metabolic pathways were significantly overrepresented within the sets of genes that were differentially expressed between conspecific populations of deer mice or between deer mice and *P. leucopus*. Specifically, gene ontology (GO) terms related to lipid catabolism ($P = 2.09 \times 10^{-8}$) and oxidation-reduction processes ($P = 4.25 \times 10^{-3}$) were disproportionately represented, underscoring the important contributions of these pathways to altitude-related differences in genome-wide transcriptional profiles within and between species.

Pathway-level analyses revealed concerted changes in gene regulation across the fatty-acid β-oxidation and oxidative phosphorylation (OXPHOS) pathways (Fig. 3 and Figs. S1 and S2). Of the 11 genes that mediate fatty-acid oxidation, 7 (64%) were significantly differentially expressed ($P < 0.05$) in the intra- or interspecific comparisons involving highland deer mice, and 6 of these remained significant after controlling for a genome-wide FDR. By contrast, only a single gene (*Acox1*) was significantly differentially expressed between the lowland deer mice and *P. leucopus*. Moreover, the direction of gene expression change was nonrandomly distributed across the pathway in the intra- or interspecific comparisons involving highland deer mice. A total of 10 genes exhibited a >20% difference in transcript abundance between the highland and lowland deer mice (mean fold difference 1.34, range 0.78–2.74), and 9 of these were up-regulated in highland deer mice (exact binomial test, $P = 0.022$; Fig. 3 and Fig. S1). Similarly, 10 genes exhibited a >20% difference in transcript abundance in comparisons between highland deer mice and *P. leucopus* (mean fold difference 1.54, range 0.86–2.81), and all of them were up-regulated in the highland deer mice (exact binomial test, $P = 0.002$; Fig. 3 and Fig. S1). Fewer genes exhibited appreciable expression differences in comparisons between the lowland deer mice and *P. leucopus* (mean fold difference = 1.20, range 0.71–1.63), and although there was a trend toward up-regulation in lowland deer mice (7 of 8 genes), this difference was not significant ($P = 0.07$). These results were corroborated by discriminate function analyses (DFAs), which demonstrated that β-oxidation transcriptional profiles can discriminate between the conspecific population samples of deer mice and between each of these populations and *P. leucopus*, correctly assigning >80% of individuals to their respective species or population of origin (Table S3). However, the highland and lowland deer mice clustered together in multivariate space

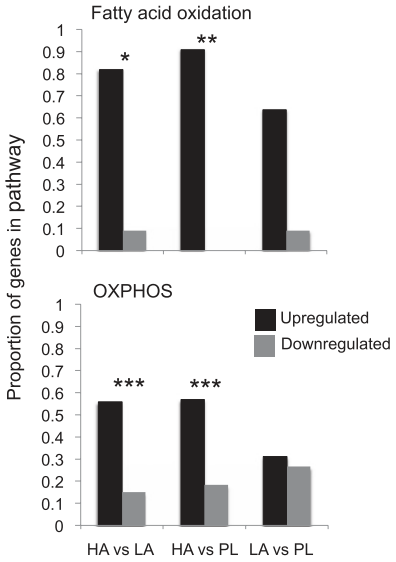

Fig. 3. Proportion of genes in the fatty-acid oxidation (*Upper*) and OXPHOS (*Lower*) pathways that are differentially expressed (>20% difference in transcript abundance) in pairwise comparisons among high-altitude deer mice (HA), low-altitude deer mice (LA) and *P. leucopus* (PL). The black bars indicate genes that are up-regulated in HA (HA vs. LA and HA vs. PL comparisons) and LA (LA vs. PL comparison), whereas gray bars indicate those that are down-regulated in the focal group. Asterisks indicate significant differences in the proportion of up- and down-regulated genes (exact binomical test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

to the exclusion of *P. leucopus* (Fig. S3), which suggests that genetic relatedness influences transcriptional profiles of the fatty-acid oxidation pathway.

Similar expression patterns were observed for the OXPHOS pathway (Fig. 3 and Fig. S2). Of the 93 genes that participate in OXPHOS, transcript abundances for 66 genes differed by >20% in the comparison between highland and lowland deer mice (mean fold difference 1.25, range 0.42–2.25). Seventy genes differed by an equivalent margin in the comparison between highland deer mice and *P. leucopus* (mean fold difference 1.96, range 0.22–15.8). Again, the direction of gene expression change was nonrandom as 78.7% ($P = 2.8 \times 10^{-6}$) and 75.7% ($P = 1.9 \times 10^{-5}$) of these genes were up-regulated in highland deer mice relative to lowland deer mice and *P. leucopus*, respectively. As with fatty-acid oxidation, the overall OXPHOS expression profiles were more similar in comparisons between lowland deer mice and *P. leucopus*. Expression levels for only 54 genes differed by >20%, and the direction of gene expression change was random, as 29 genes were up-regulated in lowland deer mice compared with 25 in *P. leucopus* ($P = 0.6835$) (Fig. 3). Despite the similarity in OXPHOS expression patterns between lowland deer mice and *P. leucopus*, discriminate function analysis demonstrated that all three groups of mice were diagnosable on the basis of their OXPHOS transcriptional profiles (Table S3 and Fig. S2).

In contrast to the β-oxidation and OXPHOS pathways, there was little evidence for large-scale changes in gene regulation in two other key pathways that contribute to metabolic flux and fuel use in vertebrates. Only three glycolytic genes (17%) were significantly differentially expressed in the intra- and interspecific comparisons, and the direction of gene expression change was randomly distributed across the pathway in all three pairwise comparisons (Fig. S1). Consistent with these results, DFA demonstrated that highland mice, lowland mice, and *P. leucopus* were indistinguishable on the basis of their glycolytic transcriptional profiles with over 50% of individuals being incorrectly

assigned to their respective species or population of origin (Table S3). However, there was a notable trend toward the down-regulation of genes involved in carbohydrate metabolism in both deer mouse populations relative to *P. leucopus*. In the tricarboxylic acid (TCA) cycle only a single gene was significantly differentially expressed, and there was no consistency in the direction of expression change between species or between the conspecific populations of deer mice (Fig. S1).

These results suggest that unique transcriptional patterns in high-altitude deer mice may increase flux through fatty-acid oxidation and OXPHOS pathways. For enzymes with large control coefficients, in vitro enzyme activity (V_{max}) can be used to estimate pathway flux capacity in vivo (45). Consistent with the gene expression patterns, activities for key enzymes in fatty-acid oxidation (β-hydroxyacyl-CoA dehydrogenase; HOAD) and OXPHOS (cytochrome *c* oxidase, COX) were significantly higher in highland deer mice compared with lowland deer mice and *P. leucopus* (Table 1). By contrast, there were no differences in the activities of key enzymes that participate in glycolysis or the TCA cycle (Table 1).

Conclusions

The stoichiometric advantage of carbohydrate metabolism under conditions of chronic O_2 deprivation has led to the suggestion that a shift in muscle metabolic fuel use in favor of carbohydrates may represent a general feature of high-altitude adaptation (13). However, empirical evidence in support of this hypothesis is based almost exclusively on studies of humans. Acclimation to hypoxia has been shown to increase carbohydrate utilization in some studies of human lowlanders and in some nonhuman animals (11, 12, 46, 47), but not in others (48, 49). In contrast, both Sherpas and Andean Quechuas show evidence for enhanced cardiac glucose uptake at the tissue level (50), and in Sherpas, the decreased ratio of phosphocreatine to ATP in cardiac myocytes suggests a greater reliance on glucose for ATP production (51). However, neither Quechuas nor Sherpas exhibit enhanced maximal aerobic capacities compared with lowlanders at similar fitness levels (52, 53), suggesting that human patterns of metabolic adjustment may be optimized to enhance O_2 economy rather than maximal aerobic capacity (54).

Questions about optimal fuel use strategies in high-altitude species with high thermoregulatory demands have received less attention. For species like deer mice, selection to maintain high capacities for prolonged aerobic thermogenesis may outweigh the benefits of an enhanced O_2 economy. Under natural conditions, deer mice endure prolonged periods of cold stress (55, 56), which is likely offset by long-term, submaximal shivering and nonshivering thermogenesis. Under these conditions, an enhanced capacity to transport and oxidize fatty acids should greatly enhance shivering thermogenic capacity and endurance (16). An increased reliance on fatty acids during shivering thermogenesis would not only preserve critical glycogen stores for periods of burst exercise (e.g., escape from predators) (10), but high rates of lipid oxidation can also power the rapid replenishment of glycogen stores following intense exercise (57). Consistent with these hypothesized benefits, we found evidence for an enhanced capacity for fatty-acid oxidation under hypoxic cold stress in high-altitude deer mice. Concomitant changes in the expression of genes that participate in fatty-acid oxidation and OXPHOS pathways in skeletal muscle suggest that this enhanced capacity is driven in part by changes in enzyme concentration (i.e., hierarchical regulation) (39). Although we have focused exclusively on mechanisms that may enhance shivering thermogenesis, rodents also derive a large proportion of their metabolic heat production from nonshivering mechanisms. A similar integrative analysis of brown adipose tissue and nonshivering thermogenic capacity would likely be a fruitful avenue for future research.

Our results suggest that high-altitude adaptation in deer mice involves the maintenance of a highly aerobic phenotype in the face of reduced O_2 availability. Elite endurance athletes and highly aerobic nonhuman mammals are also characterized by an enhanced capacity for fatty-acid oxidation under normoxic conditions (10, 16, 58–60). Under conditions of chronic O_2 deprivation at high altitude, a similar enhancement in fatty-acid oxidation capacity could promote thermogenic endurance, but would require additional physiological changes to ensure adequate O_2 flux through oxidative pathways. The elevated blood-O_2 affinity of highland deer mice helps to preserve an adequate level of tissue-O_2 delivery despite hypoxia (20, 27–30, 61) and may help to power an enhanced capacity for lipid oxidation. Thus, adaptation to hypoxic cold stress in deer mice seems to involve changes in both the functional properties and expression levels of proteins that interact in hierarchical pathways, a situation that is likely to be common in the evolution of complex physiological traits (62).

Table 1. Apparent V_{max} for enzymes in the gastrocnemius muscle of *Peromyscus* species

Enzyme	*P. maniculatus* (HA)	*P. maniculatus* (LA)	*P. leucopus* (PL)
Glycolysis			
HK	3.1 ± 0.4	2.6 ± 0.3	3.1 ± 0.3
PFK	21.2 ± 1.9	23.4 ± 2.9	28.8 ± 2.1
LDH	227.4 ± 11.5	277.9 ± 32.6	305.4 ± 28.9
β-Oxidation of fatty acids			
HOAD	**29.9± 2.0**	**22.5± 2.4**	**21.0 ± 1.4**
TCA cycle			
CS	34.0 ± 3.9	31.7 ± 3.8	26.9 ± 2.6
IDH	5.7 ± 0.6	4.7 ± 0.5	4.2 ± 0.7
OXPHOS			
COX	**14.7 ± 1.6**	**9.6 ± 1.7**	**7.7 ± 1.2**

Activities are in U/g wet weight, where U = μmoles/min ± SEM. Significant differences between high-altitude deer mice and both low-altitude populations (low-altitude deer mice and *P. leucopus*) are indicated in bold.

Materials and Methods

Experimental Animals. Mice were live trapped at one of three sampling localities: the summit of Mt. Evans, Clear Creek County, Colorado, 39° 35′ 18″ N, 105° 38′ 38″ W, 4,350 m.a.s.l. (*n* = 10 *P. maniculatus*); Nine-Mile Prairie, Lancaster County, Nebraska, 40° 52′ 12″ N, 96° 48′ 20″ W, 430 m.a.s.l. (*n* = 10 *P. maniculatus*); Denton, Lancaster County, Nebraska, 40° 44′ 36″ N, 96° 47′ 49″ W, 380 m.a.s.l. (*n* = 10 *P. leucopus*). All of the experimental animals were transferred to the animal research facility at the University of Nebraska and were housed under common conditions for 6 wk with a constant ambient temperature (25 °C) and light:dark cycle (12L:12D). During the acclimation period, all of the mice were fed a common diet ad libitum (Harlan Teklad Rodent Chow). All of the experimental protocols were approved by the University of Nebraska Institutional Care and Use Committee (IACUC) Guidelines (protocol 522).

Respirometry. We measured cold-induced maximum rates of oxygen consumption (VO_{2max}) and carbon dioxide release (VCO_{2max}) using open-flow respirometry in a hypoxic heliox atmosphere (12.6% O, 87.4 He). Rates of heat loss in heliox are several times greater than in ambient air, which makes it possible to elicit VO_{2max} without risking cold injury to experimental animals (6). All of the trials were conducted at ambient temperatures just below freezing (minimum −4 °C). Similar protocols have been used previously to elicit cold-induced VO_{2max} in *P. maniculatus* (4, 34). Heliox mixtures were obtained from a commercial supplier (Linweld).

The respirometry setup for the thermogenic trials is illustrated in Fig. S4. Briefly, heliox gas mixtures were first equilibrated with atmospheric pressure and pumped into copper coils inside a temperature control chamber using mass flow controllers (Sable Systems). The cooled heliox was then pumped into the animal chamber and a baseline (empty) chamber at a rate of ~450 mL/min. The animal and baseline chambers were constructed of thin,

airtight polypropylene with an internal volume of 180 mL, which minimized locomotion but still permitted postures normally adopted for shivering. We verified that the temperature inside the temperature-control chamber was identical to that experienced by a mouse inside the animal chamber using a thermocouple to measure excurrent air at the junction of the excurrent tube and the animal chamber (Fig. S4). Excurrent air from the animal and baseline chambers was sampled at a rate of ~130 mL/min, dried (magnesium perchlorate), passed through a carbon dioxide analyzer, scrubbed of CO_2 (ascarite), redried (drierite), and passed through an oxygen analyzer (Sable Systems; FoxBox). We monitored excurrent O_2 and CO_2 continuously, and each experimental animal was removed when values showed clear indications of dropping to baseline. We used a rectal thermometer to measure the body temperature of each mouse following the thermogenic trials, and all of the mice were hypothermic when removed from the chamber. We corrected for the influence of body mass on thermogenic capacity (VO_{2max}) using an analysis of covariance (ANCOVA) with body mass as covariate, and VO_{2max} was defined as the maximum VO_2 averaged over a continuous 5-min period. We tested for differences in mass-corrected VO_{2max} among *Peromyscus* populations using ANCOVA followed by posthoc Tukey's honestly significant difference (HSD) tests in R.

Functional Genomics. Immediately following the respirometry measurements, all of the mice (n = 10 per group) were euthanized according to IACUC guidelines, and samples of skeletal muscle (gastrocnemis) were excised and immediately frozen on liquid nitrogen. We isolated total mRNA from skeletal muscle samples using a microPolyA purist kit (Ambion) following the manufacturer's protocol. Illumina sequencing libraries were constructed following standard protocols (available upon request), and sequenced as 76 nt single-end reads on an Illumina Genome Analyzer IIx. Five individuals were multiplexed using Illumina index primers and were sequenced in a single lane of a flow cell. A total of six lanes were sequenced to obtain data for all 30 experimental animals. Image analysis and base calling was performed using Illumina pipeline software. Gene expression was estimated by mapping sequence reads to the *Mus musculus* genome, build 36.1, using the program Qseq (DNAStar). Expression values were expressed as reads per kilobase of exon model per million mapped reads (RPKM) (63) on a $\log_2$ scale, which simultaneously corrects for differences in the total number of reads sequenced per individual and length differences among transcripts. We used pairwise Student t tests to identify genes that were differentially expressed among populations and corrected for multiple tests by enforcing a genome-wide FDR of 0.05. Functional enrichment analyses were also performed in Qseq using Gene Ontology process terms associated with the *Mus* genome. We used Cytoscape (64, 65) to map expression values onto selected metabolic pathways. Pathway maps were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html). Discriminate function analyses of gene expression values were performed using JMP 501, and exact binomial tests were conducted in R. The raw sequence reads have been deposited in the NCBI sequence read archive, http://www.ncbi.nlm.nih.gov/sra (accession no. SRA051883).

Enzyme Activities. Gastrocnemius muscles were ground to a powder using a liquid N_2-cooled mortar and pestle. Approximately 20 mg of this powder was homogenized using a glass-on-glass homogenizer for 1 min on ice in 20 volumes of homogenizing buffer [100 mM potassium phosphate (pH 7.2), 5 mM EDTA, and 0.1% Triton X-100]. Assays of enzyme activity were done in triplicate and background rates were determined for each assay at 37 °C using a Spectromax Plus 384, 96-well microplate reader (Molecular Devices).

Assay conditions in millimolar concentration (mM) were as follows: for COx activities, 100 KH_2PO_4 (pH 7.0), with 0.1 reduced cytochrome c as substrate. Phosphofructokinase was assayed using 10 fructose-6-phosphate (F6P) (omitted for background), 0.28 NADH, 4 ATP, 2 AMP, 5 $MgCl_2$, 50 KCl, 5 DTT, 1 unit aldolase, 50 units triose phosphate isomerase and 8 units α-glycerophosphate dehydrogenase, in 50 triethanolamine (TEA)-HCl (pH 7.6). Conditions for assaying HOAD were: 0.1 acetoacetyl-CoA (omitted for background), 0.28 NADH, and 5 EDTA, in 100 TEA-HCl (pH 7.0). Isocitratae dehydrogenase (IDH) was measured in 40 Tris-HCl (pH 7.4), with 1.5 isocitrate (omitted for background), 2 ADP, 0.5 NADP, 1 $MnCl_2$, and 8 $MgCl_2$. Hexokinase (HK) assay conditions were 5 D-glucose (omitted for background), 8 ATP, 8 $MgCl_2$, 0.5 NADP, and 4 units glucose-6-phosphate dehydrogenase, in 50 Hepes (pH 7.6). The V_{max} of LDH was assayed after two freeze/thaw cycles of the homogenate using 1 pyruvate-Na (omitted for background), and 0.28 NADH, in 40 Tris-HCl (pH 7.4). Citrate sythase (CS) was assayed after three freeze/thaw cycles of the homogenate using: 0.5 oxaloacetate (omitted for background), 0.22 acetyl-CoA, and 0.1 dithiobisnitrobenzoic acid (DTNB) in 40 Tris-HCl (pH 8.0).

We tested for statistical outliers in enzyme V_{max} values using a Grubb's test (66) after identifying several suspect values in univariate plots. A total of three measurements were identified as outliers and removed from further analysis, with no more than one value per group being discarded (HOAD n = 1, PL; COX n = 2). After removing outliers, we tested for differences in enzyme V_{max} among *Peromyscus* population samples using a one-way ANOVA followed by posthoc Tukey's HSD tests in the program JMP 501.

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Supporting Information

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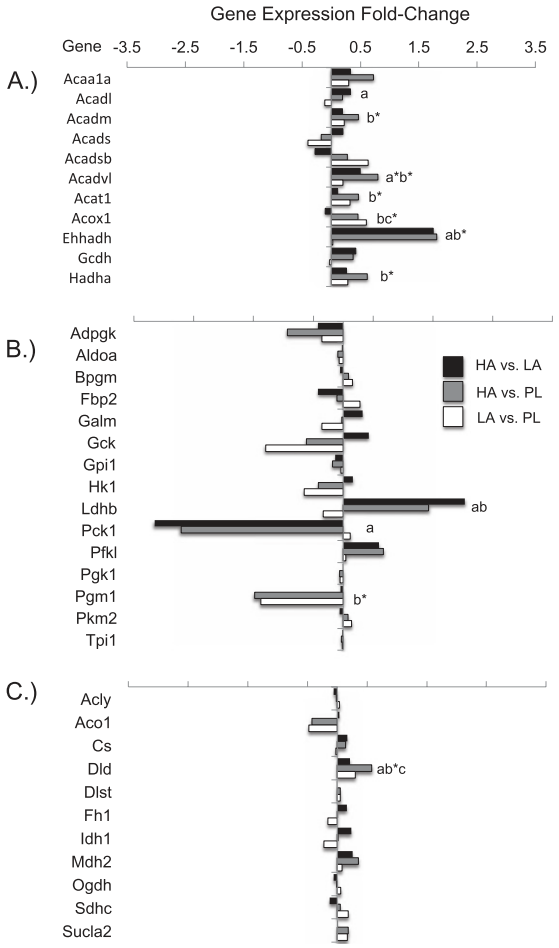

Fig. S1. Gene expression fold change for genes involved in the β-oxidation of fatty acids (*A*), glycolysis (*B*), and the tricarboxylic acid cycle (TCA) cycle (*C*). HA, highland deer mice; LA, lowland deer mice; PL, *P. leucopus*. Positive numbers indicate genes that are up-regulated in either highland deer mice (HA vs. LA and HA vs. PL comparisons) or lowland deer mice (LA vs. PL), whereas negative numbers indicate genes that are down-regulated in the focal group. Lowercase letters indicate genes that are significantly differentially expressed ($P < 0.05$) between HA and LA (*a*), HA and PL (*b*), and LA and PL (*c*). Asterisks indicate significant differences after controlling for a 5% genome-wide false discovery rate (FDR).

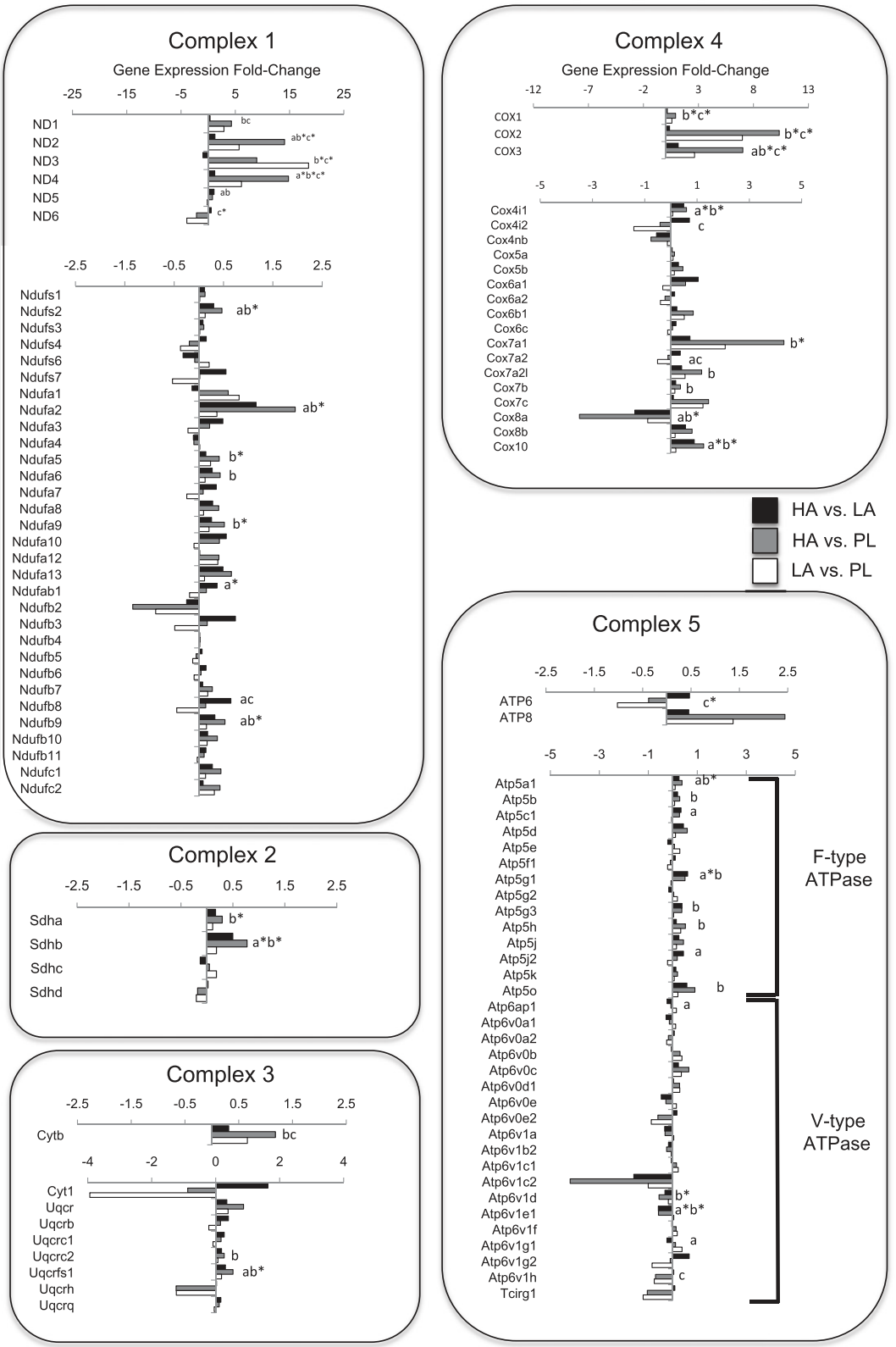

Fig. S2. Gene expression fold change for genes that participate in oxidative phosphorylation (OXPHOS) organized by mitochondrial complex. Complexes 1, 3, 4, and 5 include genes that are encoded by both the mitochondrial and nuclear genomes, and these are plotted separately with mitochondrial genes represented in the *Upper* panel for each of these complexes. HA, highland deer mice; LA, lowland deer mice; PL, *P. leucopus*. Positive numbers indicate genes that are up-regulated in either highland deer mice (HA vs. LA and HA vs. PL comparisons) or lowland deer mice (LA vs. PL), whereas negative numbers indicate genes that are down-regulated in the focal group. Lowercase letters indicate genes that are significantly differentially expressed ($P < 0.05$) between HA and LA (*a*), HA and PL (*b*), and LA and PL (*c*). Asterisks indicate significant differences after controlling for a 5% genome-wide FDR.

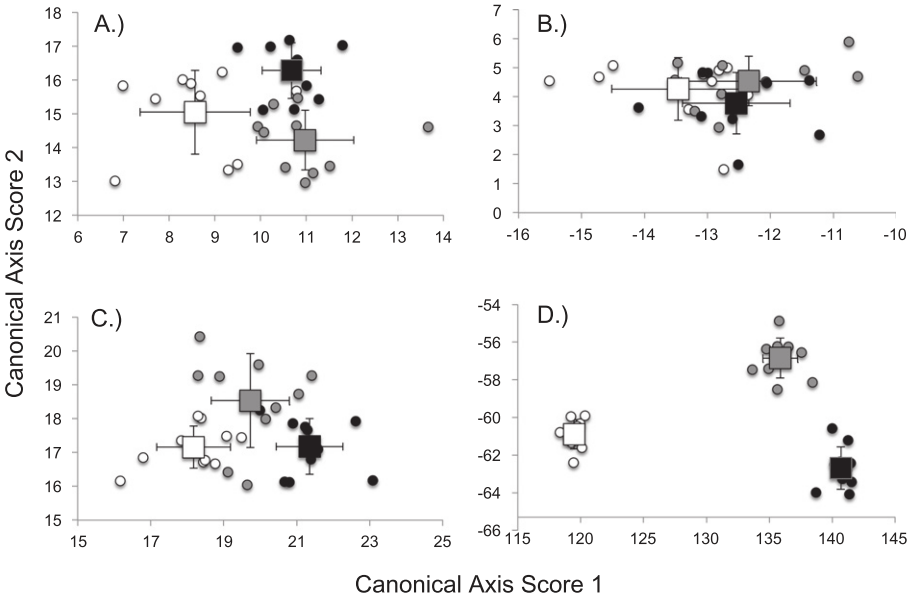

Fig. S3. Plots of canonical axis scores from discriminate function analysis of gene expression levels. Highland deer mice, lowland deer mice, and *P. leucopus* are indicated by black, gray, and white symbols, respectively. Squares represent group means ±1 SD. (*A*) β-Oxidation of fatty acids, (*B*) glycolysis, (*C*) TCA cycle, and (*D*) OXPHOS.

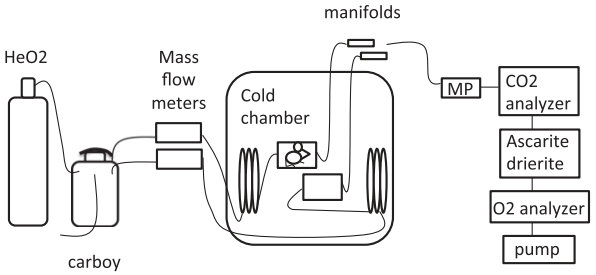

Fig. S4. Diagram of respirometry setup. Heliox flowed into a carboy at a rate sufficient to inflate a membrane sealed to the top of the carboy to ensure no mixing with ambient air. Excess gas was vented through an exit valve in the carboy. We confirmed the O_2 and CO_2 concentration within the animal and baseline chambers before each measurement session by measuring the excurrent gas from both chambers before placing a deer mouse in the animal chamber. Two mass flow meters were used to regulate flow into copper tubing and tubing leading into the animal and baseline chamber. Airflow rates were ~450 mL/min, and the chambers were constructed of thin, airtight polypropylene, with an internal volume of 180 mL. Excurrent air was vented into syringe manifolds and manually switched for subsampling by gas analyzers. Mass flow was corrected for heliox during data analysis using Eqs. 10.1 and 10.8 from ref. 1. MP, magnesium perchlorate.

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Table S1. Body mass and maximal rates of cold-induced O_2 consumption for highland deer mice (HA), lowland deer mice (LA), and *P. leucopus* (PL)

Population	Body mass, g	VO_{2max}, mL O_2*min^{-1}
P. maniculatus (HA)	21.01 ± 1.04	2.06 ± 0.15
P. maniculatus (LA)	17.71 ± 0.85	1.08 ± 0.07
P. leucopus (PL)	29.26 ± 2.12	1.82 ± 0.14

Data are presented as means ±1 SEM. Note that rates of O_2 consumption are not corrected for differences in body mass.

Table S2. Effects of body mass (g) and population of origin (HA, LA, or PL; ANCOVA with mass as a covariate) on thermogenic capacity ($VO_{2\ max}$; mL O_2*min^{-1})

Factor	df	Mean square	*F*	*P*
Body mass	1	2.036	15.175	0.00061
Population	2	1.912	14.255	6.62×10^{-5}
Error	26	0.135		

The interaction was not significant.

Table S3. Results of discriminate function analysis of gene expression profiles

	Percent misclassified		
Pathway	*P. maniculatus* (HA)	*P. maniculatus* (LA)	*P. leucopus* (PL)
Fatty acid oxidation	10	30	20
Glycolysis	60	50	50
TCA cycle	10	20	10
OXPHOS	0	0	0